TVET CERTIFICATE V in Food Processing

MICROBIAL ANALYSIS OF FOOD

FOPMA501

Conduct microbial analysis of food

Competence

Credits: 6

Learning hours: 60

Sector: Agriculture and Food processing

Sub-sector: Food processing

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Purpose statement

The competence is one of the specific competences that learner joining certificate V in Food processing are supposed to cover. It is designed to equip the learner with skills and knowledge on food microorganism analysis. At the end of the competence, the learner will be able to:

- Prepare liquid and solid culture medium, Prepare the sample and necessary dilutions, Grow and isolate specific microorganisms, enumerate microorganisms, Identify microorganisms according to Gram stain

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Learning Unit 1: Prepare Sample

L.O.1.1. Take and Handle Sample

Content/Topic 1: Sampling

What is a sample?

A collection of one or more units from a lot drawn for examination or testing purposes.

food sample units could be entire packages of food, portions of packaged food, or portions of food being prepared

- **Sampling:** is the process of collecting and testing food, ingredients, the environment or other materials.

Aim:

- ✓ Sampling is commonly used to monitor or verify the effectiveness of control measures put in place to prevent, eliminate or reduce to an acceptable level the hazards that present a risk of contamination to a food.
- ✓ Sampling can also provide assurance that incoming materials, finished products and water meet food safety standards

Sample collection

Purpose / reasons of sample Collection

The reason for which the samples a r e collected determines the options selected. A list of reasons would include:

1. A routine quality assurance program.
2. To determine the cause of a problem in a product or process.
3. Samples submitted as part of a research program.
4. Determining if a product will meet a customer specification.
5. Samples from a government surveillance or regulatory program.
6. To develop data to settle a conflict between a supplier and a customer
7. Samples of a food implicated in an outbreak of foodborne illness

How to sample

It is important to practice proper sampling techniques to avoid contaminating samples and exposing yourself to contaminants.

- Wear protective items, such as gloves and protective clothing.
- Use only clean equipment and containers to take samples.

Samples should be labelled with the information you need to link the results back to the food, ingredient or food contact surface being assessed. **Sample labels should include:**

- date and time of collection
- description of what was sampled

- lot number
- sampling site
- name of the person who collected the sample

The sample units should be representative of the lot and obtained randomly:

- each sample unit should be selected by chance and each unit in the lot should have an equal chance of being included in the sample.
- you can use a table of random number generated using a computer software to assign a number to each unit in a lot and select units to be sampled

When collecting samples:

- wash and dry hands prior to sampling
- use aseptic techniques when taking microbiological samples
- pre-packaged food samples should be collected in an original, unopened package
- use appropriate sampling containers that can withstand handling and shipping
- securely seal sample containers after filling so they cannot leak or become contaminated during further handling or transportation
- Note: Open, broken or damaged containers are not appropriate for sampling

When collecting samples for microbial testing avoid introducing microorganisms to the samples by following **aseptic sampling procedures**:

- Use only sterile equipment and containers, and properly re-sterilize sampling tools before using them again
- Make contact with the source material and the sample only with the sampling tool or the container
- Use sterile gloves if a sample must be touched with the hands. An aseptic sample should not be touched with bare hands
- Minimize exposure of the product, sampling equipment, and the interior of sampling containers to the environment. For example, avoid collecting samples in areas where dust or atmospheric conditions may contaminate the sample unless such contamination may be considered part of the sample

- Work rapidly. open sterile sampling containers only to insert the sample, and close them immediately
- Avoid unnecessary contact. The sample and sampling tool should not contact the interior, lip, or lid of the sterile container

Important points to be considered in collecting food sample:

- ✓ It is important to collect food samples that are representative of a lot or a food contact surface being assessed.
- ✓ It is also important to ensure that samples are not compromised when being collected, stored or shipped, as this could lead to inaccurate results

- ✓ The adequacy and condition of the sample or specimen received for examination are of primary importance
- ✓ If samples are improperly collected: the laboratory results will be meaningless
- ✓ Sampling protocol should be clearly defined
  - Start with description of primary food product

- ✓ Identity of the food:
  - Common/alternative name

. E.g. Maize,

  - Scientific name (Genus, species, variety)

. E.g. Zea mays

  - Plant food (entire plant/part e.g. roots)
  - Animal food (entire animal/part)
  - State of maturity (ripe immature)
  - Other details

- ✓ Need to know:
  - ➢ Number and size of sample to be collected
  - ➢ Distribution of samples
  - ➢ Stratification to be used

✓ Sample label should be permanently attached to the sample

- Common name of food
- Sample code number
- Date of receipt in Lab.

✓ **During sample collection:**

- Collection details
- Date and time of collection
- Name of collector
- Place of origin
- Sampling point/addresses (roadside stall, farm, market)
- Condition of cultivation (feed regime, altitude, irrigation)
- Purchase price
- Graphical record (Photograph, visual record with scale)
- Transport conditions (mode and conditions of transport)

✓ **Description of sample collected: after sample collection**

- Food type (Legume, fruit juice, milk product)
- Local use of foods (Famine. Festivals)
- State of food sample (solid, semisolid, viscous, or liquid)
- Process and preservation methods (canned smoked)
- Preparation method (cooking)
- Extent of preparation (raw, fully cooked, reheated)
- Extent of preparation (raw, fully cooked, reheated)
- Packing medium (brine, oil)
- Container or wrapping (can glass)
- Contact surface (can, glass)
- Label or list of ingredients (estimated by inspection)
- Batch number
- Weight of food collected/individual items
- Number of items

- Weight of common measure or portion

✓ **Things to note**:

There are many things that you can do to reduce the risk of contaminating your sample. The following points are things to consider when sampling:

➢ **Opening sample containers**

- For a bottle or cup: remove the cap or lid with a free hand and keep it in that hand during sample collection; only the exterior of the cap or lid can be touched
- For a bag: open the bag by using the tabs. Do not touch the opening of the bag
- Do not overfill the sampling container

✓ **Closing sample containers**

- For a bottle or cup: replace the cap or lid on the container without touching the inside of the cap or the mouth of the bottle and secure the cap or lid
- For a bag: pull twist ties tight, whirl the bag three revolutions, and fold the twist ties towards each other to seal the bag

➢ Deliver samples to the laboratory promptly with the original conditions maintained as nearly as possible

➢ If products are in bulk: storage procedures, choice of containers, modes of transport should be considered

➢ Use containers that are clean, dry, leak-proof, wide mouthed, sterile, and of a size suitable for samples of the product.

Content/Topic 2. **Sample Handling**

✓ During Handling

Aim: To protect the sample from changes in composition and contamination

✓ **Things to note**

- Weight and nature of edible/inedible matter (Prior to further processing (outer wilted leaves)
- Method of preparation (Cooking or not, time, temperature of preparation)
- Weight before/after cooking
- Ingredients added if any
- Method of mixing and reduction (grinding, homogenization)
- Types of storage (addition of preservatives, temp of storage)
- Methods used of take analytical samples
- Storage of analytical samples or further processing
- Name and signature of person completing record
- Date of record
- Other details

➢ **Hygienic condition:**

✓ Use aseptic technique when handling product.

✓ Before handling or analysis of sample, clean Immediate and surrounding work areas.

✓ In addition, swab immediate work area with commercial germicidal agent.

Content/Topic 3. Sampling techniques

Three main types of sampling strategy:

- Random
- Systematic
- Stratified

Within these types, you may then decide on a; point, line, area method.

Random sampling

- Least biased of all sampling techniques, there is no subjectivity - each member of the total population has an equal chance of being selected

- Can be obtained using random number tables
- Microsoft Excel has a function to produce random number

Advantages and disadvantages of random sampling

Advantages:

- Can be used with large sample populations
- Avoids bias

Disadvantages:

- Can lead to poor representation of the overall parent population or area if large areas are not hit by the random numbers generated. This is made worse if the study area is very large
- There may be practical constraints in terms of time available and access to certain parts of the study area

Systematic sampling

Samples are chosen in a systematic, or regular way.

- They are evenly/regularly distributed in a spatial context, for example every two meters along a transect line
- They can be at equal/regular intervals in a temporal context, for example every half hour or at set times of the day
- They can be regularly numbered, for example every 10th house or person

Advantages and disadvantages of systematic sampling

Advantages:

- It is more straight-forward than random sampling
- A grid doesn't necessarily have to be used, sampling just has to be at uniform intervals
- A good coverage of the study area can be more easily achieved than using random sampling

Disadvantages:

- It is more biased, as not all members or points have an equal chance of being selected
- It may therefore lead to over or under representation of a particular pattern

Stratified sampling

This method is used when the parent population or sampling frame is made up of sub-sets of known size. These sub-sets make up different proportions of the total, and therefore sampling should be stratified to ensure that results are proportional and representative of the whole.

Advantages and disadvantages of stratified sampling

✓ **Advantages:**

- It can be used with random or systematic sampling, and with point, line or area techniques
- If the proportions of the sub-sets are known, it can generate results which are more representative of the whole population
- It is very flexible and applicable to many geographical enquiries
- Correlations and comparisons can be made between sub-sets

✓ **Disadvantages:**

- The proportions of the sub-sets must be known and accurate if it is to work properly
- It can be hard to stratify questionnaire data collection, accurate up to date population data may not be available and it may be hard to identify people's age or social background effectively.

Content/Topic 4. Sources of errors in sampling

- It is essential that all those involved in the sampling process are familiar with the objectives of the work and are clear about their roles.
- This will identify aspects that are unclear or impracticable and require modification to avoid errors.

Major sources of errors in Sampling

source	Example	Precaution
Food sample identification	Poor labeling of samples	Maintenance of documentation throughout sampling and analytical process
Nature of sample	Samples do not conform to the defined sampling protocol	Explicit instructions , sampling protocol, training of sample staff
Transport and handling	Samples contaminated, degraded or depleted during transport, loss of samples	Protocol specifies condition to be maintained, supervision
Analytical sample preparation	Incorrect mixing or homogenization	Proper supervision in laboratory Laboratory quality assurance
Analytical sample storage	Incorrect storage of samples	Proper laboratory techniques and supervision

L.O.1.2. Storing and shipping samples

Content/Topic 1: Precautions during sample transportation

Samples for microbial testing can be compromised by the temperature they are exposed to and the time that goes by before they are tested. To preserve their integrity, you should store and ship the samples at appropriate temperatures and within the time frames recommended by the laboratory

To prevent contamination, deterioration, and other damage that could compromise the integrity of a sample during transportation:

- Before shipping, store samples in a manner to maintain their integrity
- Measure and record temperature of sample units before placing them into the shipping container
- Use sound, clean, dry shipping containers, coolers, coolant and packaging materials
- Pack samples tightly to prevent shifting
- Transport frozen or refrigerated samples in an insulated shipping container of rigid construction and packed with suitable coolant material to maintain their frozen or refrigerated state. Avoiding direct contact with the coolant material
- Ship samples collected as quickly as possible
- Keep ground samples in glass or plastic containers with air and water tight covers.
- Samples not analyzed immediately should be left in cold storage to minimize spoilage and other chemical reactions.
- Samples for lipid analysis – store under nitrogen at low temperature to prevent oxidation and unsaturated lipids
- Light may initiate oxidation so store in dark containers.
- For lipid analysis, antioxidants may be added if they won't interfere with the analysis
- It is therefore desirable to store a number of identical analytical samples
- Minimize the number of staff involved in taking portions from them.

The method of transporting should be one which results in a minimum The sample of microbial growth or death in the sample. received in the laboratory must be representative of the product at the time the sample was collected.

L.O.1.3. Sample Preparation

Topic 1.Purpose of sample preparation

- ✓ to meet certain set standard
- ✓ to estimate the shelf-life of the product
- ✓ to determine quality of the food
- ✓ for public health purposes

Once the samples are at the Laboratory, they must be prepared for analysis. Frozen samples are frequently allowed to thaw overnight in a refrigerator.

In some instances, time is critical so the samples might be placed under cold running water. Care should be taken that the package does not leak and permit entry of water.

