ISBN: 978-93-91768-69-0

A LABORATORY MANUAL FOR UNDERGRADUATES IN AGRICULTURAL MICROBIOLOGY, MICROBIOLOGY AND BIOTECHNOLOGY

Dr. Prita Shamrao Borkar

Bhumi Publishing

First Edition: 2022

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IN

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MICROBIOLOGY AND BIOTECHNOLOGY

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Published by:

Bhumi Publishing,

Nigave Khalasa, Kolhapur 416207, Maharashtra, India

Website: www.bhumipublishing.com

E-mail: bhumipublishing@gmail.com

Book Available online at:

https://www.bhumipublishing.com/books/

PREFACE

Microbiology, like many other branches of science has advanced rapidly and has paved roads in several areas of Science and Technology and served mankind. With the exploration of knowledge on microorganisms, renaissance in Biology has been taken place leading to exploitation of microorganisms in diversified field for the welfare of Society. Microorganisms are just like double edged sword which can be either boon or bane. These microorganisms, if employed and utilized efficiently, play an important role in the bio-world. With increasing understanding of microbial world, man is able to mould and harness microorganisms for the benefit of humans. The present Book highlights some of the areas where they are immensely important.

This Practical manual is based on the course curriculum of various universities in the subject Microbiology, Biotechnology and Agricultural Microbiology at undergraduate level. The subject matter is presented in simple and lucid way keeping the view of the beginners in the concerned subject. The text is fully illustrated with neat diagrams, figures and data is presented in precise form in tables to make the subject more understanding. All the experiments in this manual will help students perform microbiology practicals routinely without any hurdles in their laboratories. In the end suggested readings are provided for the benefit of students knowing more about the subject. The author will be happy to receive valuable opinion which will go a long way for further improvement of the book.

The author is highly grateful to Dr. D U Gawai, Principal, NES Science College, and Nanded for his motivation, guidance and support. I owe my gratitude to Prof. C N Khobragade (untimely death) for his blessings in my entire professional career.

My sincere thanks to Prof. C. S. Ukesh madam, Shri Shivaji Science College, Amravati for reading the manuscript and providing suggestions for improvement. The author is indebted to Dr. B. D. Gachande, Head of the Department, Department of Botany, Microbiology and Biotechnology and all the associates for their graceful suggestions. I am thankful to my parents, my family members and all the persons directly and indirectly in shaping the book in the present form. Author is highly thankful to Publications for the neat get up and timely publication of the book.

- Dr. Prita Shamrao Borkar

CONTENT

EXPERIMENT INDEX

Chapter - 1
General Laboratory Practices

General sterility and cleanliness:

- ❖ It is expected to remove foot wares before entering Microbiology Laboratory.
- ❖ It is necessary to wear Laboratory Aprons and neatly tie your hair and fold handcuffs.
- ❖ Sterility and cleanliness of the laboratory is necessary to ensure the integrity of samples and analytical procedures and microbiological processes.
- ❖ Traffic through the laboratory is restricted to those doing work in the laboratory, especially when analytical and microbiological work is being done.
- ❖ The tabletops are wiped with 70 percent ethanol or Dettol, before and after use.
- ❖ Antimicrobial soap is available at various laboratory sinks to facilitate hand washing before and after laboratory work.
- ❖ Clean and sterile glassware that is free of detergent residue is crucial to ensure valid results in microbiology.
- ❖ Dirty dishes are placed separately after use and are not to be stored on tabletops.
- ❖ Dishes are washed with hot water and laboratory-grade phosphate-free detergent.
- ❖ Dishes are rinsed with tap water and then deionized water.

Chapter - 2
Common Glassware

1. Measuring Cylinder

A graduated cylinder, measuring cylinder or graduate is a piece of laboratory equipment used to accurately measure the volume of a liquid. Water displacement can be used to find out the volume of a solid. Graduated cylinders are generally more accurate and precise for this purpose than flasks and beakers.

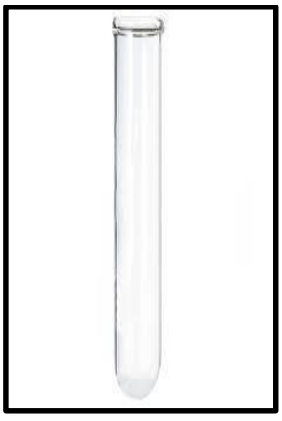
A traditional cylinder is usually narrow and high (so as to increase the accuracy of volume measurement) and has a plastic or glass stem and a "spout" for easy pouring from the measured liquid.

Certain types of cylinders have ground glass joints instead of a "spout", so that they can be closed with a stopper or connect directly with other elements of a manifold; they are also known as mixing cylinders. With this kind of cylinder, the metered liquid does not pour directly, but is often removed using cannula.

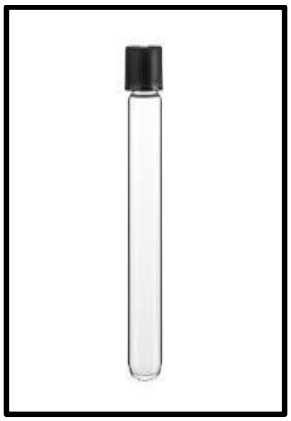
The measuring cylinder is normally and most commonly used in the laboratories.

2. Test Tube, Culture Tube and Screw-Capped Tubes

1. These are made up of glass, one end of which is closed and the other end open.
2. If the side wall is open end is slightly curved outside, it is called a **test tube**; if the side wall is smooth, it is called **culture tube**. When the side wall of the tube has screws so that a plastic cap may be fitted, it is called a **screw-capped tube**.
3. These are used in microbiological laboratories.
4. These tubes are used for testing the chemical reactions as well as determining pH, etc.
5. Culture tubes are used for preparation of agar slants and maintenance of microorganisms for lab work. The open end is plugged with a non-absorbent cotton plug.
6. Sometimes the microorganisms are purified and preserved in screw capped tubes.



Test Tube



Culture Tube

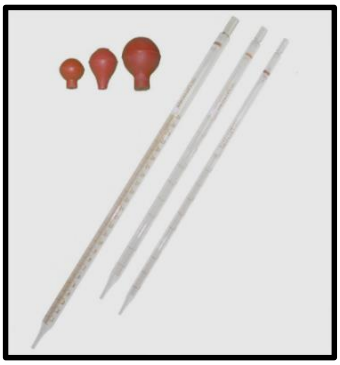
Screw Capped Tube

3. Petri Dish

1. R.J. Petri, a student of the most renowned bacteriologist Robert Koch first devised this dish, hence called **'Petri dish'.**
2. It consists of two shallow glass dishes, the upper half or lid and the lower half or bottoms half.
3. For isolation and cultivation of different types of microorganisms these dishes are used in all microbiological laboratories.
4. According to the diameter its varies.
5. Molten agar medium is aseptically poured on the bottom half of the sterilized Petri dish and then covered with the upper half.
6. The Petri dishes are sterilized by putting them in a Petri dish container and in an oven/autoclave.
7. Now, disposable sterile plastic Petri dishes are also available commercially.

4. Pipette

1. It is a cylindrical and graduated glass apparatus.
2. One end (lower side) tapers, while the other end (mouth piece) is normal. The middle portion is wider or of the same size as the mouth end.
3. It is graduated with numbers 1, 2, ……25.
4. It has different measuring capacity such as 0.1, 0.5, 1, 5, 10, 25 ml, etc. hence measures different quantities.
5. It is used for transferring the appropriate amount of liquid in other containers.
6. It should be sterilized in an oven/autoclave before use by keeping it in a pipette container after plugging it with cotton.
7. For safety, liquid should be sucked by attaching pipette-sucker at the normal end of pipette.
8. Pipettes should be sterilized by keeping them first in a steel container and then sterilized at 121^{o}C for 30 minutes.



Glass Pipette

Pasteur Pipette

5. Erlenmeyer Flasks

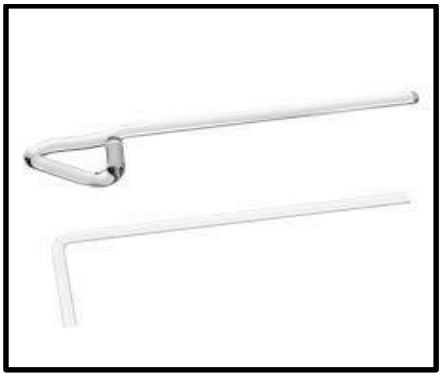
1. The flask was first devised by Erlenmeyer; hence it is called Erlenmeyer flask.
2. It has a narrow beak at top with an opening and a broad bottom.
3. The flasks of different sizes, hence measure different volumes such as 100, 250, 500, 1000, 2000 ml liquid.
4. The flasks are of round bottom or flat bottom.
5. Sometimes the flasks are also graduated to represent the volume of liquid.
6. Certain modifications are made in Erlenmeyer flask according to requirement, for example a beak is fabricated near the neck of the flask to connect to other equipment with rubber tubing. Such flasks are called side-arm flasks.
7. During sterilization, a cotton plug is inserted in the mouth of the flask.
8. It must be sterilized before microbiological usage.

6. Volumetric Flask

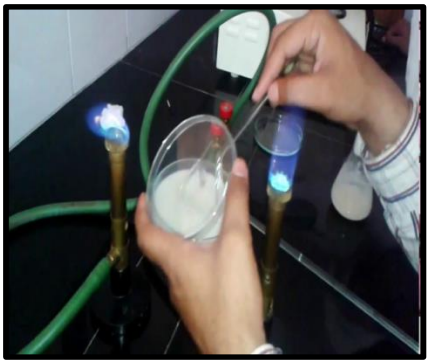
1. It is used to prepare solutions of accurate strength.
2. Its upper part is cylindrical and narrow, and marked at a point. This mark denotes the water level to be maintained at this point.
3. The lower half is rounded and voluminous.
4. Its base is flat so that it may be properly placed on the surface**.**

7. Glass Spreader

1. Glass spreader is made by bending a glass rod and making a L or V shaped structure.
2. It is used to spread culture of microorganisms on agar surface present in liquid medium.
3. The long arm is held in hand and the small arm is flame-sterilized and placed on agar surface.
4. It is brought forth and back so that microorganisms present in liquid may be dissociated and evenly spread on the entire surface of agar.



Glass Spreader

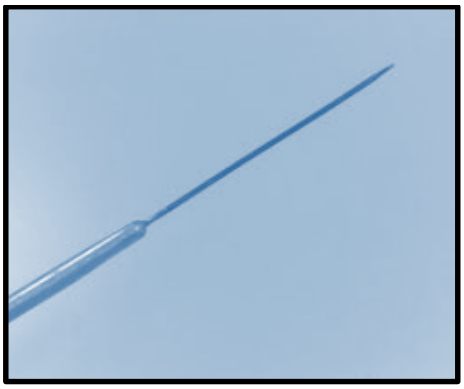


Method of Spreading

Chapter - 3
Tools used in Microbiology Laboratory

1. Inoculation Needle and Inoculation Loop

1. These are the most commonly used tools. Inoculation needle/loop is made up of a long platinum/ wire fixed into a metallic rod.
2. A wire loop has a handle with a steel screw shaft in which a nichromeor metallic rod is fixed. The wire is too wrapped around a small round object such as a pen, etc. to form a loop by twisting it mechanically. The loop should be such as to retain a small circular film in it by dipping in solution. For this purpose, a proper size (5-7cm) of the wire is recommended.
3. The straight wire or straight needle has a wire in place of a loop. The open or free end of which is blunt. Both loops are straight wire are to be sterilized either by using Bunsen burner or hot heating coil wire till the needle or loop become red hot. After the loop or wire is cooled, these are generally used in transferring culture from liquid broth.
4. The needle straight wire is used for transferring culture from solid medium. Even smaller amount of liquid culture can be manipulated by using a straight needle. Straight wire is often used for stab cultures.
5. The loop and wire are also used for picking small quantities of solid materials from a microbial colony, and can be used to inoculate either a liquid or a solid medium. Both the loop and straight wire must be flamed immediately after use so that contamination is avoided.



Inoculation Needle

Loop

2. Bunsen Burner and Spirit Lamp

1. The basic device for providing dry heat for incineration in the laboratory is the Bunsen burner. It is named after the name of R.W. Burner. It is called a spirit lamp due to the use of spirit for burning purpose.
2. The gas enters the burner at the base, and its supply is regulated externally by the gas cock. As the gas streams upwards through a get inside the base, air is polluted in through the air intake holes just above the base.
3. The amount of air can be controlled by rotating a sleeve that fits over the top end of the barrel.

4. The proper method of lighting the burner is to close off the air supply, turn on the gas and light. The flame will be large and yellow. Gradually open the air intake until the flame takes a blue color.
5. The inoculation needle/loop is sterilized before inserting into culture tubes or petri plates.
6. It is also used during transfer and purification of microbial cultures. Before opening the cotton plug, the mouth of culture tubes/flasks should be flamed so that the microorganisms if any present on the mouth get killed.
7. Sterilization of tools by using Bunsen burner/spirit lamp is called incineration.

Bunsen Burner

Spirit Lamp

Chapter - 4

General Cleaning and Sterilization of Glassware

1. Cleaning Glassware

The conventional method of washing glassware involves soaking glass in a chromic acid-sulfuric acid bath followed by tap water rinses, distilled water rinses, and finally double-distilled water rinses. Due to the corrosive nature of chromic acid, the use of this procedure has been eliminated except for highly contaminated or soiled glassware. Adequate cleaning of most glassware for tissue culture purposes can be achieved by washing in hot water (above 70 °C) with commercial detergents, rinsing with hot tap water (above 70 °C), and finally rinsing with distilled and double-distilled water. However, highly contaminated glassware should be cleaned in a chromic acid-sulfuric acid bath or by some other proven method such as (1) ultrasonic cleaning, (2) washing with sodium pyrophosphate, or (3) boiling in metaphosphate (Alcon ox), rinsing then boiling in a dilute hydrochloric acid solution, and then finally re rinsing. Cleaned glassware should be inspected, dried at 150 °C in a Hot air oven, capped with aluminium foil, and stored in a closed cabinet.

The following general procedure is recommended for cleaning glassware that contains media and cultures after completion of experiments and all data have been collected:

1. Autoclave all glassware with media and cultures still in it. This kills any contaminating microorganisms that may be present.
2. After the autoclaved media has cooled, but while it is still in a liquid state, pour it into biohazard plastic bags or thick plastic bags, seal, then discard.
3. Wash all glassware in hot soapy water using a suitable bottle brush to clean the internal parts of the glassware. Any glassware that is stained should be soaked in a concentrated sulfuric acid-potassium dichromate acid bath for 4 hr, then rinsed 10 times before washing it with soapy water.
4. All glassware should be rinsed three times in tap water, three times in deionized water, three times in double-distilled water, dried, and stored in a clean place.
5. Wash all the new glassware in a similar manner.

2. Methods of sterilization

Sterilization:

It is defined as the process where all the living microorganisms, including bacterial spores are killed. Sterilization can be achieved by physical, chemical and physicochemical means. Chemicals used as sterilizing agents are called chemi-sterilants.

There are four different sterilization principles:

1. Sunlight and Heat sterilization
2. Radiation sterilization
3. Filtration sterilization
4. Chemical sterilization (Chemical method)

a. Physical Methods of Sterilization:

A. Dry Heat Sterilization:

Heat sterilization is the most widely used and reliable method of sterilization, involving destruction of enzymes and other essential cell constituents like cell wall, cell membrane. The process is more effective in a hydrated state where under conditions of high humidity,

hydrolysis and denaturation occur, thus lower heat input is required. Under dry state, oxidative changes take place, and higher heat input is required.

This method of sterilization can be applied only to the thermo stable products.

Sunlight*:*

The microbicidal activity of sunlight is mainly due to the presence of ultraviolet rays in it. It is responsible for spontaneous sterilization in natural conditions. In tropical countries, sunlight is more effective in killing germs due to combination of ultraviolet rays and heat. By killing bacteria suspended in water, sunlight provides a natural method of disinfection of water bodies such as tanks and lakes. Sunlight is not sporicidal; hence it does not sterilize.

Heat:

Heat is considered to be the most reliable method of sterilization of articles that can withstand heat. Heat acts by oxidative effects as well as denaturation and coagulation of proteins. Those articles that cannot withstand high temperatures can still be sterilized at lower temperature by prolonging the duration of exposure.

Red heat:

Articles such as bacteriological loops, straight wires, tips of forceps and searing spatulas are sterilized by holding them in Bunsen flame till they become red hot. This is a simple method for effective sterilization of such articles, but is limited to those articles that can be heated to redness in flame.

Flaming:

This is a method of passing the article over a Bunsen flame, but not heating it to redness. Articles such as scalpels, mouth of test tubes, flasks, glass slides and coverslips are passed through the flame a few times. Even though most vegetative cells are killed, there is no guarantee that spores too would die on such short exposure. This method too is limited to those articles that can be exposed to flame. Cracking of the glassware may occur.

Hot air oven:

This method was introduced by Louis Pasteur. Dry heat sterilization involves heating at atmospheric pressure and often uses a fan to obtain uniform temperature by circulation. Heat at 180 ºC for half an hour, 170 ºC for 1 hr., or 160 ºC for 2 hrs are the usual temperature range in this method. Times are the periods during which an object is maintained at the respective temperature.

The heat is transferred to the article by radiation, conduction and convection. The oven should be fitted with a thermostat control, temperature indicator, meshed shelves and must have adequate insulation.

Articles sterilized: Metallic instruments (like forceps, scalpels, scissors), glassware (such as Petri-dishes, pipettes, flasks, all-glass syringes), swabs, oils, grease, petroleum jelly and some pharmaceutical products.