TOPIC 2.Sample Preparation

Some practical equipment requirements for handling and preparation of laboratory and analytical samples

- ✓ Trays (for carrying foods)
- ✓ Chopping boards (polythene, wood)
- ✓ Oven thermometer, meat thermometer
- ✓ Waring blender
- ✓ Pestle and mortar
- ✓ Ball mill
- ✓ Hammer mill

Topic 2. Sample preparation techniques

- ✓ Preparation of analytical portions
  - If the particle size or bulk is too large for analysis, it must be reduced in bulk or size for analysis
  - Documentation of sample preparation is very important
  - Separate edible/inedible portions, record descriptions and weigh all parts
  - Measure portion sizes, weights, volumes, density etc.
- ✓ Homogeneous foods
  - Solids:

-Friable: crumble and mix.

-Sticky: freeze and crush at low temperature.

- Hygroscopic: take portions rapidly into pre-weighed sealable containers for weighing

- ✓ Emulsions.
  - Take by weight rather than volume; warm and mix.

- ✓ Liquids with suspended solids.
  - Homogenize, or sample during gentle mixing.
- ✓ Reduction by quartering:
  - The principle is that the quarter should be representative of the whole
  - Any symmetrical food should be cut into quarters, and one-quarter of each batch taken for processing for analysis
  - Large items, if symmetrical, can be reduced in size by this technique
  - Oval or elongated foods (e.g. potato or cucumber) should be cut into eighths, and two-eighths taken for a quarter,
  - Food lots of small items (flour, rice, legumes, small fruits, chopped mixed units).
  - The bulk is tipped into a uniform pile on a clean, inert surface
  - Turned over several times with a polythene or glass spatula.
  - The pile is leveled and then divided into four equal segments.
  - Two opposing segments are taken and the other two discarded.
  - The remaining segments are mixed and further reduced in the same way
  - Foods consisting of fairly large, separate, but similar portions, such as loaves of bread or joints of a meat, should be quartered and sampled then processed for analysis.
  - Segmented foods sampling e.g. packets of biscuits, cartons of eggs, batches of bread rolls.

Take every fourth item to form a composite sample.

- For sliced loaves, take every fourth slice and one end slice,

which then must be thoroughly crumbed before further reduction.

Examples of analytical sample preparations

1.Nuts.

- Batches of nuts should be ground separately with a pestle and mortar, then mixed together thoroughly in a bowl.
- # An analytical portion should be taken for inorganic analyses and the remaining mixture should be homogenized mechanically for further analyses.

2. Eggs: - Fresh. Fresh eggs should be shelled and mixed briskly with a fork; after analytical portions are taken for inorganic analyses, the remainder is homogenized mechanically.

- Dried. Dried eggs should be handled as flour.

3. Fruit.

- ✓ Large fruits (e.g. pineapples or watermelons) and medium-sized ones (e.g. apples) must be quartered.
- ✓ Small fruits (e.g. cherries) should be quartered by the method used for particulate foods.
- ✓ Quarters should be coarsely chopped and combined, and unhomogenized analytical portions should be taken for immediate vitamin C and inorganic analyses.
- ✓ The remaining mixture can then be homogenized to produce an analytical sample for other analyses.

4. Meats and fish (raw, cooked and processed).

- ✓ The fat and muscle of some meats are more conveniently analysed separately and the results combined to produce the final values.
- ✓ The edible portion of each unit is chopped coarsely with a sharp knife (fish is flaked with a fork) and mixed thoroughly in a bowl with a spatula.
- ✓ A portion is removed, frozen and crushed in a polythene bag, and used for inorganic analyses.
- ✓ The remainder of the analytical sample is minced and mixed thoroughly again; portions are taken for further analyses.
- ✓ Care must be taken to avoid fat separation during mixing

5. Leafy vegetables and vegetable inflorescences.

- ✓ Small leafy vegetables should be mixed together in a bowl, chopped coarsely and mixed again briefly.
- ✓ A large portion should be taken for inorganic analysis and another portion into metaphosphoric acid for vitamin C analysis.
- ✓ Large tight-leaved vegetables (e.g. cabbage, iceberg lettuce) must be quartered.
- ✓ " All large leafy vegetables must be chopped coarsely and mixed, and this must be done very quickly "
- ✓ After the mixing, analytical portions should be taken for analyses of vitamin C, vitamin A, carotenes, vitamin E and inorganic nutrients

- ✓ The remainder can be chopped further. Stalks are often difficult to reduce and may have to be chopped separately and reintegrated into the food sample.

6. Prepared composite foods and dishes.

- ✓ This is the form in which most foods are consumed.
- ✓ Items should be briefly homogenized, carefully mixed, then rehomogenized.
- ✓ It can be assumed that laboratory homogenization will not introduce any contamination greater than that arising during domestic or commercial food preparation.
- ✓ Care is required to blend in the individual pieces of muscle, fat, vegetables, etc., which may be found in mixed prepared foods.
- ✓ Portions for vitamin C assay are best taken from the mixed homogenate before it is rehomogenized.
- ✓ If the prepared foods are hot, speed is essential to prevent moisture loss.
- ✓ Total meals or diets can be handled in the same way.

LEARNING UNIT.2 Culture Microorganisms (total count; yeast and molds and total coli form.)

L.O.2.1. Inoculate plates

Content/Topic 1,Key concept:

- **Culture Medium**: Nutrients prepared for microbial growth
- **Inoculum:** Suspension of microorganisms
- **Inoculation:** Culture Media Introduction of microbes into culture medium
- **Culture:** Microbes growing in/on culture medium
- **A pure culture** contains only one species or strain
- **Mixed culture** contains several species
- **Contaminated culture** contains unwanted species of organisms
- **Colony:** visible growth of microbes on the surface of a solid medium Culture Media
- **A colony** is a population of cells arising from a single cell or spore or from a group of attached cells
- **A colony** is often called a colony-forming unit (CFU)

Content/Topic 2.Growth Media

- ✓ **Growth Media**

To study bacteria and other microorganisms, it is necessary to grow them in controlled conditions in the laboratory. Growth media contain a variety of nutrients necessary to sustain the growth of microorganisms.

There are two commonly used physical forms of growth media:

- ✓ liquid media and
- ✓ solid growth media.

A liquid medium is called a **broth**.

Solid growth media usually contains **agar**, which is a mixture of polysaccharides derived from **red algae.**

Agar is used as a solidification agent because it:

(1) is not broken down by bacteria,

(2) contains no nutrients that can be used by bacteria and

(3) melts at high temperatures, and yet is solid at temperatures used for most bacterial growth.

Solid growth media is used in the following forms:

- ✓ agar plates,
- ✓ agar slants and
- ✓ agar deeps.

To make agar deeps or agar slants, melted agar is poured into a test tube and then allowed to solidify vertically (agar deep), or at a slant (agar slant). Agar plates are made by pouring melted agar into a **petri dish**.

Broths can be used to determine growth patterns in a liquid medium, and for certain types of inoculations and metabolic tests. They are also the method of choice for growing large quantities of bacteria. **Agar slants** are commonly used to generate stocks of bacteria. **Agar plates** can be used to separate mixtures of bacteria and to observe colony characteristics of different species of bacteria. **Deeps** are used for several different types of differential metabolic tests (e.g., the gelatinase test).

Growth media can be categorized based on their chemical constituents, or the purpose for which they are used:

- ✓ **Complex growth media** contain ingredients whose exact chemical composition is unknown (e.g. blood, yeast extract, etc.).
- ✓ **Synthetic (also called chemically defined**) growth media are formulated to an exactly defined chemical composition.
- ✓ **A general purpose growth medium** (e.g. tryptic soy agar (TSA) or Luria broth (LB) is used to grow a wide variety of non-fastidious bacteria. This type of medium is often a complex growth medium.
- ✓ **A selective growth medium contains** chemicals that allow some types of bacteria to grow, while inhibiting the growth of other types. An example of a purely selective growth medium is PEA, phenyl ethyl alcohol agar, which allows Gram positive bacteria to grow while inhibiting the growth of Gram negative bacteria.
- ✓ **A differential growth medium** is formulated such that different types of bacteria will grow with different characteristics (e.g. colony color). An example of a differential growth medium is **blood agar,** which differentiates among bacteria based on their ability to break down red blood cells and hemoglobin. Blood agar is also a complex growth medium because it contains blood.

A growth medium can be both selective and differential. For example, EMB (eosin methylene blue agar) inhibits the growth of Gram positive bacteria. Gram negative bacteria that grow on this medium are differentiated based on their ability to ferment the sugars lactose and sucrose.

Note: The Gram staining procedure divides bacteria into 2 main groups: Gram-positive bacteria and Gram-negative bacteria, based on their cell wall structure.

Characteristics of Bacterial Growth

Even on general purpose growth media, bacteria can exhibit characteristic growth patterns. On agar plates, bacteria grow in collections of cells called **colonies**. Each colony arises from a single bacterium or a few bacteria. Although individual cells are too small to be viewed, masses of cells can be observed. Colonies can have different **forms, margins**, **elevations** and **colors**. Observing colony characteristics is one piece of information that microbiologists can use to identify unknown bacteria.

- ✓ **Inoculation** is the purposeful introduction of bacteria into a **sterile** growth medium. A material is sterile when it has no living organisms present; **contamination** is the presence of unwanted microorganisms.
- ✓ **Aseptic techniques** are practices that prevent the contamination of growth media. When working in a microbiology laboratory, you must always remember that bacteria are present on all surfaces in the lab, as well as on your own hands and clothing. Aseptic techniques are designed to prevent the transfer of bacteria from the surrounding environment into a culture medium. These techniques require care and concentration. Pay attention to what you are doing at all times!

Aseptic techniques include the following practices:

1. Minimize the time that cultures and growth media are open to the environment.
2. Disinfect the work area before and after use.
3. Do not touch or breathe into the sterile culture media or the stock cultures.
4. Loops, needles, pipets, etc. should be sterilized before they are used.
5. When working with tubes, the tube caps should not be placed on the table top; they should be held in your hand while inoculating.
6. When removing the caps from test tubes, flame the lip of the test tube after the cap is removed. This heats the air inside the tube, so the air moves out of the tube, preventing contaminants from entering the tube.
7. Information about the use of the Bunsen burner can be found in the General Introduction in the Lab Manual.

Content /Topic 2.The 5I's of culturing Microbes

A**. Inoculation:** the introduction of a sample into a container of media to produce a culture of observable growth

B. **Incubation:** Under conditions that allow growth

C**. Isolation:** Separating one species from another

Isolation techniques include:

- ✓ Streak plate technique
- ✓ Pour plate technique and
- ✓ Spread plate technique

D. **Inspection**: Inspection of growth characteristics

The cultures are observed macroscopically for obvious growth characteristics (color, texture, size) that could be useful in analyzing the specimen contents. Slides are made to assess microscopic details such as cell shape, size, and motility. Staining techniques may be used to gather specific information on microscopic morphology

E**. Identification**.

How does one determine what sorts of microorganisms have been isolated in cultures?

Certainly the combination of microscopic and macroscopic appearance can be valuable in differentiating the smaller, simpler procaryotic cells from the larger, more complex eukaryotic cells.

However, bacteria are generally not identifiable by these methods because very different species may appear quite similar. For them, we must include techniques that characterize their cellular metabolism. These methods, called **biochemical tests**, can determine fundamental chemical characteristics such as nutrient requirements, products given off during growth, presence of enzymes, and mechanisms for deriving energy.

A major outcome is to pinpoint an isolate down to the level of species. Summaries of accumulated data are used to develop profiles of the microbe or microbes isolated from the sample. Information can include relevant characteristics already taken during inspection or additional tests that further describe and differentiate the nature of microbes isolated. Other types 39 of specialized tests include biochemical tests to determine

- **Inoculation:**

The three main aims of inoculation of media are:

- to grow only the organism or organisms that are present in the original sample and not introduce any adventitious agent (aseptic technique)
- to protect yourself, your co-workers and the environment from any pathogenic microbes (aseptic technique and safety rules)
- to grow the microbes in such a way that further testing can be performed on the isolates (growth conditions and testing requirements).

General Procedure for inoculating media

1. Sterilize an inoculating loop or needle in the flame of a Bunsen burner. The portion of the loop or needle that will contact the stock culture or the growth medium must turn bright orange for effective sterilization. For the most rapid sterilization, place the loop at the top of the inner blue cone of flame—this is where the temperature of the Bunsen burner is the hottest. Remove the loop from the flame after it is properly heated- keeping the loops in the flame for too long will eventually cause them to crack.

2. If you are picking a colony from a plate, cool the inoculating loop on agar that does not contain any bacterial colonies.

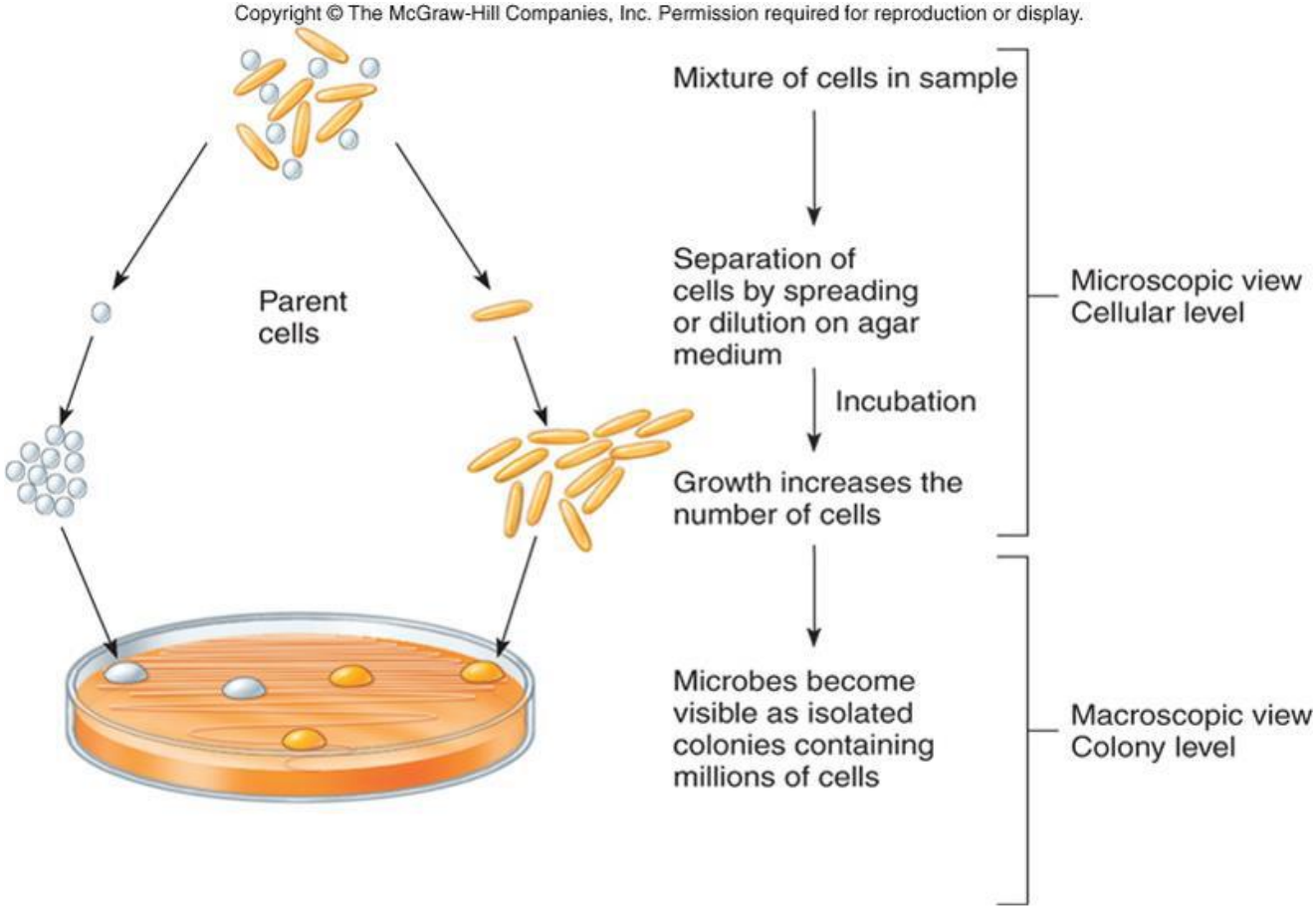
3. Pick a small amount of bacteria (you do not need much). If you are inoculating a tube of broth or an agar slant, remove the cap of the tube (do not set the cap down on the table) and flame the lip of the tube. Throughout the procedure, hold the tube at an angle to reduce the probability of particles entering the opening. Insert the loop into the tube and transfer bacteria to the growth medium. Be careful that only the sterilized part of the loop touches the tube or enters the growth medium.

4. Flame the lip of the test tube before replacing the cap.

5. Sterilize the inoculating loop again.

- **Isolation**

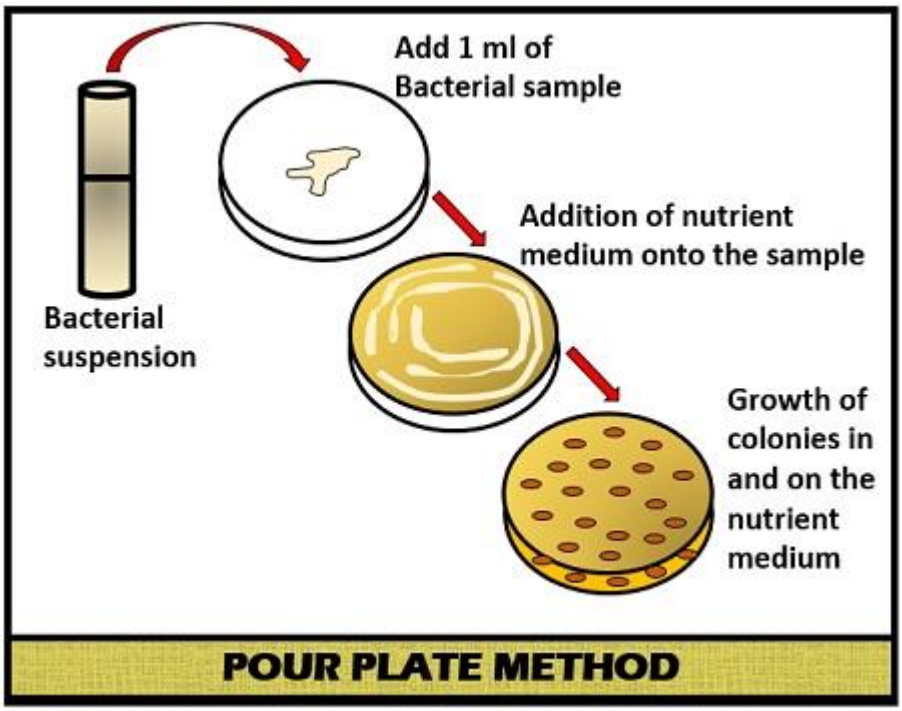
- If an individual bacterial cell is separated from other cells and has space on a nutrient surface, it will grow into a mound of cells -a colony.
- A colony consists of one species.



Isolation techniques include:

- ✓ streak plate technique
- ✓ pour plate technique
- ✓ spread plate technique

A. Pouring Method: Pouring is a simple method for the separation of bacteria. In the pouring method, the bacterial suspension which carries the huge bacterial population is taken.



By the help of pipette, take 1ml of a bacterial sample into the sterile Petri plate. For the growth of bacteria, there should be some nutrient source like carbon and nitrogen. Therefore, the most common Agar nutrient medium is firstly prepared and then added to the Petri plates containing the bacterial sample.

For the uniform distribution of the sample and the media, rotate the plates in the clockwise and anticlockwise direction. Before keeping the Petri plates in the incubator, allow the culture plates to solidify.

For the proper growth of bacteria, keep the culture plates in the incubator at the temperature of 35-37 degrees Celsius for the maximum period of 48 hours. After the incubation period of 24-48 hours, we can see the growth of bacterial colonies.

In the pouring method, the isolation of bacteria becomes difficult because of suspended growth of bacteria in the solid media. Some bacteria appear on the surface of the solid nutrient medium, and some appear under the surface of a solid nutrient medium.

In the pouring method, there is an overgrowth of bacterial colonies from which the isolation of pure culture is very difficult. This technique is least preferred for the separation of pure culture.

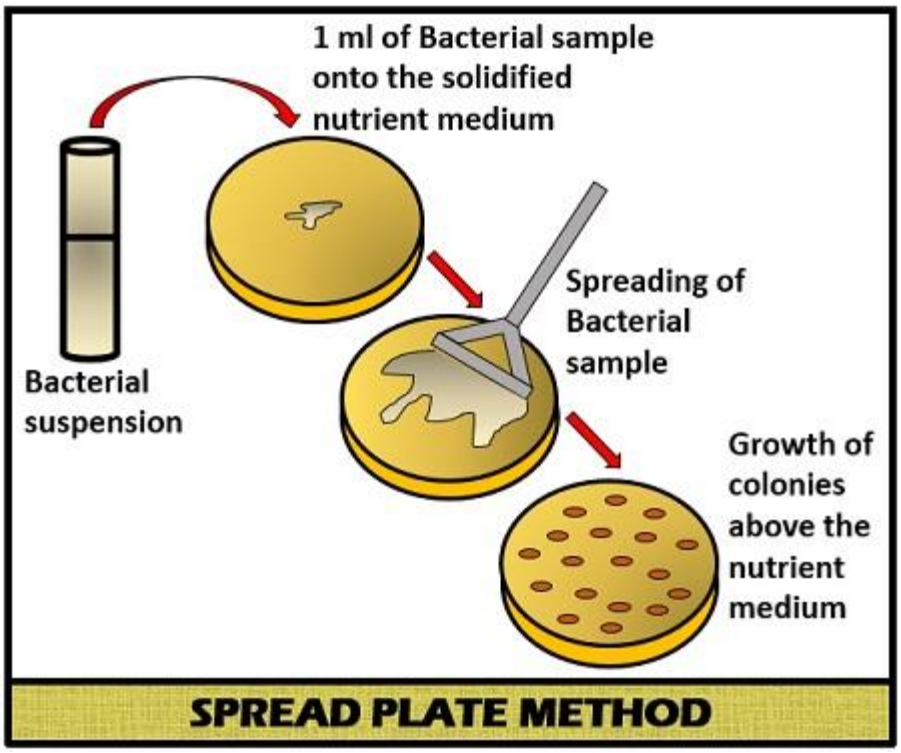
The procedures of pour plate techniques used in preparing culture media

1. prepare the dilution of the test sample expected to contain between 30-300 CFU/ ml.
2. Inoculate labeled empty petri dish with specified ml (0.1 0r 1.0ml)
3. Collect one bottle of sterile molten agar (containing 15ml of melted plate count agar or any other standard culture medium from the water bath about 45°C
4. Hold bottle in right hand, remove the cap with little finger of the left hand
5. Flam the neck of the bottle and replace the cap
6. Lift the lid of the petri dish slightly with the left hand and pour the sterile molten agar into petri dish and replace the lid
7. Flame the neck of the bottle and replace the cap
8. Gently rotate the dish to mix the culture and the medium thoroughly and to ensure that the medium covers the plate evenly. Do not slip the agar over the edge of the petri dish
9. All the agar to completely gel without disturbing it, it will take approximately 10 minutes
10. Seal and incubate the plate in an inverted position at 37°C for 24-48 hours
11. Calculate CFU/ml using the formula: CFU/ml= CFU* dilution factor 81/ aliquot

Aliquot: the volume of diluted specimen, which should be either 0.1 or 1.0ml.

B. Spreading Method

Spreading method is again a very simple method to perform isolation of bacteria. It differs from the pour plate method, add the nutrient medium first before the addition of the bacterial sample.



The nutrient medium is added to the sterile Petri plates and then allowed to solidify. After the solidification of the nutrient media, add 1ml of the bacterial suspension over the surface of solid media.