Dry heat destroys bacterial endotoxins (or pyrogens) which are difficult to eliminate by other means and this property makes it applicable for sterilizing glass bottles which are to be filled aseptically.

B. Moist Heat Sterilization:

Moist heat may be used in three forms to achieve microbial inactivation. Moist heat sterilization involves the use of steam in the range of 121-134 ºC. Steam under reduced pressure is used to generate high temperatures needed for sterilization.

Autoclaves use pressurized steam to destroy microorganisms, and are the most dependable systems available for the decontamination of laboratory waste and the sterilization of laboratory glassware, media, and reagents. Sterilization in an autoclave is most effective when the organisms are either contacted by the steam directly or are contained in a small volume of aqueous (primarily water) liquid. Under these conditions, steam at a pressure about 15 psi; attaining temperature (121^{o}C) kills all organisms and their endospores in about 15 minutes.

Principle:

A basic principle of chemistry is that when the pressure of a gas increases, the temperature of the gas increases proportionally. For example, when free flowing steam at a temperature of 100 ^{o}C is placed under a pressure of 1 atmosphere above sea level pressure - that is, about 15 pounds or pressure per square inch (psi), the temperature rises to 121 ^{o}C. In this way steam is a gas, increasing its pressure in a closed system which increases temperature. As the water molecules in steam become more energized, their penetration increases substantially.

To destroy the infective agents associated with spongiform encephalopathy (prions), higher temperatures or longer times are used; 135 ^{o}C or 121 ^{o}C for at least one hour are recommended.

C. Radiation Sterilization:

Many types of radiation are used for sterilization like electromagnetic radiation (e.g., gamma rays and UV light), particulate radiation (e.g. accelerated electrons). The major target for this radiation is microbial DNA. Gamma rays and electrons cause ionization and free radical production while UV light causes excitation.

Gamma Ray Sterilizer:

Gamma rays for sterilization are usually derived from cobalt-60 source, the isotope is held as pellets packed in metal rods, each rod carefully arranged within the source. This source is housed within a concrete building with 2 m thick walls. Articles being sterilized are passed through the irradiation chamber on a conveyor belt and move around the raised source.

Ultraviolet Irradiation:

The optimum wavelength for UV sterilization is 260 nm. A mercury lamp giving peak emission at 254 nm is the suitable source of UV light in this region.

D. Filtration Sterilization:

Filtration process does not destroy but removes the microorganisms. It is used for both the clarification and sterilization of liquids and gases as it is capable of preventing the passage of both viable and non-viable particles.

Membrane filters with pore sizes between 0.2- 0.45 μm are commonly used to remove particles from solutions that can't be autoclaved. It is used to remove microbes from heat labile liquids such as serum, antibiotic solutions, sugar solutions, and urea solution. Various applications of filtration include removing bacteria from ingredients of culture media, preparing suspensions of viruses and phages free of bacteria, measuring sizes of viruses, separating toxins from culture filtrates, counting bacteria, clarifying fluids and purifying hydrating fluid. Filtration is aided by using either positive or negative pressure using vacuum pumps. The older filters made of earthenware or asbestos are called depth filters.

The major mechanisms of filtration are sieving, adsorption and trapping within the matrix of the filter material. Sterilizing grade filters are used in the treatment of heat sensitive injections and ophthalmic solutions, biological products and air and other gases for supply to aseptic areas.

They are also used in industry as part of the venting systems on fermenters, centrifuges, autoclaves and freeze dryers. Membrane filters are used for sterility testing.

E. Chemical Sterilization:

The chemical compounds used can be:

a) Gas Sterilization

b) Liquid Sterilization

Generally, chemical sterilization procedures have the disadvantages of presenting health hazards to users (e.g., poisonous, flammable).

Gaseous Sterilization:

The chemically reactive gases such as formaldehyde, (methanol, HCHO) and ethylene oxide $(CH_2)_2O$ possess biocidal activity. Ethylene oxide is a colorless, odorless, and highly flammable gas.

a. Ethylene Oxide Sterilizer:

An ethylene oxide sterilizer consists of a chamber of 100-300-Litre capacity and surrounded by a water jacket. Air is removed from sterilizer by evacuation, humidification and conditioning of the load is done by passing sub-atmospheric pressure steam, then evacuation is done again and preheated vaporized ethylene oxide is passed. After treatment, the gases are evacuated either directly to the outside atmosphere or through a special exhaust system.

Ethylene oxide (& formalin) autoclaves have the following disadvantages: -

i. Difficult to operate.
ii. Unsuitable for hospitals but used in industry (e.g. for sterilizing disposable materials that cannot tolerate high temperatures)

b. Low Temperature Steam Formaldehyde (LTSF) Sterilizer:

An LTSF sterilizer operates with sub atmospheric pressure steam. At first, air is removed by evacuation and steam is admitted to the chamber.

Liquid Sterilization:

Per acetic Acid Liquid Sterilization:

Peracetic acid was found to be sporicidal at low concentrations. Also, it shows no harmful health or environmental effects. It disrupts bonds in proteins and enzymes and may also interfere with cell membrane transportation through the rupture of cell walls and may oxidize essential enzymes and impair vital biochemical pathways.

Hydrogen Peroxide Sterilization:

This method disperses a hydrogen peroxide solution in a vacuum chamber, creating a plasma cloud. This agent sterilizes by oxidizing key cellular components, which inactivates the microorganisms. The plasma cloud exists only while the energy source is turned on. When the energy source is turned off, water vapor and oxygen are formed, resulting in no toxic residues and harmful emissions. The temperature of this sterilization method is maintained in the 40-50 °C range, which makes it particularly well suited for use with heat-sensitive and moisture-sensitive medical devices. The instruments are wrapped prior to sterilization, and can either be stored or used immediately.

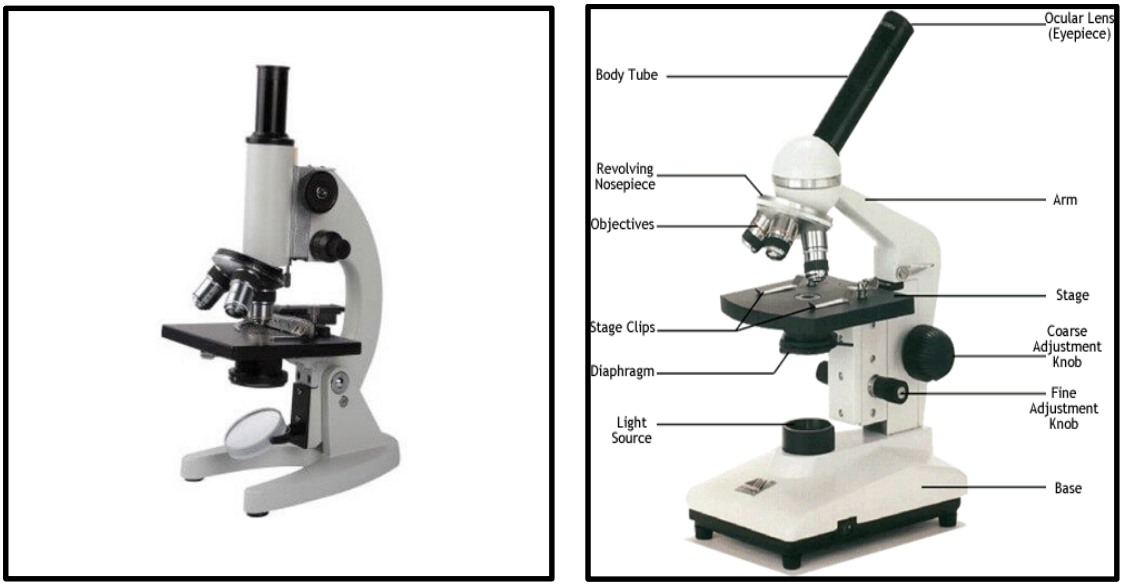
Chapter - 5

General Laboratory Equipment, Their Uses and Precautions in Handling

1. The Compound Microscope

The parts of microscope:

1. **Eyepiece Lens:** The lens at the top that you look through. They are usually 10X or 15X power.
2. **Body Tube:** Connects the eyepiece to the objective lenses.
3. **Arm:** Supports the tube and connects it to the base.
4. **Base:** The Bottom of the microscope, used for support.
5. **Illuminator:** A steady light source (110 volts) used in place of a mirror.
6. **Stage:** The flat platform where you place your slides. Stage clips hold the slides in place. If your microscope has a mechanical stage, you will be able to move the slide back and forth or side wise by turning two knobs. One moves it left and right, the other moves it up and down.
7. **Revolving Nosepiece or Turret:** This part holds two or more objective lenses and can rotate to easily change power of the objectives.
8. **Objective Lenses:** Usually you will find 3 or 4 objective lenses on a microscope. They usually consist of 4X, 10X, 40X and 100X powers.
9. **Rack Stop:** This is an adjustment that determines how close to the objective lens can get to the slide.
10. **Condenser Lens:** The purpose of the condenser lens is to focus the light onto the specimen.
11. **Diaphragm or Iris:** Many microscopes have a rotating disk under the stage. This diaphragm has different sized holes and is used to vary the intensity and size of the cone of the light that is projected upward into the slide.