For the uniform distribution of bacteria over the surface of solid media, take the spreader of T or L-shape to spread the bacterial suspension evenly. After that, incubate the culture plates for 35-37degrees Celsius for 24 to 48 hours.

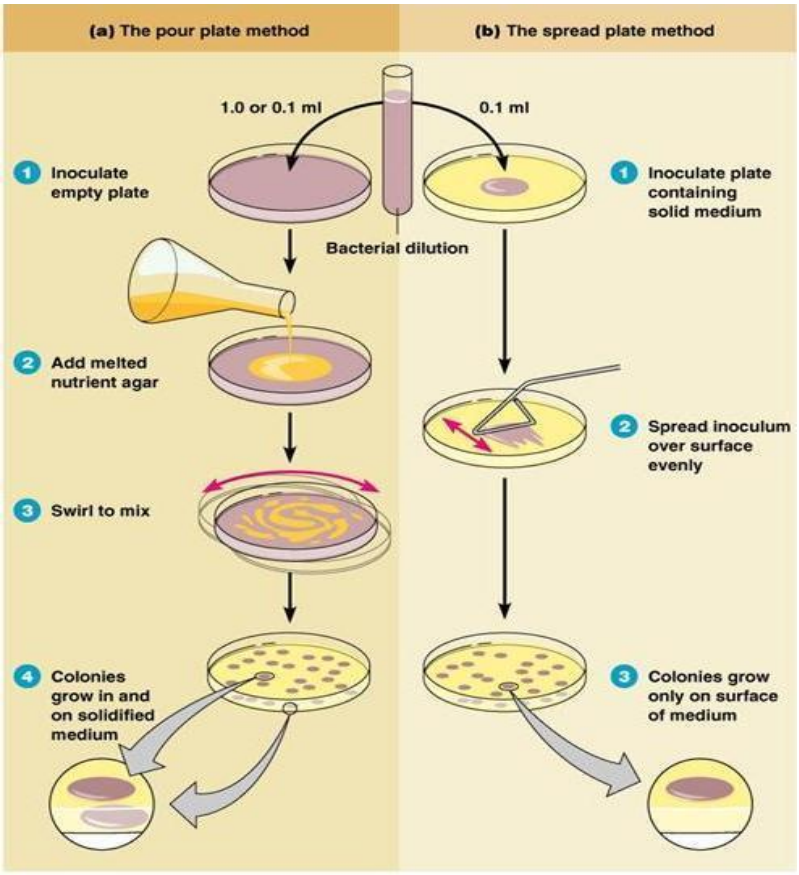
We can see a number of bacterial colonies after the incubation period. In the spreading method, we can select the isolated colonies for the culturing of bacteria. For the isolation of pure culture, the spreading method is not very popular.

Procedure of Spread Plate Technique

1. Make a dilution series from a sample.
2. Pipette out 0.1 ml from the appropriate desired dilution series onto the center of the surface of an agar plate.
3. Dip the L-shaped glass spreader into alcohol.

4. Flame the glass spreader (hockey stick) over a Bunsen burner.
5. Spread the sample evenly over the surface of agar using the sterile glass spreader, carefully rotating the Petridish underneath at the same time.
6. Incubate the plate at 37°C for 24 hours.
7. Calculate the CFU value of the sample. Once you count the colonies, multiply by the appropriate dilution factor to determine the number of CFU/mL in the original sample.

Pour Plates versus Spread Plates



(the volume of diluted specimen (aliquot) is either 0.1 or 1.0 mL)

The difference between pour-plate method and spread-plate method are as follows:-

1.Procedure:

For pour plate-

- Inoculum from a sample is placed in the center of sterile Petri dish using a sterile pipette. Molten cooled agar (approx. 15mL) is then poured into the Petri dish containing the inoculum.

For spread plate-

- Inoculum from a sample is pipette out and spread evenly on sterile nutrient Agar by spreader.

2.Amount of Inoculum:

For pour plate*:*

- Inoculum is more i.e **1ml**

For spread plate-

- Inoculum is less i.e. **0.1ml**

3. Growth of colonies:

For pour plate-

- Colonies grow in and on solidified medium.

For spread plate-

- Colonies grow only on surface of medium.

4 Mixing of inoculum and medium:-

For pour plate-

- After pouring molten agar on inoculum the plate is gently swirl.

For spread plate-

- Inoculum is spread on surface of medium (agar) by sterile glass rod spreader.

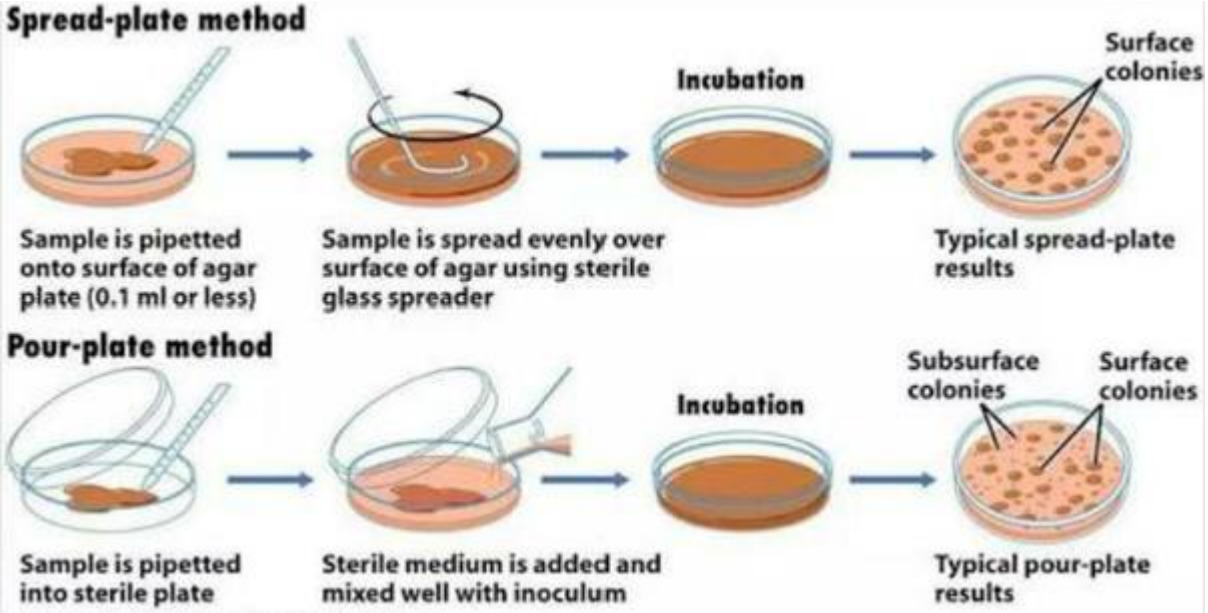
5.Surface area covered by sample:-

In pour plate-

- More surface area is covered as the sample is spread throughout the media.

In spread plate-

- Sample is spread only to a limited area i.e. only on the surface of agar.



6. Uses: -

For pour plate-

- It is used to determine CFU/ml or PFU/ml.

For spread plate-

- It is used to isolate specific clonal colonies.

7.Advantage: -

Pour plate-

Spread plate-

1. Picking surface colony will not interrupt other colonies by digging out of agar.
2. Get even distribution of colonies.

8. Disadvantage: -

For pour plate-

1. Picking subsurface colonies can interrupt other colonies by digging out of agar.

2. Don't get even distribution of colonies.

For spread plate-

1. It doesn't allow growth of microaerophilic.
2. It doesn't allow growth of obligate anaerobes.

9.Benefits:

For pour plate-

- It is beneficial to isolate certain bacteria which are motile and don't grow as colony.

For spread plate-

- It is beneficial for isolation of bacteria from soil or water.

10.Application: -

For pour plate-

- This technique is used in Bacteriologic examination of milk, bacterial number of urine to signify urinary tract infection.

For spread plate-

- This technique used in enrichment, selection and screening experiment.

10.Prevention: -

1. ***Aseptic condition must be there:***

For pour plate-

- As contamination can occur on medium when adding the dilution of bacteria to it.

For spread plate-

- As contamination can occur when spreading the bacteria on surface of medium.

2. ***While handling agar and alcohol:***

For pour plate-

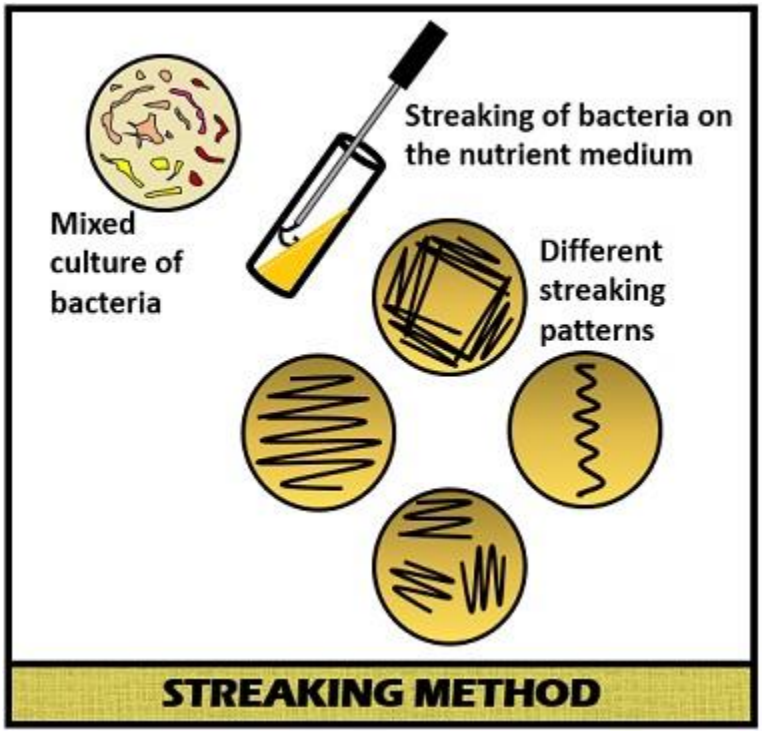
- Some organisms may die if the agar is too hot.

For spread plate-

- Care should be taken while sterilizing glass spreader

C. Streaking Method

Streaking method is very popular and the most widely used method for the isolation of pure culture. In this take the nutrient medium into the sterile Petri plates and allow it to solidify.



After that take the inoculating loop and sterilize it on the flame until it gets red hot. Then, take the inoculum by the help of sterilized inoculating loop and streak over the solid nutrient media by keeping the plate close to the flame, to avoid contamination. After streaking of bacteria, incubate the culture plates for 24-48 hours at a temperature of 35-37degrees Celsius in the incubator.

In the streaking method, there is a limited population of bacteria, from which the isolation of pure culture is quite easier than the pour plate and the spread plate method. In this, one can separate the individual colony of bacteria for the isolation and culturing of bacteria

Procedure

Quadrant Streaking for isolation into pure culture

1. Sterilize the inoculating loop in the bunsen burner by putting the loop into the flame until it is red hot. Allow it to cool.

2. Pick an isolated colony from the agar plate culture and spread it over the first quadrant (approximately 1/4 of the plate) using close parallel streaks or Insert your loop into the tube/culture bottle and remove some inoculum. You don't need a huge chunk.

3. Immediately streak the inoculating loop very gently over a quarter of the plate using a back and forth motion

4. Flame the loop again and allow it to cool. Going back to the edge of area 1 that you just streaked, extend the streaks into the second quarter of the plate **(area 2).**

5. Flame the loop again and allow it to cool. Going back to the area that you just streaked (area2), extend the streaks into the third quarter of the plate **(area 3).**

6. Flame the loop again and allow it to cool. Going back to the area that you just streaked (area3), extend the streaks into the center fourth of the plate **(area 4).**

7. Flame your loop once more.

ADVANTAGES

- ✓ **It is useful for isolating aerobic microorganisms.**
- ✓ **Cultures are never exposed to high melted agar temperature**
- ✓ To produce isolated colonies of an organism (mostly bacteria) on an agar plate.
- ✓ This is useful when we need to separate organisms in a mixed culture (to purify/isolate particular strain from contaminants) or
- ✓ When we need to study the colony morphology of an organism.
- ✓ To identify the organism: biochemical tests to identify bacteria are only valid when performed on pure cultures.

DISADVANTAGES

- ✓ There is a higher probability of contamination prior to isolation.
- ✓ Streaking plate method can be used for qualitative and not quantitative studies because it cannot be used for the enumeration of the approximate number of bacteria in the given sample.
- ✓ The amount of inoculums added is not measured quantitatively
- ✓ Colonies are not formed on streak plate since except in the fourth quadrant

L.O.2.2. Incubate inoculated plates

Incubation is the process of keeping something at the right temperature and under the right conditions so it can develop.

An **incubator** is a device used to grow and maintain microbiological **cultures** or cell **cultures**. The **incubator** maintains optimal temperature, humidity and other conditions such as the CO_2 and oxygen content of the atmosphere inside.

- ✓ **Incubation conditions:**
  - Temperature
  - Aerobic or anaerobic oxygen
  - Time
  - Humidity

Notes about Labeling and Incubating Plates

1. Always label your plates/tubes BEFORE you do your inoculations. You can use Sharpies on the plates, but wax markers ONLY on tubes. When labeling tubes, label the tube itself—don't label the cap!

2. Make sure you label the **bottom** of the plates (the part of the plate that holds the agar).

3. Place plates inverted (upside down) for incubation. This prevents condensation from falling on the surface of the agar and disrupting the streaking pattern

Learning unit 3. Measure microorganisms in food

L.O.3.1.Identify colonies

Content/Topic1. Characteristics of colonies morphology

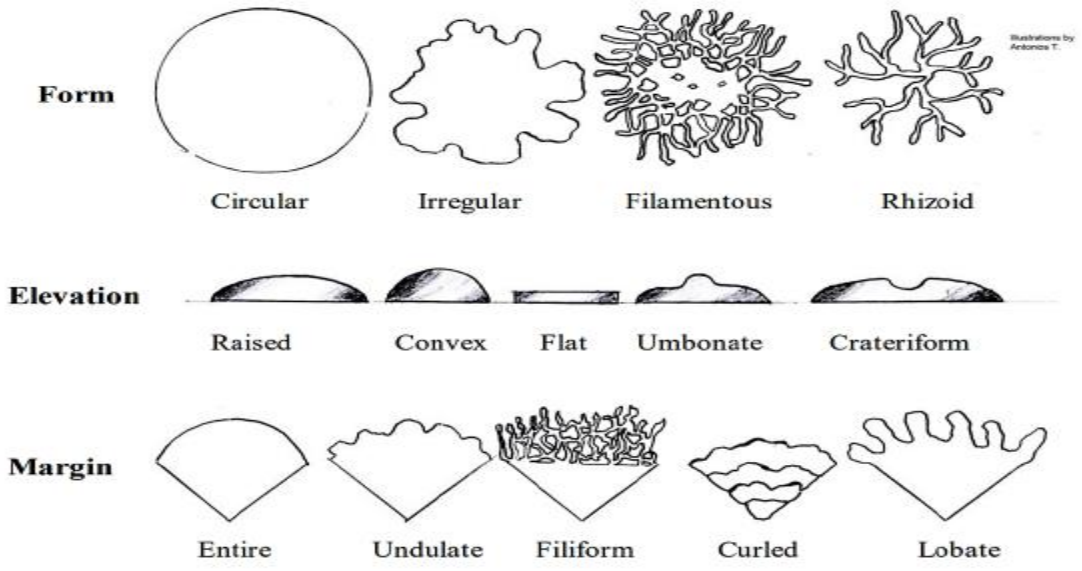
These are the characteristics used to accurately and consistently describe the morphology of a bacterial colony:

- Size.
- Shape.
- Color (also known as pigmentation)
- Texture.
- Height (a.k.a. elevation)
- Edge (a.k.a. margin)

1.Colony Shape: It includes form, elevation and margin of the bacterial colony.

- ✓ **Form of the bacterial colony:** – The form refers to the shape of the colony. These forms represent the most common colony shapes you are likely to encounter. e.g. Circular, Irregular, Filamentous, Rhizoid etc.
- ✓ **Elevation of bacterial colony:** This describes the "side view" of a colony. These are the most common. e.g. **Flat, raised, umbonate (having a knobby protuberance), Crateriform, Convex, Pulvinate (Cushion-shaped)**

- ✓ **Margin of bacterial colony:** The margin or edge of a colony may be an important characteristic in identifying an organism. Common examples are **Entire (smooth), irregular, Undulate (wavy), Lobate, Curled, Filiform** etc. Colonies that are irregular in shape and/or have irregular margins are likely to be motile organisms. Highly motile organism swarmed over the culture media. Such as Proteus spp.



2.Size of the bacterial colony: The size of the colony can be a useful characteristic for identification. The diameter of a representative colony may be measured in millimeters or described in relative terms such as pin point, small, medium, large. Colonies larger than about 5 mm are likely to be motile organisms.

3.Appearance of the colony surface: Bacterial colonies are frequently shiny and smooth in appearance. Other surface descriptions might be: dull (opposite of glistening), veined, rough, wrinkled (or shriveled), glistening

4.Consistency/Texture: Several terms that may be appropriate for describing the texture or consistency of bacterial growth are:

- ✓ Dry
- ✓ Moist
- ✓ viscid (sticks to loop, hard to get off)
- ✓ brittle/friable (dry, breaks apart)
- ✓ mucoid (sticky, mucus-like).

5.Color of the colonies (pigmentation): Some bacteria produce pigment when they grow in the medium e.g., green pigment produces by *Pseudomonas aeruginosa*, buff colored colonies of *Mycobacterium tuberculosis* in **L.J medium**, red colored colonies of *Serratia marcescens.*

6. Density (Opacity) of the bacterial colony: Is the colony transparent (clear), opaque (not transparent or clear), translucent (almost clear, but distorted vision–like looking through frosted glass), iridescent (changing colors in reflected light

7. **Surface (glistening or dull)**

L.O.3.2.Perform colony counting

Content/Topic 1.Tools for counting colonies

Counting colonies is traditionally performed manually using a pen and a click-counter. This is generally a straightforward task, but can become very laborious and time-consuming when

many plates have to be enumerated. Alternatively, semi-automatic (software) and automatic (hardware + software) solutions can be used.

- ✓ Pen and click-counter
- ✓ Software for counting cfus
- ✓ Automated system (MATLAB)
- ✓ Alternative units (Most probable number)

Software for counting CFUs:

Colonies can be enumerated from pictures of plates using software tools. The experimenters would generally take a picture of each plate they need to count and then analyse all the pictures (this can be done with a simple digital camera or even a webcam). Since it takes less than 10 seconds to take a single picture, as opposed to several minutes to count CFU manually, this approach generally saves a lot of time. In addition, it is more objective and allows extraction of other variables such as the size and colour of the colonies.

- **OpenCFU**: is a free and open-source program designed to optimize user friendliness, speed and robustness. It offers a wide range of filters and control as well as a modern user interface. OpenCFU is written in C++ and uses Open CV for image analysis.
- **NICE:** is a program written in MATLAB that provides an easy way to count colonies from images.
- **ImageJ** and **CellProfiler:** Some ImageJ macros and plugins and some CellProfiler pipelines can be used to count colonies. This often requires the user to change the code in order to achieve an efficient work-flow, but can prove useful and flexible. One main issue is the absence of specific GUI which can make the interaction with the processing algorithms tedious.

In addition to software based on traditional desktop computers, apps for both Android and iOS devices are available for semi-automated and automated colony counting. The integrated camera is used to take pictures of the agar plate and either an internal or an external algorithm is used to process the picture data and to estimate the number of colonies

Automated systems:

Many of the automated systems are used to counteract human error as many of the research techniques done by humans counting individual cells have a high chance of error involved.

Due to the fact that researchers regularly manually count the cells with the assistance of a transmitted light, this error prone technique can have a significant effect on the calculated concentration in the main liquid medium when the cells are in low numbers.

Completely automated systems are also available from some biotechnology manufacturers. They are generally expensive and not as flexible as standalone software since the hardware and software are designed to work together for a specific set-up. Alternatively, some automatic systems use the spiral plating paradigm.

Some of the automated systems such as the systems from **MATLAB** allow the cells to be counted without having to stain them. This lets the colonies to be reused for other experiments without the risk of killing the microorganisms with stains. However, a disadvantage to these automated systems is that it is extremely difficult to differentiate between the microorganisms with dust or scratches on blood agar plates because both the dust and scratches can create a highly diverse combination of shapes and appearances.

- **MATLAB** (*matrix laboratory*) is a multi-paradigm numerical computing environment and proprietary programming language developed by MathWorks. MATLAB allows matrix manipulations, plotting of functions and data, implementation of algorithms, creation of user interfaces, and interfacing with programs written in other languages.

Alternative units:

Instead of colony-forming units, the parameters **Most Probable Number (MPN) and Modified Fishman Units (MFU)** can be used. The Most Probable Number method counts viable cells and is useful when enumerating low concentrations of cells or enumerating microbes in products where particulates make plate counting impractical. Modified Fishman Units take into account bacteria which are viable, but non-culturable.

❖ **Manual colony counter**

The primary trick in counting colonies is to count each colony dot once. One approach is to set the Petri dish on a grid background and count the colonies in each grid cell, moving in a

methodical pattern through all of the cells. Marking counted colonies on the back of the Petri dish can also be a helpful approach. Generally, you will need to count at least three plates; only use plates containing 30 to 300 colonies to make robust inferences, suggests the Microbiology Network, a firm that provides consulting services to labs and manufacturers. Plates with colonies that are too numerous to count or with too few colonies need to be re-plated from a new dilution.

- **Automated counting**

Human error adds to the time involved in counting colonies manually. To improve both accuracy and efficiency, place the Petri dish in an automated colony counting device. Automated colony counters take an image of the dish, separate out the colonies from the background and then use an algorithm to count the colonies on the plate. The algorithms can have difficulties differentiating colonies when two or more colonies are touching at the edges, so this is an area of ongoing software development

L.O.3.3. Enumeration of microorganisms in foods

Content/Topic 1. Method for enumerating microorganisms in foods

Enumeration methods for microorganisms can be classified into:

- ✓ **Direct methods** where the numbers of micro-organisms are counted as in the case of Direct Microscopic Count (DMC), Standard Plate Count (SPC), Filtration, Most Probable Number **(MPN)**
- ✓ **Indirect methods** where overall microbial load is correlated to the metabolic activity of the micro-organisms as in dye reduction tests.

1. Direct Microscopic Count (DMC)

- ✓ A specific volume of a bacterial suspension (0.01 ml) is placed on a microscope slide with a special grid.

- ✓ Stain is added to visualize bacteria.
- ✓ Cells are counted and multiplied by a factor to obtain concentration.

Advantages:

• No incubation time required.

Disadvantages:

• Cannot always distinguish between live and dead bacteria.

• Motile bacteria are difficult to count.

• Requires a high concentration of bacteria (10 million/ml) stands for microscopic factor

Precautions recommended during direct microscopic count

Never dust a lens by blowing on it. Saliva will inevitably be deposited on lenses and is harmful, even in minute amounts. Never use facial tissues to clean lenses. They may contain glass filaments which can scratch lenses. Linen or chamois may be used for cleaning but may not be as convenient as lens tissue.

Never leave microscope tubes open. Always keep them closed with dust plug, eyepiece, or objective, as appropriate.

Avoid touching lenses. Even light fingerprints, especially on objectives, can seriously degrade image quality.

Avoid getting immersion liquid on non-immersion objectives; it can damage the lens mounting glue.

Use the following

procedure to properly clean lenses of microscopes or other optical equipment. Crumple a piece of lens tissue to create many folds to trap dirt without grinding it into the lens. Do not touch the part of the tissue that will be applied to the lens; excessive touching transfers natural oils from the fingers to the lens tissue. Apply a small amount of lens cleaning solution to the lens tissue and blot the tissue against absorbent material to prevent

fluid from entering the lens mount. Wipe the lens very lightly to remove gross dirt that was not blown away by the rubber bulb. If necessary, repeat the cleaning process with a new piece of lens tissue and with slightly more pressure to remove oily or greasy residue.