The compound Microscope

2. Balance

For chemical or biological experiments, an accurate amount of chemical should be weighed by using a balance. However, no experiment can be conducted without a balance. There are several types of balances used for weighing such as single pan, chemical or analytical, and electrical balances. The balances are used according to requirement and the amount is more than 100 g; a chemical or electrical balance is used to weigh 10 mg but its total weighing capacity is 100 g. Besides, the ultra-micro balance can weigh the materials of 0.01 µg to 2 mg.

Electric Balance:

1. It works in the presence of electricity that shows a digital display of weights.
2. It contains a single pan, the weight of which is counterbalanced by weights and set at zero.
3. The material to be weighed is placed on the pan of balance and required counterweights are removed by using the knobs. Soon the digital scale moves down and up.
4. Always remove the counter weights corresponding to the weight of materials.

Analytical balance:

Analytical balances are used for accurate weighing of reagents and media. They are checked and calibrated annually. Balances must rest on a firm, level surface. Balance trays are wiped off daily with water or a surface disinfectant such as 70 % ethanol.

Electronic balance

Electronic balances have become standard equipment for many high schools and various departments in college and universities. They allow the user to quickly and accurately measure the mass of a substance to a level of accuracy impossible for traditional balances to achieve. This is especially important in experiments that require precise amounts of each substance to achieve the desired results. The popularity of the electronic balance is also due to its extreme ease of use for any skill level.

1. Place the electronic balance on a flat, stable surface indoors. The precision of the balance relies on minute factors and wind, shaky surfaces, or similar forces will cause the readings to be inaccurate.
2. Press the "ON" button and wait for the balance to show zeros on the digital screen.
3. Use tongs or gloves to place the empty container you will use for the substance to be measured on the balance platform. Fingerprints and other greases from your hands add mass and must be avoided for accurate measurements.
4. Press the "Tare" or "Zero" button to automatically deduct the weight of the container from future calculations. The digital display will show zero again, indicating that the container's mass is stored in the balance's memory.
5. Carefully add the substance to the container. Ideally this is done with the container still on the platform, but it may be removed if necessary. Avoid placing the container on surfaces that may have substances which will add mass to the container such as powders or grease.

6. Place the container with the substance back on the balance platform if necessary and record the mass as indicated by the digital display.

3. Hot Plate

Laboratory hot plates are normally used for heating solutions to 100 ^{o}C or above when inherently safer steam baths cannot be used. Any newly purchased hot plates should be designed in a way that avoids electrical sparks. However, many older hot plates pose an electrical spark hazard arising from either the on-off switch located on the hot plate, the bimetallic thermostat used to regulate the temperature or both. Laboratory personnel should be warned of the spark hazard associated with older hot plates.

1. In addition to the spark hazard, old and corroded bimetallic thermostats in these devices can eventually fuse shut and deliver full, continuous current to a hot plate.
2. Do not store volatile flammable materials near a hot plate
3. Limit use of older hot plates for flammable materials.
4. Check for corrosion of thermostats. Corroded bimetallic thermostats can be repaired or reconfigured to avoid spark hazards.

4. pH Meters

In 1909, Sorenson used the term pH to denote the hydrogen ion concentration of the solution. It is defined as a negative log of hydrogen ions (H^+) concentration: pH= $-\log_{10}(H^+)$. The pH is the degree of acidity and alkalinity of a solution on a scale 1 to 14. The pH values 1 to 7 show the acid values, pH 7 neutrality and pH 7 to 14 alkalinity. pH of water is 7 at 25 ^{o}C. The acid is proton donor and base is the proton acceptor (acid=base+H^+) i.e. acid dissociates and produces hydrogen ion concentration (H^+).

Maintenance of pH values is an important parameter for the growth and process of any organism.

1. The pH is measured by using a pH meter which is an instrument calibrated against a series of buffers of known pH values.
2. A standard pH meter has two electrodes, one glass electrode and the other mercury-mercurous chloride (calomel) or silver-silver chloride reference electrode. The reference electrode is immersed in a saturated KCl solution.
3. A normal pH meter consists of a glass electrode made up of a thin glass membrane which is selectively permeable to H^+.
4. Thus, the function of electrode is to form conductive bridge between the metallic element and the sample solution in which two electrodes are placed.
5. Now-a-days pH meters with combined electrodes are also available.

6. By using a pH meter, pH of an unknown solution is measured.
7. Each piece of equipment has a daily logbook; record all calibrations in the appropriate logbook.

Precautions:

A pH meter should be handled carefully so that its electrode may not be broken. The electrodes should always be kept immersed in distilled water so that electrodes may not be non-functional.

5. Laminar Air Flow

Laminar flow is an apparatus consisting of an air blower in the rear side of the chamber which can produce air flow with uniform velocity along parallel flow lines. There is a special filter system of high efficiency particulate air filter (HEPA) which can remove particles as small as 0.3mm. In front of the blower, there lies a mechanism through which air blown from the blower produces air velocity along parallel flow lines.

Principle:

The laminar is based on flow of air current of uniform velocity along parallel flow lines which help in transferring microbial cultures in aseptic conditions. Air is passed through the filters into the enclosure and the filters do not allow any kind of microbe to enter into the system. Inside the chamber one fluorescent tube and the other UV tube are fitted. Two switches for these tubes and a separate switch for regulation of air the air flow is fitted outside the apparatus.

Due to uniform velocity and parallel flow of air current, pouring of media, plating, slant preparations, streaking etc. without any kind of contamination are performed. Initially dust particles are removed from the surface of the laminar flow with the help of smooth cloth containing alcohol.

1. Switch on the UV light for a period of 30 minutes so as to kill the germs, if any present in the area of working space.
2. The front cover sheet of the apparatus is opened to keep the desired material inside. The air blower is set at the desired degree so that the air inside the chamber is expelled because the air inside the chamber may be contaminated /may bring contaminants.
3. Sit properly in front of the chamber again; wipe the working table with alcohol to reduce the contaminants. All the work related to pouring, plating streaking, etc. are to be carried out in the flame zone of the burner or spirit lamp.

In the microbiology laboratory, a horizontal type of laminar airflow is used to supply the air through a filter.

Precautions:

1. Remove the shoes before entering the Lab to operate the apparatus.
2. Wash the hands with detergent or soap. One should not talk inside the chamber while performing microbial culture transfer, failing which chances of contamination may be more which may come either through mouth, sneezing or air.

6. Incubator

An incubator is an instrument that consists of a copper/steel chamber, around which warm water or air is circulated by electrical current or by means of small gas flame. The temperature of the incubator is kept constant due to its control by using thermostat. The incubator is made up of a double walled chamber adjusted to a desired temperature. It is done by using an external knob controlling the thermostat system. The gap between two walls is insulated to check heat conduction. A thermometer is inserted from the top for recording the temperature. Now sophisticated incubators are available with humidity and oxygen control systems.

Principle:

An incubator is based on the principle that microorganisms require a particular set of parameters for their growth and development. When organisms are provided with the optimal condition of temperature, humidity, oxygen, and carbon dioxide levels, they grow and reproduce.

1. Temperature greatly influences microbial growth. Therefore, this instrument is generally designed that can allow the desired microorganism to grow at a particular temperature.
2. It is operated to allow the microbial growth on a suitable medium under proper temperature. In an incubator, the variation in temperature should not be more than one degree.
3. Small square type incubators are better than large ones. If a lower temperature than the room is required, the water is circulated around the chamber to pass through an ice chest.
4. Similarly, other parameters like humidity and airflow are also maintained through different mechanisms that create an environment similar to the natural environment of the organisms.
5. They are also provided with adjustments for maintaining the concentration of CO_2 to balance the pH and humidity required for the growth of the organisms

Precautions:

1. The door of the incubator should be opened only when necessary.
2. As microorganisms are susceptible to temperature change, the fluctuations in temperature of the cabinet by repeatedly opening the door should be avoided.
3. The required parameters growth of the organism should be met before the culture plates are placed inside the cabinet.
4. The plates should be placed upside down with the lid at the bottom to prevent the condensation of water onto the media.
5. The inside of the incubators should be cleaned regularly to prevent the organisms from settling on the shelves or the corners of the incubator.
6. While running the incubator for an extended period of time, sterile water should be placed underneath the shelves to prevent the culture media from drying out.

7. If the tubes are to be incubated for a long time or at higher temperature, the medium may become too dry due to excessive evaporation. In such cases cotton plugs should be pushed inside the neck of the tube. The tube should be covered by a rubber cap so as to cover the plug.
8. If the Petri dishes are to be incubated for a long time. They may be placed in a moist chamber with a damp sterile cotton wool at the bottom.

7. BOD incubator (Biological Oxygen Demand)

BOD Incubator also known as Biological Oxygen Demand incubator. BOD Incubator is additionally recognized as a low-temperature incubator or refrigerated incubator because it produces a temperature limit between 5 °C to 60 °C or including cooling and heating capacities under one unit. Biological Oxygen Demand (BOD) or Biochemical Oxygen Demand is the amount of dissolved oxygen needed (i.e., demanded) by aerobic biological organisms to break down organic material present in a given water sample at certain temperature over a specific time period. In laboratories, the BOD incubator also used to study the germination, insects, and culturing of bacteria.

The BOD value is most commonly expressed in milligrams of oxygen consumed per litre of sample during 5 days of incubation at 20 ^{o}C and is often used as a surrogate of the degree of organic pollution of water.

Principle:

Power is supplied by channels MCB. Temperature is fixed within a digital PID temperature controller, normally at 20 °C. The machine is kept working for 5 days. The cooling system starts only after fixing the temperature. An Axial fan distributes air inside the container. The temperature sensor senses the prevailing temperature and provides data to the PID controller, which furthermore grips the set heat constant till the aspired time.

1. Before running a BOD incubator make sure the incubator is connected with the power supply.
2. After that turn on the main switch on the mainboard then turn on the switch on the cabinet.
3. After that set the desired temperature on the controller by pressing the set knob and soft key.
4. Control the temperature every day as by the following procedure.
5. Record the temperature which is displayed on the controller.
6. Monitor the temperature displayed on the digital screen. The temperature should not deviate by 2 degrees centigrade.

Precautions:

1. Always disconnect the BOD incubator from the socket when it is not in use.
2. To avoid downtime and maintain the working condition of the incubator, periodic servicing is important.

3. Before running a BOD incubator read the instruction manual carefully and don't overuse it.
4. During cleaning the incubator make sure the incubator is disconnected from the power supply.
5. Clean the BOD incubator regularly to maintain its working performance.
6. Monitor the temperature changes in the BOD incubator on a monthly basis.

8. Refrigerator

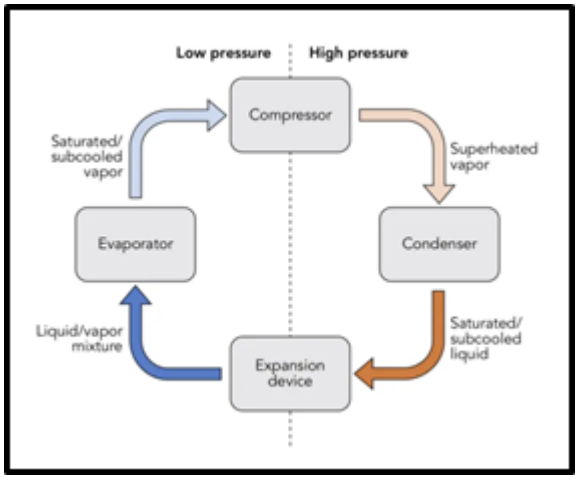
Scottish professor William Cullen designed a small refrigerating machine in 1755. Cullen used a pump to create a partial vacuum over a container of diethyl ether, which then boiled, absorbing heat from the surrounding air. A refrigerator is used for a wide variety of purposes such as maintenance and storage of stock cultures between sub-culturing periods and storage of sterile media to prevent dehydration.

It is also used as a repository for thermolabile solutions, antibiotics, serums, and biochemical reagents.

Principle:

Refrigerators work on the principle of thermodynamics cycles and the second law of thermodynamics. Thermodynamic cycle is essentially a closed cycle in which the working substance undergoes a series of processes and is always brought back to its initial state. The vapour compression refrigeration system (VCRS) is most commonly used in refrigeration. In a vapour compression refrigeration system, refrigeration is obtained as the refrigerant evaporates by absorbing latent heat of vaporization from the evaporator (storage space) and liquid refrigerant converts into vapour.

A refrigerator consists of several components like Compressor, Condensers, Expansion devices, evaporator and some accessories for proper functioning like controls (temperature controlling devices), filters, defrost system etc. for proper working of the device it is necessary to proper matching of all the components as per the requirement and function. Every component performs their own function so it is necessary to assemble all in proper sequence.



A good refrigerant must have some qualities like it should be non-toxic, non-explosive, non-corrosive; non-inflammable and leakage should be non-inflammable etc. Different

refrigerants are used as per their requirements. For example: R-12 is used in domestic refrigerators and water coolers.

Precautions:

1. Have a trained professional install your refrigerator lean up any spills immediately to prevent the growth of bacteria.
2. Ensure the refrigerator is turned off before you clean any electrical components or disassemble the ice or water dispenser unit to clean it.
3. Slightly dampen a soft lint-free cloth or paper towel with water and then you use it on the Refrigerator.
4. Do not use any kind of detergent to clean the refrigerator because that can discolour or damage the refrigerator.

9. Autoclave

Sterilization is the process that eliminates living organisms from media, solutions, substances or objects. The killing action of heat on the organisms can be done by using an increase in steam in a closed system. The water molecules become aggregated resulting in increase in their penetration. The water boils at 100 ^{o}C and the steam accumulates in a closed container resulting in increase in pressure.

Principle:

The autoclave works on the principle of moist heat sterilization where **steam under pressure** is used to sterilize the material present inside the chamber. The high pressure increases the boiling point of water and thus helps achieve a higher temperature for sterilization. Water usually boils at 100 °C under normal atmospheric pressure (760 mm of Hg); however, the boiling point of water increases if the pressure is to be increased.

Similarly, the high pressure also facilitates the rapid penetration of heat into deeper parts of the material, and moisture present in the steam causes the coagulation of proteins causing an irreversible loss of function and activity of microbes.

This relationships between pressure and temperature is shown below:

Pressure (lb/inch^2)	**Temperature (^{o}C)**
0	100
5	109
10	115.5
15	121.5
20	126.5
25	130.5
30	135.5
40	141.5

1. The autoclave is usually made up of gun a metal sheet which is supported in an iron case.
2. It is closed by swing door which is fastened by radical bolts tightly.
3. In microbiology laboratories, system jacketed horizontal type autoclave is necessary.
4. The steam is passed from below at the base. The side walls are heated by the steam jacket. It has a provision to record the pressure.
5. There is a possibility to regulate the pressure using a pressure meter. It consists of a safety valve that guard against accidents. It is based on moist heat that is used in sterilization.

The autoclave is usually operated at 15 lb/inch2 steam pressure for 30 min., as demonstrated in the table given above. This temperature for 30 minutes is enough to kill all the spores and cells of microorganisms.

Precautions:

1. The level of water should be checked before operating.
2. The air should be completely evacuated and the steam must have access to the materials to be sterilized.
3. The cotton or glass beads must be sterilized in a glass container closed with foil. The heat sensitive substances should not be sterilized by autoclaving.
4. Check the sterility of autoclave regularly by using chemical strips which turns green, at 115 ^{o}C, when kept for 25 min.
5. The condensate holding tank is drained daily or as needed.
6. If the autoclave does not reach the specified temperature, service the autoclave and re-sterilize all glassware and reagents that were insufficiently sterilized.
7. The autoclaves are operated using deionized water.
8. The use of bacilli spores, after autoclaving is considered perfect if no growth is obtained on thioglycollate medium or cooked meat medium.

12. Hot Air Oven

A hot air oven is a laboratory instrument that uses dry heat to sterilize laboratory equipment and other materials. Hot air oven also known as forced air circulating oven. We can sterilize Glassware (like petri dishes, flasks, pipettes, and test tubes), Powder (like starch, zinc oxide, and sulfadiazine), Materials that contain oils, Metal equipment (like scalpels, scissors, and blades) by using hot air oven.It is used to sterilize N95 masks and packaging items in the Life Science and Microbiology Laboratory.

To destroy microorganisms and bacterial spores, a hot air oven provides extremely high temperatures over several hours. Hot air oven is generally

used for sterilization of glassware, metal devices and other articles which are deteriorated/ degraded by autoclaving. Although sterilization can be made by an incubator, glassware requires dry heat instead of moist heat. It kills the microbes by oxidizing their chemical constituents. It is less effective as compared to moist heat.

Principle:

Sterilization by dry heat is performed by conduction. The temperature is consumed by the surface of the objects, then moves towards the core of the object, coating by coating. The whole object will ultimately attain the temperature needed for sterilization to take place. Dry heat causes most of the injury by oxidizing particles. The primary cell components are damaged and the organism dies. The temperature is kept for about an hour to eliminate the most ambitious of the resistant spores.

Now-a-days, ovens based on microwaves are also sold in the market but generally, are not in much use in laboratories.

1. The oven generally consists of double walled chamber, the gap between two walls is insulated. It is heated from below by using electric current and heating elements are arranged in a manner to heat the inside chamber uniformly.
2. There is an in-built thermostat when required, it helps in regulating the temperature. The calibration knob sets the desired temperature.
3. For sterilization, the holding time depends upon the temperature. If the temperature of the oven is 160 ^{o}C, the holding time should be 1 hour but at 180 ^{o}C it should be 30 minutes. The holding time can be a little more for better sterilization.