2. Filtration:

- ✓ Used to measure small quantities of bacteria.

• **Example**: Fecal bacteria in a lake or in ocean water.

- ✓ A large sample (100 ml or more) is filtered to retain bacteria.
- ✓ Filter is transferred onto a Petri dish.
- ✓ Incubate and count colonies.

3. Most Probable Number (MPN):

- ✓ Used mainly to measure bacteria that will not grow on solid medium.
- ✓ Dilute a sample repeatedly and inoculate several broth tubes for each dilution point.
- ✓ Count the number of positive tubes in each set.
- ✓ **Statistical method**: Determines 95% probability that a bacterial population falls within a certain range.

4. Standard Plate count (SPC)

- ✓ Most frequently used method of measuring bacterial populations.
- ✓ Inoculate plate with a sample and count number of colonies.
- ✓ Standard Plate Count is carried out to estimate the number of viable bacteria in a food sample.
- ✓ Bacteria growing aerobically in the mesophilic range of temperature are detected in SPC.
- ✓ A high SPC of the food product indicates poor sanitary conditions of the food processing plant and a low SPC indicates the good quality of the processed food in terms of bacterial load, however, SPC does not indicate the absence of pathogens in the finished food product.
- ✓ SPC is based on the serial dilution of the food sample in sterilized dilution blanks followed by plating on the nutrient media.
- ✓ The dilution of sample is required because usually food samples contain a high number of bacteria, which are difficult to count. If undiluted sample is plated, a lawn will form on the plate and the individual isolated colonies that can be counted will not be formed.
- ✓ After serially diluting the representative sample of food, plating is carried out by pour plate or spread plate technique.
- ✓ In pour plate technique, the dilution is added to the sterile petri plate followed by the addition of molten nutrient agar.

- ✓ The diluent and agar are mixed and plates are incubated after solidification for the growth of bacterial colonies. Pour plate technique is the preferred technique for carrying out SPC, however at times, spread plate technique which involves the spreading of the dilution on the surface of solidified nutrient media may also be used. The SPC of the food sample is reported as colony forming units per ml(cfu/ml).
- ✓ The term colony forming unit is used because the bacterial colony obtained on the agar plate may not be produced by a single bacteria but bacterial clumps may also give rise to the colony hence cfu/ ml and not cells/ ml is used for reporting

Assumptions:

• Each colony originates from a single bacterial cell.

• Original inoculum is homogeneous.

• No cell aggregates are present.

Advantages:

• Measures **viable** cells

Disadvantages:

• Takes 24 hours or more for visible colonies to appear.

• Only counts between 25 and 250 colonies are accurate.

• Must perform **serial dilutions** to get appropriate numbers/plate

A. Pour Plate:

- ✓ Introduce a 1.0 or 0.1 ml inoculum into an **empty** Petri dish.
- ✓ Add liquid nutrient medium kept at 50^{o}C.
- ✓ Gently mix, allow to solidify, and incubate.

Disadvantages:

• Not useful for heat sensitive organisms.

• Colonies appear under agar surface.

B. Spread Plate:

- ✓ Introduce a 0.1 ml inoculum onto the **surface** of Petri dish.
- ✓ Spread with a sterile glass rod.

Advantages: Colonies will be on surface and not exposed

Serial dilution is a technique that is used to produce very dilute solutions without the necessity of measuring very small quantities of liquids. It is a series of stepwise dilutions, in

which one first dilutes a solution, then dilutes the dilution, then dilutes the dilution of the dilution and so forth. The dilution factor at each step is usually constant, resulting in a geometric progression of concentration. An example of a serial dilution is seen below. In this example, each dilution is a 10-fold dilution (transferring 1 ml into 9 ml of H2O results in a 1/10 dilution; i.e., 1 ml in a total volume of 10 ml).

Serial dilutions are often used in standard plate counts because the number of bacteria in a sample (water, food, or a medical sample such as a urine or a fecal sample) is unknown. The sample is diluted to obtain a number of **CFUs** that supplies statistically significant results, yet is still easily countable. The general recommendation for a countable plate is between **30 – 300 CFUs/plate**. After dilutions are prepared, a set amount of liquid (typically between 0.1- 1 ml) is spread out over the surface

of an agar plate, and then incubated to allow for bacterial growth.

CFU counts from these diluted plates are used to calculate the number of bacterial cells/ml in your original (undiluted) sample.

Example:

Calculate the number of bacteria (CFU) per milliliter or gram of sample by dividing the number of colonies by the dilution factor The number of colonies per ml reported should reflect the precision of the method and should not include more than two significant figures.

The CFU/ml can be calculated using the formula:

cfu/ml = (no. of colonies x dilution factor) / volume of culture plate

For example, suppose the plate of the 10^{6} dilution yielded a count of 130 colonies. Then, the number of bacteria in 1 ml of the original sample can be calculated as follows:

Bacteria/ml = (130) x (10^{6}) = 1.3×10^{8} or 130,000,000.

CFU/mL Practice Problems - CFU/mL Calculation Examples

Problem 1:

Five ml of Bacterial Culture is added to 45 ml of sterile diluent. From this suspension, two serials, 1/100 dilutions are made, and 0.1 ml is plated onto Plate Count Agar from the last dilution. After incubation, 137 colonies are counted on the plate. Calculate CFU/mL of the original Sample?

Answer:

First thing we need to know is the Dilution Factor, or how much the original sample is diluted:

here Initially 5mL in 45mL = Final Volume / Sample volume = 50/5 = 10.

Then two serial dilutions of 1/100.

Total Dilution Factor = 10 * 100 *100 = 10^5

CFU/mL = cfu/ml = (no. of colonies x dilution factor) / volume of culture plate

= (137 * 10^5)/0.1

=1.37*10^8

So Total colony forming units = 1.37*10^8 CFU/mL

Converting CFU/mL to Log value

For example,

Total colony forming units = 1.37*10^8 CFU/mL and you want to convert it into Log value,

Just take Log(CFU/mL)

Here, log (1.37*10^8) = 8.1392492

To work the problem, you need 3 values---a colony count from the pour or spread plates, a dilution factor for the dilution tube from which the countable agar plate comes, and the amount of the dilution that was plated on the agar plate.

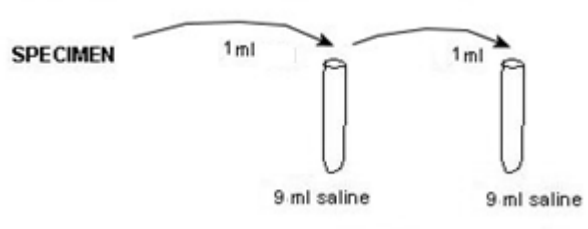
STEP 1: Determine the appropriate plate for counting

Look at all plates and find the one with 30-300 colonies (or plaques), preferably. Greater than 300 and less than 30 is a high degree of error. Air contaminants can contribute significantly to a really low count and a high count can be confounded by error in counting too many small colonies. Use the total dilution for the tube from where the plate count was obtained. If duplicate plates (with same amount plated) have been made from one dilution, average the counts together.

STEP 2: Determine the total dilution for the dilution tubes

Dilution = amount of specimen transferred divided by the [amount of specimen transferred + amount already in tube].

Determine the dilution factor for each tube in the dilution series. Multiply the individual dilution of the tube **X** previous total dilution. **To calculate this dilution series:**



Dilution factor for each tube in a set = $\frac{\text{amount of sample}}{\text{amount of sample + amount of diluent in tube}}$

But after the first tube, each tube is a dilution of the previous dilution tube.

Total dilution factor = previous dilution of tube **X** dilution of next container

Examples for above dilution series

1 ml added to 9ml = 1/10 for 1st tube

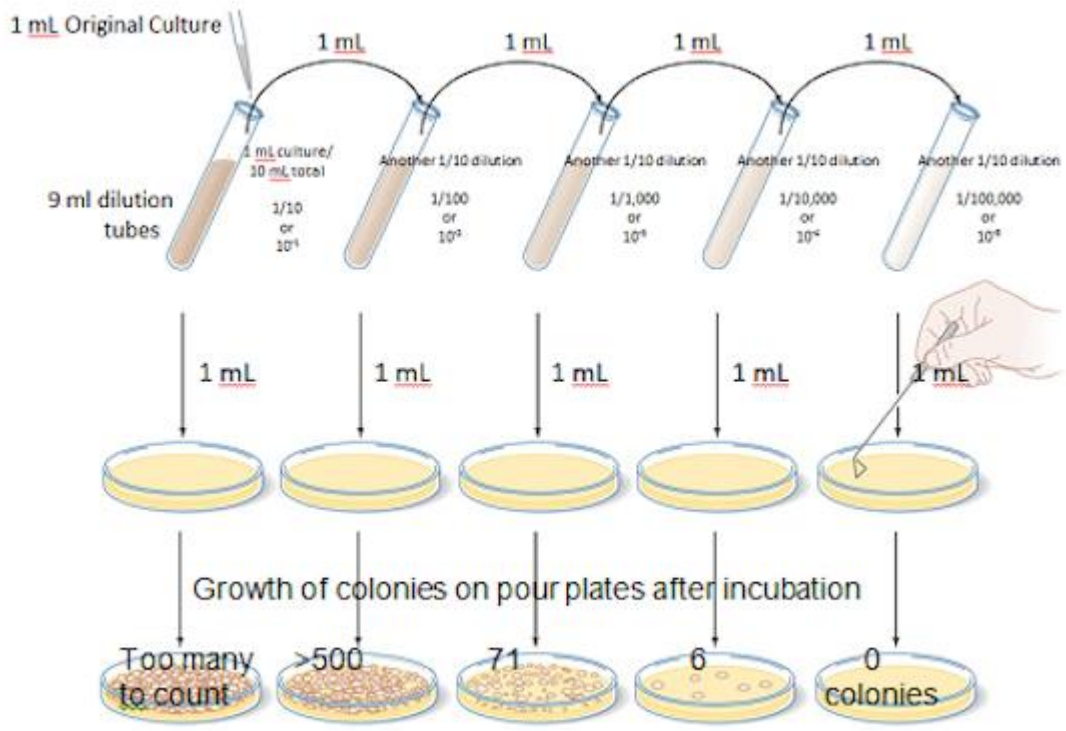
1ml added to 9ml = 1/10 for 2nd tube

previous dilution of 1/10 (1st tube) **X** 1/10 (2nd tube) = total dilution of 1/100 ($=10^{-2}=1/10^{2}$)

STEP 3: Determine the amount plated

The amount plated is the amount of dilution used to make the particular pour plate or spread plate.

There is nothing to calculate here: the value will be stated in the procedure, or it will be given in the problem.



Solving above problems

1. The countable plate is the one with 71 colonies.
2. The total dilution of 3rd tube from which above pour plate was made = 1/10 **X** 1/10 **X** 1/10 = $1/10^3$
3. The amount used to make that pour plate = 1ml

71 colonies

_____ = 71 **X** 10^3 = 7.1 **X** 10^4 (scientific notation) **OR** 71,000/ml

$1/10^3$ **X** 1

Note: Rules for scientific notation

For a number to be in correct scientific notation, the following conditions must be true:

1. The **coefficient** must be greater than or equal to 1 and less than 10.
2. The base must be 10.
3. The exponent must show the number of decimal places that the decimal needs to be moved to change the number to standard notation. A negative exponent means that the decimal is moved to the left when changing to standard notation.

Precautions:

1) The sample taken for analysis should be representative of the whole stock. If a sample taken for analysis is small, it will give erroneous results.

2) Blending of the sample should be done carefully so as to prepare homogeneous suspension.

3) Dilutions should be mixed properly before each transfer.

4) Molten agar should not be very hot (40-42O C is optimum temperature) at the time of pouring.

5) Plates should be incubated in an inverted position so as to avoid moisture condensation and consequently lawn formation on the surface of agar. 6) Diluent taken may differ with the type of food product. Sodium citrate (2%) may be used for fatty foods such as cheese. Normal saline, buffers or even distilled water may also be used as diluent.