Precautions:

1. The glass materials should be wiped and dried before keeping inside the chamber in the oven, otherwise it may break.
2. After the holding time is over, the glassware should not be taken out immediately; rather, the temperature should be bearable to remove the glassware unless you will damage or break the glassware.
3. The air, within the oven, should be circulated by a fan to ensure that all parts are kept at required temperature; the articles should be placed properly so as not obstruct the flow of air.
4. Most of the materials are not fit with hot air ovens such as surgical dressings, rubber items, or plastic material; they can cause a meltdown at low temperatures.

11. Water Bath

A hot water bath or Laboratory Hot water bath is one of the essential instruments of a laboratory. It's normally used for incubation of test samples underwater at constant temperature (hot or cold) over a long period of time. It is mainly used in clinical and microbiology laboratories, for chemical reactions, sample thawing, corrosion tests and bacteriological examinations etc.

It consists of an insulating box made up of steel fitted with electrode heating coil. The design configurations, sizes, and dimensions of a hot water bath varies. The container size of a laboratory water bath varies from 12 liters to 32 liters for a standard model and 50 -100 liters for

a large size water bath. Water bath is is used to provide constant temperature to a sample. The temperature is controlled through the thermostat.

In some of the water baths, the platform rotates, it is called a water-bath shaker. It is more useful to microbiologists because it provides a uniform heat to the cell cultures meant for incubation. The main use of water-bath is the incubation of samples at a desired and constant temperature.

1. Used for incubation of cell culture.
2. Also used as a heat source for flammable chemicals.

Principle:

Laboratory water bath has a temperature sensor, which transfers water temperature to resistance value, and is amplified and compared by an integrated amplifier. Then output the control signal, and efficiently control the average heating power of the electric heating tube and maintain water in constant temperature.

1. Before use make sure water is at the desired level in a water bath. If not, fill it with tap water at the desired level and then turn on the switch.
2. After that set the desired temperature by using the temperature controller and allow the water to warm to that temperature.
3. Monitor the temperature from the thermometer.
4. After reaching the desired temperature, now insert your test samples in it for incubation.

Precautions:

1. Keep changing water daily and keep clean from the inside to prevent the encrustation of important components in a water bath.
2. When using the water bath, keep the lid closed so that the water does not evaporate.
3. Measure the inside and outside temperature of the water bath once a week.
4. Make sure the thermometer does not stick to the wall of the water bath.

Water Bath

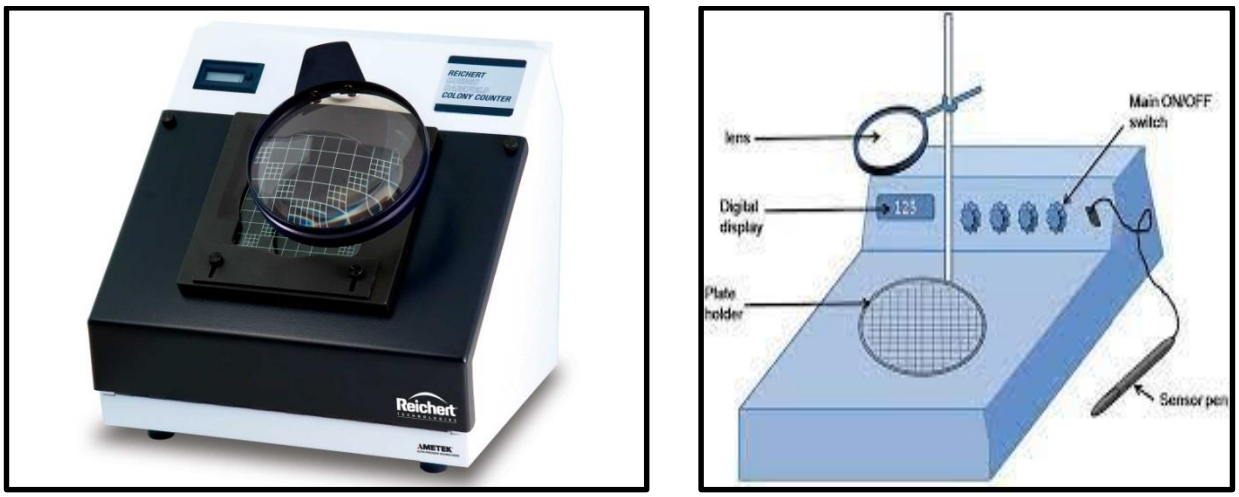
Water-bath shaker incubator

12. Quebec Colony Counter

A device used for counting the small, or closely growing colonies on the surface of media. It is used to automatically count the number of cells or colonies present on the Petri plate. Cell counters are capable of determining the number of cells, whether they are viable, and analyzing the cell width distribution. Colony counters count the number of colonies of microorganisms that have grown on an agar plate prepared from a sample. The colony counters can use fluorescent labels or the contrast between light and dark areas on the plates to make their count.

The true Quebec darkfield principle is used with this counter. When in contrast against the dark background, bacteria colonies are bright and can be distinguished quite easily from other structures in the agar. Round dishes up to 100 mm in diameter may be centred because the dish holder is adjustable. Case is 10 x 11 x 10.5 cm size. Counter comes with a 40-watt lamp, a tilt leg that may be adjusted to provide the most convenient viewing angle, and a white ruled Wolffhuegel guide plate.

The number of colonies are read out with the support of digital reading meters.



Quebec's Colony counter

Key Features of Reichert Quebec® Darkfield Colony Counter:

1. Glare-free Darkfield illumination for sharp contrast
2. Light spreads uniformly on the counting plate for easily distinguishing bacterial colonies
3. Adjustable culture dish holder for accommodating small culture dishes
4. Adjustable rod for easy focusing
5. Lens rotates for easy loading of culture dishes
6. Built-in tilt leg for height/angle adjustment
7. Optional 1.5X auxiliary magnifying lens for up to 3X magnification

Chapter - 6
Micrometry (Measurement of the size of the Microorganisms)

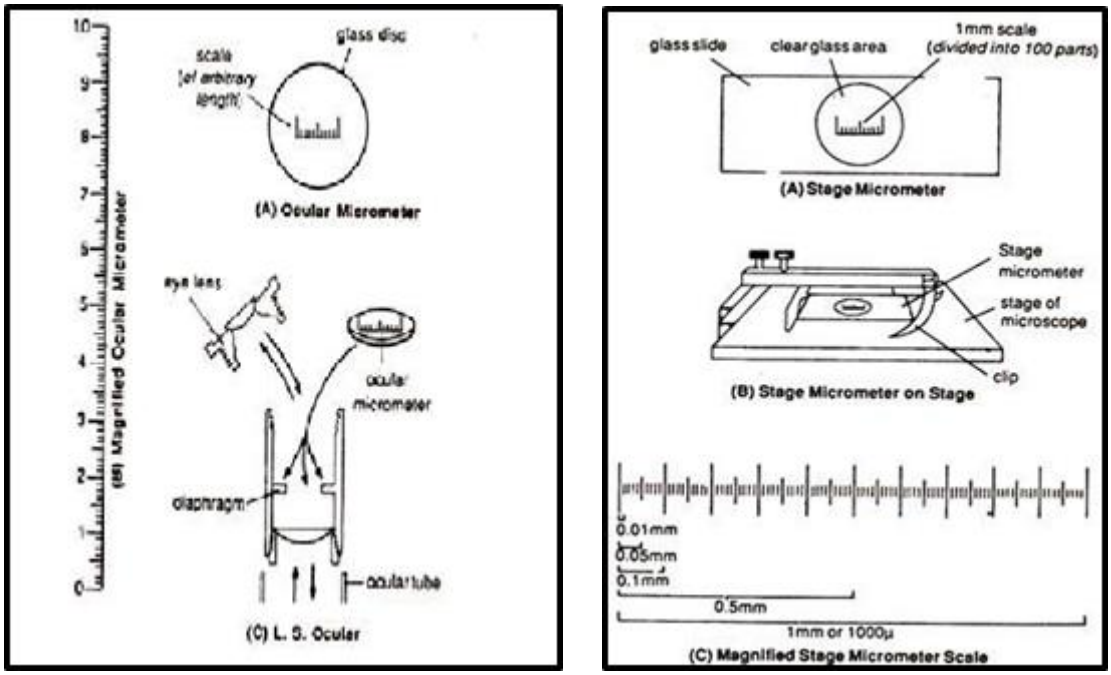
Micrometry is the science which deals with measurement of the dimensions or size of an object/ microorganisms being observed under the microscope. This method employs some special types of measuring devices which are so oriented that these can well be attached to or put into the microscope and observed. The object, to be measured, is calibrated against these scales.
An object observed under a microscope by the 10X objective and the 10X eyepiece, we say that the image that we are able to perceive is 10 × 10 = 100 times of the object i.e. image is magnified 100 times.
There are usually two types of micrometers, i.e. stage micrometer and ocular micrometer.

Stage Micrometer

As the name suggests it is for the measurement usually kept on the stage of the microscope where an object is placed. This Micrometer is a slide and has a mount of very finely graduated scale. The scale measures only 1 mm and has a least count of 0.01 mm, i.e. 1 mm region is divided into 100 divisions. As 1 mm has 1000µ, one division of stage micrometer is equivalent to 10µ.

Ocular Micrometer

This micrometer is placed inside the eyepiece. The upper eye lens is unscrewed and the ocular meter is put into the tube of eyepiece, and the eye lens is again replaced in its original position. There are usually 50 or 100 divisions in the ocular meter which are engraved on the glass.



Ocular and Stage Micrometer

Experiment 1

Aim:

To measure the dimensions of common microorganisms by calibration and standardization of microscope using stage micrometer and ocular micrometer.

Theory:

Microorganisms are microscopic objects that are visible only with the help of a microscope. Sometimes it is necessary to measure its dimensions (length, breadth and diameter) for its identification process. But, determination of the size of a microorganism is not an easy task. Micrometry refers to the measurement of dimensions of the desired microorganisms under a microscope which uses two micro-scales known as 'micrometers'. At first, the diameter of the microscopic field must be established with the help of these micrometers namely ocular micrometer and stage micrometer. Ocular micrometer with microscopic graduations etched on their surfaces is a circular glass disc that fits into the circular shelf inside the eyepiece of the microscope. It has 100 equally spaced divisions marked as 0 to 10. Depending on the objective being used, the distance between these graduations will vary that determines the size of the field. The other micrometer, stage micrometer, is clipped to the stage of the microscope. In the centre of the stage micrometer a known 1mm distance is etched into100 equally spaced divisions making each division equal 0.01 mm or 10 µm.

Requirements:

Microscope, stage micrometer, ocular meter, slide of the microorganism to be measured.

Procedure:

To work out the measurements per ocular divisions, the stage micrometer is kept under low power of microscope and is observed through the eyepiece having an ocular meter. Suppose we have a 10X objective and 10X eyepiece fitted in the microscope with a tube of 170 mm length.

At this magnification the number of ocular divisions coinciding the stage micrometer are observed and then calculated for microns per ocular divisions, e.g., let us assume that 22 ocular divisions coincide with 12 divisions of stage micrometers.

Divisions on ocular and Stage superimposed

One division of Stage= 0.01mm

One division of ocular= 12(0.01) /22× 1000µ = 5.5µ.

Or, it can also be calculated by following formula:

One division of ocular = Number of stage micrometer divisions/Number of ocular meter divisions × 10

In the case mentioned above it will be

One division of ocular= 12/22× 10µ = 5.5µ.

In this way the microscope is calibrated for different combinations of eyepieces and objective lenses and is kept for record.

Take three readings in this way, and the mean value of these readings will be the actual value of one part of the ocular meter.

Record your data in the following table.

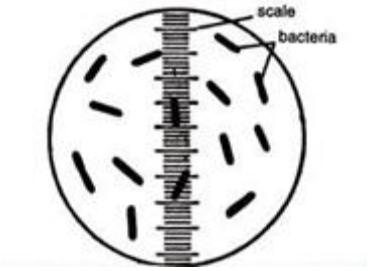
Observations:

Sr. No.	Number of divisions on Stage micrometer	Number of divisions on Ocular micrometer	One div of Ocular = Stage/ocular X 10 µ	Result
1.	**12**	**22**	**12/22x10**	**5.5 µ**
2.				
3.				

Measurement of the Microorganisms:

When the microscope is calibrated, then the object or slide of microorganism to be measured is kept on the stage of the microscope and is observed through the eyepiece with ocular. The object/ microorganism is measured in the particular magnification by ocular divisions and then is changed into microns by multiplying ocular divisions with the calibrated value of one ocular division in that particular magnification.

Suppose the diameter of a microorganism observed spans 6 divisions on ocular, then the size in microns of the microorganism will be:



Suppose the diameter of a microorganism observed spans 6 divisions on ocular, then the size in microns of the microorganism will be:

6 × 5.5µ = 33µ

In this way the dimensions of microorganisms are measured using objectives with different magnifications.

Chapter - 7
Culture media and their preparation

Cultivation of Microorganisms:

For cultivating microbes in the laboratory, we require culture media. The various mixtures of nutritive substances used for the laboratory cultivation of microorganisms are collectively known as culture media. The culture media serve as soil in which bacteria are grown for the purpose of study.

Culture Media:

Culture media must contain all the essential nutrient components required by the organism for its growth and reproduction. A suitable source of energy, building materials and growth factors must be supplied in adequate amounts. Since microorganisms show a considerable variation in their nutritional requirements, no single medium is suitable for growth of all of them.

The different types of culture media used for microbial growth falls into three groups:

1. Defined or synthetic media:

These are the media prepared from chemical compounds. They are highly purified and specific, exact amounts are dispensed in double distilled water to prepare media. An investigator working in another laboratory can duplicate the media.

2. Complex or non-synthetic media:

Media that are prepared from ingredients that have not been precisely defined. It contains hydrolysed proteins and vitamin extracts. This type of medium cannot be duplicated by another worker in another laboratory. Peptone is usually produced by boiling beef, by the hydrolysis of its protein. Casein peptone and milk peptone are also used in complex media as the source of amino acids and nitrogen.

All liquid media, whether complex or synthetic may be converted to solid media by adding either gelatin (a protein) or agar-agar, (a complex polysaccharide) extracted from red marine algae. The use of agar has an advantage. Most bacteria are unable to hydrolyse this complex molecule into more simple components. Since hydrolysed gelatin liquifies at room temperature, the use of agar is recommended as it remains solid while microbes are growing on its surface.

3. Living cells:

These are used for the cultivation of viruses. For example, fertilized eggs incubated for 8 to 12 days are able to support the growth of many viruses. Many cell lines are being used for the cultivation of viruses.

In another classification culture media are grouped into following four types:

1. **Natural media:**
   Includes substances occurring in nature such as 1) Milk 2) Eggs 3) Blood 4) Extract of plant and animal tissues.

2. **Derived media:**
   Includes known substances but the chemical composition of each is unknown. Examples are 1. Nutrient broth (prepared by boiling beef to extract nutrients and adding an amino acid-nitrogen source.) 2. Nutrient agar 3. Gelatin agar.
3. **Chemically defined media:**
   Exact chemical composition known.
4. **Special media:**
   Include combinations of the other three types of media.

There are four categories of media chiefly used in Microbiology laboratory:

They are:

1. Enrichment
2. Selective
3. Differential and
4. Propagation.

1. **Enrichment media:**

These media are prepared with ingredients that enhance the growth of certain microbes. Enrichment media encourage the growth of the suspected pathogen so that it will become the most predominant type of microbe in the culture. Two types of enrichment media are blood agar and chocolate agar.

2. **Selective media:**

They are prepared with ingredients that inhibit the growth of unwanted microbes which might be in the sample. The inhibitor may be an antibiotic, salt or other chemical. Mixed culture of microbes originally grown in enrichment media may be inoculated into selective media to isolate the desired microbe. SS Agar, DCA are selective agar.

3. **Differential media:**

They are designed to differentiate the microbes from one another. Different bacterial species may produce distinct colony colours and appearance when grown on differential agar. While in differential broth cultures, the media change colour. Differential media are used to confirm the identity of a microbe that has already been isolated by culturing in enrichment and selective media. Mac Conkey's Agar, BGLB Agar are selective and differential media.

4. Propagation media:

They are used to propagate, or keep microbes growing for a long lime. Samples grown on these media may be taken for analysis. The most common propagation media are Nutrient broth and agar.

Preparation of Media:

There are three main steps in the preparation of media:

(a) Preparation as solutions of chemicals and adjusting the pH.

(b) Dispensing the media, and

(c) Sterilization.

A broth is prepared by dissolving the appropriate amount of the components in distilled water and pH is adjusted by the addition of either dilute NaOH or HCl. The broth is dispensed

into clean rimless 'Pyrex' test tubes which are plugged with non-absorbent cotton wool plugs. The test tubes are placed in wire baskets which are covered with grease proof paper.

The media are sterilized by autoclaving at a temperature of 121 °C and a pressure of 15 lb/in^{2}for 15 minutes. But medium containing heat- sensitive substances are sterilized either by filtering the solution at room temperature, using bacteria-proof filters or by a process called Tyndallization. In this method, the liquids are steamed for one hour a day on three consecutive days and the liquids are incubated at 25-30°C. During the first steaming, all the heat sensitive vegetative cells are killed, leaving only the spores. During the first incubation period, most of the spores germinate into vegetative cells. These vegetative cells are killed by the second steam period.

In the second incubation period, the rest of the spores germinate into vegetative cells which are killed by the third steaming period. In this way, the liquids are sterilized without temperature rising above 100°C.