2. Indirect Methods of Measurement

1. Turbidity:

- ✓ As bacteria multiply in media, it becomes turbid.
- ✓ Use a spectrophotometer to determine % transmission or absorbance.
- ✓ Multiply by a factor to determine concentration.

Advantages:

• No incubation time required.

Disadvantages:

• Cannot distinguish between live and dead bacteria.

• Requires a high concentration of bacteria (10 to 100 million cells/ml).

2. Metabolic Activity:

- ✓ As bacteria multiply in media, they produce certain products:
- Carbon dioxide
- Acids
- ✓ Measure metabolic products.
- ✓ Expensive

3. Dry Weight:

- ✓ Bacteria or fungi in liquid media are centrifuged.
- ✓ Resulting cell pellet is weighed.
- ✓ Doesn't distinguish live and dead cells.

The Factors affecting microbial count

The following are useful factors that affect microbial count:

(1) The sampling method used.

(2) The distribution of the microorganisms in the food sample.

(3) Nature of the food microflora.

(4) Nature of the food material biological component.

(5) Nutritional status of the culture media used.

(6) Incubation temp and the time used for the test.

(7) The pH, H20-activity and redox-potential of the medium.

(8) Extrinsic factor; - temperature, water vapor, partial pressure of gases of incubation.

Content/ Topic 2 . Preparation of Bacterial Smears and Gram Staining

Key Terms: Microbial smear**,** stain (dyes), Gram stain, bacterial smear, simple stain, differential stain, Gram positive, Gram negative, Gram variable, capsule, spirochete, flagella, negative staining, silver stain

Microbial smear: It is a very small amount of microbial growth (broth or solid) spreaded on a clean slide and drying by air.

Fixation: The process of passing the smear after drying several times over benzene burner to fix the microbes on slide and prepare it for staining.

Note: The reason of fixation process is to kill the microbes, fix the microbe cells to the slide and prevent their removal during washing steps.

Steps of microbial smear preparation:

1.Handle a clean slide by its edge, label the target place at the bottom side o the slide by drawing a circle with a diameter about 2 cm using a marker.

2.Sterile the loop until reaching the red heat.

3.If the bacterial culture was broth, shake the culture and transfer loopful of broth to the center of the slide and spread over the target circle. While if the bacteria were grown on solid medium, place loopful of water on the slide then transfer inoculums to the water and homogenize the smear.

4.Sterile the loop.

5.Leave the smear to dry at room temperature (by air) .

6.After drying, Pass the slide over the flame to fix the smear (avoid prolonged heating of the slide)

Note: It is preferred that the microbial culture used in the staining process is a recent one, since the old one gives false results.

Staining is any procedure that applies colored chemicals called **dyes** to specimens. Dyes impart a color to cells or cell parts by becoming affixed to them through a chemical reaction.

In general, they are classified as:

1.Basic dyes (cationic dye): these dyes are positively charged because the chromophore is the positive ion, so they attract to the bacterial cell wall which is negatively charged , e.g: methylene blue, safranine , crystal violet , malachite green

2, Acidic dyes (anionic dyes): these dyes are negatively charged because the the chromophore is the negative ion, so they not attract to the bacterial cell wall which is negatively charged , therefore they do not stain the cell , this process is called " **negative staining** "

E.g : nigrocin , india ink , erocin , rose Bengal These stains do not penetrate the bacterial cell wall but they make the background around the cells dark or opaque, so these stains show the shape

and size of cells and the extracellular structures such as capsule or flagella

Negative Versus Positive Staining

Two basic types of staining technique are used, depending upon how a dye reacts with the specimen. Most procedures involve a **positive stain,** in which the dye actually sticks to the specimen and gives it color. A **negative stain,** on the other hand, is just the reverse (like a photographic negative). The dye does not stick to the specimen but settles around its outer boundary, forming a silhouette. In a sense, negative staining —stains‖ the glass slide to produce a dark background around the cells. **Nigrosin** (blue-black) and **India ink** (a black suspension of carbon particles) are the dyes most commonly used for negative staining.

The cells themselves do not stain because these dyes are negatively charged and are repelled by the negatively charged surface of the cells.

The value of negative staining is its *relative simplicity* and the *reduced shrinkage* or distortion of cells, as the smear is not heat-fixed. A quick assessment can thus be made regarding cellular size, shape, and arrangement. Negative staining is also used to accentuate the capsule that surrounds certain bacteria and yeasts.

Simple versus Differential Staining

Positive staining methods are classified as simple, differential, or special. Whereas **simple stains** require only a single dye and an uncomplicated procedure, **differential stains** use two different-colored dyes, called the *primary dye* and the *counterstain,* to distinguish between cell types or parts. These staining techniques tend to be more complex and sometimes require additional chemical reagents to produce the desired reaction.

Most simple staining techniques take advantage of the ready binding of bacterial cells to dyes like malachite green, crystal violet, basic fuchsin, and safranin. Simple stains cause all cells in a smear to appear more or less the same color, regardless of type, but they reveal such bacterial characteristics as shape, size, and arrangement.

Simple stains: In this process only one stain is used for staining. The most commonly used stains for simple staining are crystal violet, methylene blue , carbol fuchsia. This method of staining is useful determining basic morphology and the presence or absence of certain kinds of granules.

Steps of simple staining:

1.Prepare a fixed smear .

2.Stain the smear with crystal violet by putting a couple of the stain drops and let for 1 min.

3.Wash off by tap water gently, leave it to dry at room temperature (by air) .

4.Put one drop of cedar oil on the fixed smear , examine directly under oil immersion lens .

Types of Differential Stains

A satisfactory differential stain uses differently colored dyes to clearly contrast two cell types or cell parts. Common combinations are red and purple, red and green, or pink and blue. Differential stains can also pinpoint other characteristics, such as the size, shape, and arrangement of cells. Typical examples include **Gram, acid-fast, and endospore stains**.

1.Gram Stain

- ✓ This is the most important staining technique for the bacterial cultures.
- ✓ The basis of gram staining is the difference in the cell wall structure of the two types of bacteria : Gram positive cell walls have a higher content of peptidoglycan and a lower content of lipids while gram negative cells have higher concentration of lipids and lower concentration of peptidoglycan. During gram staining, thebacterial smear is stained with crystal violet and iodine followed by destaining with alcohol. Lipids present in the cell wall of gram negative cells retaining the crystal violet and hence the purple color. In this process, more than one stain and some chemical solutions are used, it is the most important stain in bacteriology gets dissolved in alcohol forming pores through which crystal violet leaks out, while peptidoglycan in the cell wall of gram positive shrink due to alcohol, thus named according to " Hans Christian Gram " in 1884

Key Points: Gram Positive vs. Gram Negative Bacteria

- Most bacteria can be broadly classified as Gram positive or Gram negative.
- Gram positive bacteria have cell walls composed of thick layers of peptidoglycan.
- Gram positive cells stain purple when subjected to a Gram stain procedure.
- Gram negative bacteria have cell walls with a thin layer of peptidoglycan. The cell wall also includes an outer membrane with lipopolysaccharide (LPS) molecules attached.
- Gram negative bacteria stain pink when subjected to a Gram stain procedure.

- While both Gram positive and Gram negative bacteria produce exotoxins, only Gram negative bacteria produce endotoxins.

GRAM STAINING PROCEDURE

- ✓ For all steps in the gram staining procedure, add enough of the solution to cover the areas of the slide that have bacteria on them. You do not need to flood the entire slide.
- ✓ All staining should be done over a staining tray. Be sure to put newspaper under the tray in case of spillage.
- ✓ Gloves should be worn while staining and removed before working with the microscope.

Materials

- ✓ Clean microscope slides
- ✓ Staining trays and newspaper
- ✓ Gram stain reagents: crystal violet, Gram's iodine, safranin, 95% ethanol
- ✓ Water bottle (for rinsing)
- ✓ Bacterial cultures: *Escherichia coli, Staphylococcus aureus, Micrococcus luteus, Pseudomonas aeruginosa, Corynebacterium xerosis,* and *Neisseria sicca*

Steps of Gram staining:

1.Prepare a fixed smear.

2.Cover the smear with crystal violet **(primary stain**) and let it stand for 1 min .

3.Wash off the stain with tap water gently : All cells are **purple** after this step. Stopping here would be a simple stain

4.Cover the smear with Gram's iodine (**mordant)** solution and let it stand for 1 min.:

Gram's iodine forms a complex with crystal violet

5.Wash off the stain with tap water gently :

All cells are **purple** after this step

6.Cover the smear with 95% alcohol (**Decolorize)** by putting a couple of drops of the solution

on the smear and let it stand for 30 sec: Gram positive cells retain crystal violet and remain **purple**. Gram negative cells lose crystal violet and are now **colorless**

7. Wash off the stain with tap water gently : Water rinse stops the decolorization process

8.Cover the smear with safranin (the counter stain) let it stand for 1 min : Safranin is a pink/red dye

9.Wash off the stain with tap water gently and let it dry by air : Be careful not to wipe off the bacteria: Gram positive cells remain **purple**; gram negative cells are now **pink/red**

10.Observe your slide under the microscope: For each organism, determine morphology, arrangement and Gram reaction.

Stains and solutions used in Gram staining:

1. Crystal violet: it stains all cells with purple color, it called (primary stain)

2. Gram's iodine solution : it combines with crystal violet and forms an insoluble complex (IC.V. complex) . This complex is not removed from G+ve bacterial cells, but it removed from G-ve bacterial cells by alcohol solution, therefore it called " mordant solution "

3. 95% alcohol : it is an organic solvent which is used in concentration about 95% and removes or decolorizes the purple color of the primary stain from G-ve cells, but it can't removes this stain from G+ve cells . This process called " decolorization " and ethanol called " decolorizer agent " .Aceton can be used as alternative decolorizer .

4. Safranin : this stain is used to re-stain the cells which lost the primary stain after treating with alcohol. These cells are colored with red color . This stain is called " counter stain " or " secondary stain " .

Factors affecting the efficiency of Gram staining process:

1.Thickness of bacterial smear.

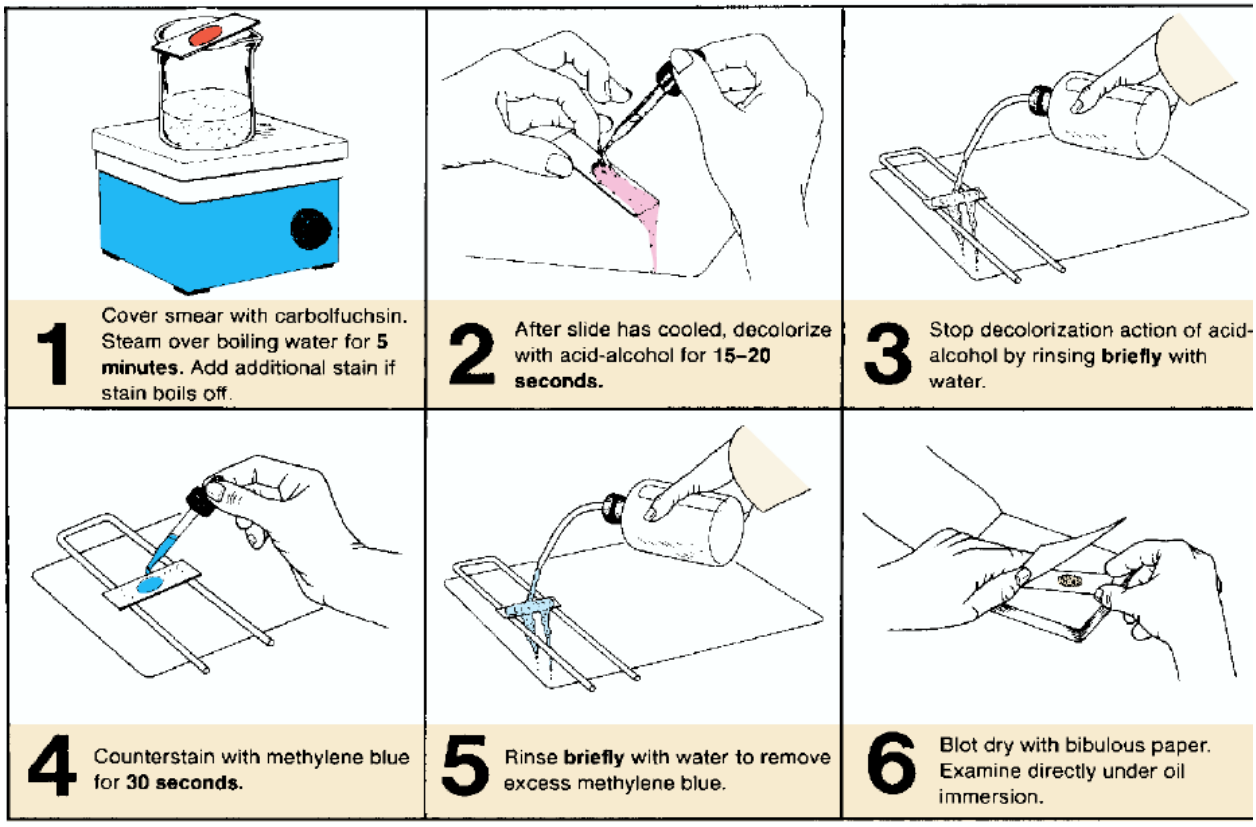
2.Concentration and purity of the solutions and reagents .

3.The nature and age of bacterial culture .

4.The amount of washing water.

2.Acid – fast stain

The acid-fast stain, like the Gram stain, is an important diagnostic stain that differentiates acid-fast bacteria (pink) from non acid-fast bacteria (blue). This stain originated as a specific method to detect Mycobacterium tuberculosis in specimens. It was determined that these bacterial cells have a particularly impervious outer wall of mycolic acid that holds fast (tightly or tenaciously) to the dye (carbol fuchsin) even when washed with a solution containing acid or acid alcohol. This stain is used for other medically important mycobacteria such as the leprosy bacillus and for Nocardia, an agent of lung or skin infections.



3.Endosperm stain

The **endospore stain** (spore stain) is similar to the acid-fast method in that a dye is forced by heat into resistant bodies called spores or endospores. This stain is designed to distinguish between spores and the cells that they come from (so-called **vegetative cells**). Of significance in medical microbiology are the gram-positive, **spore-forming members** of the genus *Bacillus* (the cause of anthrax) and *Clostridium* (the cause of botulism and tetanus).

4.Special stains are used to emphasize certain cell parts that are not revealed by conventional staining methods.

5.Capsule staining is a method of observing the microbial capsule, an unstructured protective layer surrounding the cells of some bacteria and fungi. Because the capsule does not react with most stains, it is often negatively stained with India ink, or it may be demonstrated by special positive stains. The fact that not all microbes exhibit capsules is a useful feature for identifying pathogens. One example is *Cryptococcus,* which causes a serious fungal infection in AIDS patients.

Summary of Common Bacterial Staining Techniques

1.Simple Stains

- ✓ Crystal Violet, Methylene Blue, Safranin
- ✓ Used to provide color to otherwise transparent bacterial cells
- ✓ Can be used to determine cell size, morphology and arrangement

2.Gram Stain

- ✓ Primary stain – crystal violet
- ✓ Mordant – iodine;
- ✓ decolorizer- 95% Ethanol
- ✓ Counterstain – Safranin
- ✓ Common differential stain
- ✓ Gram reaction (positive or negative) reflects cell wall properties
- ✓ Also used to determine cell size, morphology and arrangement

3.Acid-Fast Stain

- ✓ Primary stain – Carbol fuchsin
- ✓ Decolorizer – acid alcohol
- ✓ Counterstain – Methylene blue
- ✓ A differential stain used to detect bacteria with
- ✓ mycolic acid cell walls (genera *Mycobacterium* and *Nocardia*)
- ✓ Developed to detect the bacterial species that causes tuberculosis
- ✓ Acid-fast organisms resist decolorization with acid-alcohol

4.Endospore Stain

- ✓ Primary stain: Malachite green

- ✓ Counterstain: safranin
- ✓ Endospores resist staining with basic stains
- ✓ Endospores stain with malachite green;
- ✓ vegetative cells stain with safranin

5.Capsule Stain (Negative staining)

- ✓ Uses an acidic stain: (Congo red or Nigrosin) and a basic stain: (crystal violet or safranin)
- ✓ Negative stains are neither heat-fixed nor rinsed
- ✓ The background of the slide is stained by acidic stains (capsule remains unstained)
- ✓ The cells within the capsule are stained with Basic stains
- ✓ Examples of encapsulated cells: *Bacillus anthracis*, *Streptococcus pneumoniae*, and *Klebsiella pneumonia*

6.Flagella Stain

- Silver nitrate
- Used to see bacterial flagella that are too slender to be seen with other staining techniques
- Silver nitrate makes flagella appear larger than they are
- Can be used to determine arrangement of flagella for identification.
- Ex: *Proteus vulgaris* has peritrichous flagella

7.Spirochete stain

- Silver nitrate
- Used to visualize slender spirochetes like *Treponema pallidum*

Content/Topic 3. Microscope

- Microscope is a high precision optical instrument that uses a lens or a combination of lenses to produce highly magnified images of small specimens or objects especially when they are too small to be seen by the naked(unaided)eye.
- It magnifies the image of such small objects thus making them visible to the human eye.
- Microscopes are used to observe the shape of bacteria, fungi, parasites, …

- Microscopy is the use of a microscope or investigation by a microscope.

Who invented the microscope?

- ✓ There is no one person who invented the microscope as several different inventors experimented with theories and ideas and developed different parts of the concept as they evolved to what is today's microscopes.
- ✓ However, a Dutchman, Anton van Leeuwenhoek, is considered the father of microscopes because of the advances he made in microscope design and use.
- ✓ In 1674, Anton was the first to see and describe bacteria, yeast, plants, and life in a drop of water

Uses of microscopes:

- **Education**–chemistry, biology, botany, zoology.
- **Medical**–microbiology, hematology, pathology, entomology, dermatology, dental usage, veterinary use, every day analysis to advanced research. From medical schools to labs to hospitals.
- **Industry**–inspection of electronic assembly components and many different materials such as metals, textiles, plastics,etc. Used in agriculture, wineries, breweries, and for fine engravings and mining inspection. Used by jewelers and geologists.
- **Science**–for the study of archeology, oceanography, geology, metallurgy, and numerous other fields.
- **Government**–many areas for public health and safety such as water quality, pharmaceuticals, forensics, asbestos, lab work, military applications, etc.

Types of Microscopes

1.Light (bright field) microscopes: they rely on light to observe the magnified image of a specimen or object

- ✓ **Compound** (high power microscopes),
  - ✓ **Stereo** or **dissecting** (low power microscopes).

2.Electron microscopes: they use a beam of electrons (instead of a beam of light) and electromagnets (instead of glass lenses) for focusing

Parts of the Compound Microscope

The key microscope parts are illustrated and explained below:

1.Structural components

•**Head/Body** houses the optical parts in the upper part of the microscope

•**Arm** connects to the base and supports the microscope head. It is also used to carry the microscope.

•**Base** of the microscope supports the microscope and houses the illuminator

2.Optical components

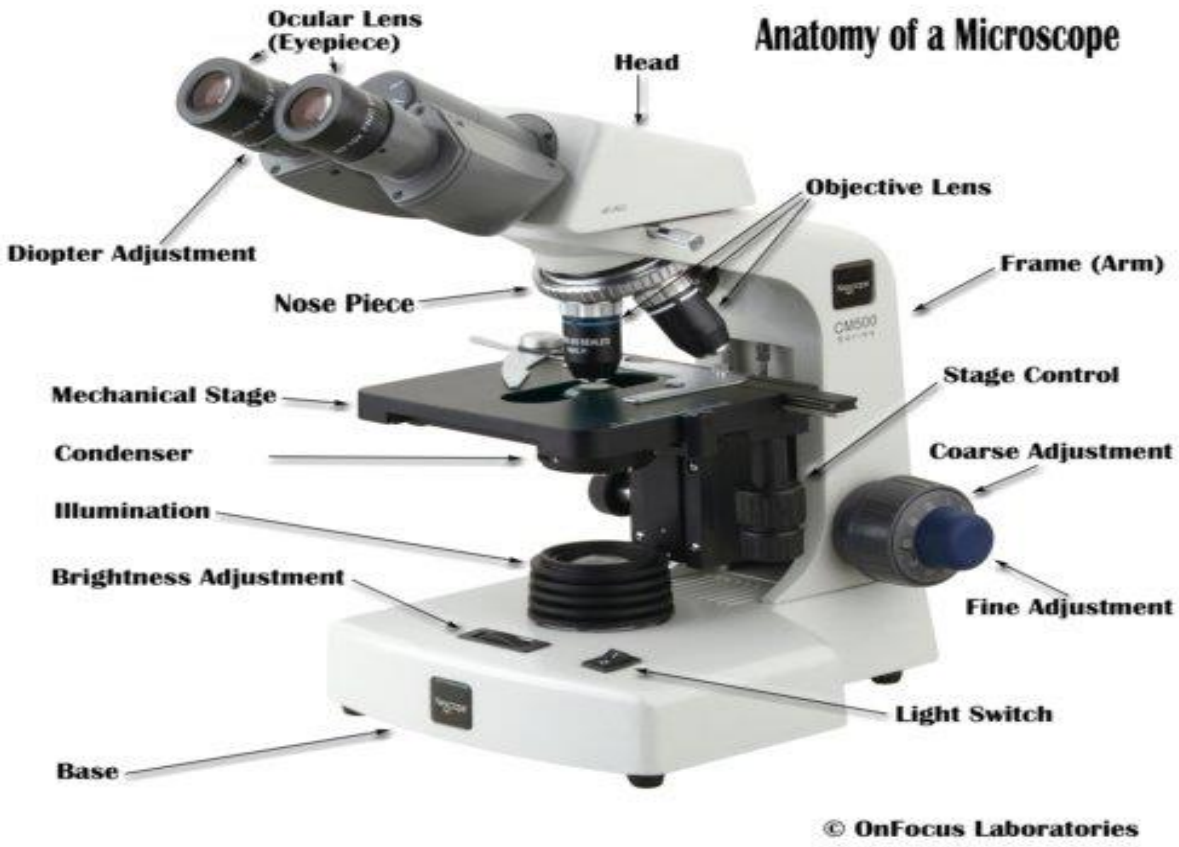
There are two optical systems in a compound microscope: Eyepiece Lenses and Objective lenses.

- **Ocular (eyepiece)**: what you look through to view your slide. Our microscopes are **binocular**, which means they have two eyepiece tubes and both eyes are used (monocular microscopes have only one tube). Typically, ocular lenses magnify an image 10X, although some have other magnifications (ex: 12X). Binocular microscopes allow for adjustment of the distance between pupils so that both eyes can be used to observe one image.
- **Objective lenses** are the lenses close to the stage. Usually microscopes will have 3-5 lenses of different magnifications (ours have 4—4X, 10X, 40X and 100X magnifications). Objective lenses are located on a rotating turret to allow for changes in magnification.

Other components

- **Coarse adjustment knob**: the larger (and outermost) of the two focusing knobs—moves the stage toward or away from the objectives to bring the image into focus at low power.
- **Fine adjustment knob**: smaller knob within the coarse adjustment knob—used for "fine-tuning" an image. Only fine focus can be used when the 40X and 100X objectives are in place.
- **Stage**: the platform where the slide to be viewed is placed. A **mechanical stage** holds the slide in place and allows for the movement of the slide to view different areas.
- **Illuminator** (light source): found at the base (bottom) of the microscope below the stage.

- **Condenser /iris diaphragm assembly:** found directly beneath the stage. This assembly can be raised and lowered using a knob at the side of the microscope. For our purposes, the assembly should be positioned very close to the bottom of the stage. The condenser is a lens that focuses the light from the illuminator onto the specimen. The iris diaphragm controls the amount of light that passes through the specimen. The iris diaphragm can be opened and closed by twisting the ridged ring of the assembly.



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