Maintenance of Pure Culture:

After obtaining the pure culture of a particular microbe, it may be grown and maintained as a pure culture in different ways:

1. The most common practice is to grow the culture on suitable medium until it reaches the stationary phase of growth, and then stored in a refrigerator. If they are to be kept alive for a long period all culture be subcultured timely i.e., must be transferred to a fresh sterile medium. Thus, by successive transfer, a culture may be kept for an indefinite time period.

2. A second method involves freezing of young culture and desiccating it under vacuum. The pure culture will remain viable for a long period of time if they are mixed with sterile blood serum, sterile skimmed milk, before freezing and drying. These dried cultures are kept in the sealed, evacuated tubes and are stored in cool places.

3. This method of maintaining pure culture is most suitable for spore forming species. The microorganisms are grown in pure culture in suitable media. A suspension of microorganisms is then transferred to cotton stoppered tubes of sterilized dry soil. The spores remain viable, though dormant, for long periods of time, in dry soil. The organism can be grown after a desired period, by transferring the soil into a suitable medium and incubating it under physiological conditions.

Media Preparation

When the broth medium is supplemented with agar-agar powder, it is called agar medium, for example nutrient agar medium (NA) for cultivation of bacteria, potato dextrose agar (PDA) medium for cultivation of fungi.

Experiment 2

Aim:

Preparation of solid and liquid media for the growth of microbes

A. Nutrient Agar media

Nutrient Agar is a general purpose, nutrient medium used for the cultivation of microbes supporting growth of a wide range of non-fastidious organisms. Nutrient agar is popular because it can grow a variety of types of bacteria and fungi, and contains many nutrients like water-soluble substances, vitamins, growth factors, carbohydrates, organic nitrogen compounds and salts needed for the bacterial growth.

***Requirements*:**

Nutrient agar medium
HCL 1 N
NaOH 1N
pH meter
Distilled water
Autoclave
Petri dishes
Glass rod
Beaker
Measuring cylinder

Composition of Nutrient Agar:

Peptone:	5g
Beef extract/yeast extract:	3g
NaCl:	5g
Agar:	15g
Distilled water:	1000ml

The pH is adjusted to neutral (7.4) at 25 °C.

Sodium chloride in nutrient agar maintains a salt concentration in the medium that is similar to the cytoplasm of the microorganisms.

Procedure:

1. Accurately weigh the nutrient agar medium components and transfer them into a beaker containing 500 ml distilled water.
2. Gently heat the contents with slight agitation to dissolve the medium.
3. Add more distilled water to make the volume to 1 litre.
4. Measure pH of the medium by using a pH meter and adjust the pH to 7 by adding drops of either HCl or NaOH solution.
5. Pour the medium into two or more Erlenmeyer flasks, put on cotton plugs, cover the plug with aluminium foil/paper and autoclave at 121^{o}C at 15lbs pressure for 15 minutes.
6. Sterilized media are then allowed to cool and are subsequently poured from the flask into sterilized petri dishes, test tubes, or other appropriate containers, if required or store at room temperature.

Uses of Nutrients Agar

1. It is frequently used for isolation and purification of cultures.

2. It can also be used for producing the bacterial lawns needed for antibiotic sensitivity tests.
3. Bacterial lawns for plaque formation for the study of bacteriophages.

B. Potato Dextrose Agar (PDA) medium

Requirements:

Potato tubers: 200 g
Dextrose: 20 g
Agar: 15 g
Peptone: 2 g
Distilled water: 1 litre
HCl: 1N
NaOH: 1N
Knife
Muslin cloth
Heater
Beaker (1 litre capacity)
Erlenmeyer flask (500 ml capacity): 02

Procedure:

1. Take potato tubers, peel off and weigh 200 g.
2. Chop the tubers into small pieces with the help of a knife.
3. Transfer the chopped potatoes into a beaker containing about 100 ml of distilled water.
4. Boil the contents with the help of a heater for about 20 minutes.
5. Decant supernatant, filter with fourfold of the muslin cloth and collect the filtrate into a beaker. This filtrate is called potato extract.
6. Transfer dextrose (20 g), Agar (15 g) and Peptone (2 g) into the extract and gently heat and shake to dissolve the ingredients.
7. Finally transfer this medium into a measuring cylinder of 1 litre capacity and make the volume to 1 litre by adding more distilled water.
8. Measure the pH of the medium and adjust to 5.6 by using 1N HCl or NaOH. Drop-wise.
9. Pour the medium into two or more Erlenmeyer flasks, put cotton plug, cover the plug with aluminium foil/paper and autoclave at 121^{o}C for 15 minutes.
10. Sterilized media are then allowed to cool somewhat and are subsequently poured from the flask into sterilized petri dishes, test tubes, or other appropriate containers, if required or stored at room temperature.

C. Preparation of agar deep tubes for cultivation of bacteria:

When a culture tube containing autoclaved agar, medium, is placed in an upright position, it gives deep agar tubes after solidification.

1. Follow the steps as described for preparation of media NA or PDA.
2. Pour the culture tubes culture medium placed on the Test tube Stands.
3. Place the tubes in an upright position. After 30-45 minutes the medium solidifies which is called agar deep tubes.
4. Use them soon if required or store for further use.

D. Preparation of agar slants in culture tubes:

When the autoclaved molten agar is poured into the culture tubes and the latter is placed in a slanting position, it gives agar slants after solidification.

Requirements:

Any agar medium (e.g. PDA or NA)
Culture tubes
Test tube stand
Heater
Aluminium foil or paper

Procedure:

5. Prepare agar medium as described above, following steps 1-10, or use already prepared and stored medium (if the stored medium is to be used, melt it by using a heater with gentle agitation).
6. Dispense 8-10 ml of medium into each tube and put a cotton plug. However, the amount of medium may be increased or decreased according to the volume of culture tubes.
7. Transfer all the tubes into a test tube stand or iron basket and autoclave at 121^{o}C for 15 minutes.
8. Take out the culture tubes when temperature cools down and place them in a slanting position by giving a support. Wait for about 30 minutes.
9. Thereafter, the medium is solidified and agar slants are prepared.
10. Use the slants for culture transfer if required or store them for further use.

E. Media for isolating Soil Microorganisms:

Nutrient Agar is generally used for isolation of soil microorganisms however, pH is adjusted to 6.6 to 7.0, using bromothymol blue as an indicator. Another media commonly used is Soil extract Agar for the isolation of Bacteria.

For isolation of Bacteria

a. Composition of Soil extract Agar

Glucose: 1.0g
K_2HPO_4: 0.5g
Agar: 15.0g
Soil Extract (Stock*): 100ml
Tap water: 900ml

*1000g of sieved garden soil is mixed with 1000ml of tap water and steamed in an autoclave for 30 minutes. A small amount of $CaCO_3$ is added and the whole mixture is filtered through a double filter paper.

Dissolve the agar in 900ml of water by steaming it for an hour or more. Add 100ml of the stock soil extract solution. Then add glucose just prior to pouring. Check the pH of the medium, it should be 6.8 pH.

For isolation of Fungi

PDA is generally used for isolation of soil fungi, however there are other specific media for the isolation of fungi.

a. Composition of Czapek-Dox Agar (Thom and Raper,1945)

$NaNO_3$: 3.00g

K_2HPO_4:	1.00g
$MgSO_4.7H_2O$:	0.05g
KCl:	0.05g
$FeSO_4.7H_2O$:	0.01g
Sucrose:	30.0g
Agar:	15.0g
Distilled water:	1000ml

One gram of yeast extract per litre may be added. Original Czapek's sucrose nitrate agar contains only 2g $NaNO_3$ per litre.

b. Composition of Rose-Bengal Agar (Martin,1950)

Glucose:	10.00g
Peptone:	5.00g
K_2HPO_4:	1.00g
$MgSO_4.7H_2O$:	0.05g
Streptomycin:	30.00mg
Agar:	15.0g
Rose-bengal:	0.035g
Distilled water:	1000ml

The antibiotic is sterilized separately and added aseptically to the sterilized medium.

For isolation of Actinomycetes

a. Starch Casein Agar

Soluble Starch:	10.0g
Vitamin free Casamino acids:	0.3g
$CaCO_3$:	0.02g
$Fe_3SO_4.7H_2O$:	0.01g
KNO_3:	2.0g
$MgSO_4.7H_2O$:	0.05g
NaCl:	5.0g
Agar:	18.0g
Aged Sea Water:	1000ml
pH:	7.1

b. Composition of Kenknight and Munaier's Medium

Dextrose:	1.00g
K_2HPO_4:	0.10g
$NaNO_3$:	0.10g
KCl:	0.10g
$MgSO_4.7H_2O$:	0.10g
Agar:	15.0g
Distilled water:	1000ml

Asparagine mannitol agar and soil extract agar can also be used for the isolation of actinomycetes.

For isolation of Yeasts

a. Composition of Malt extract Agar

Malt extract: 30.0g
Mycological peptone: 5.0g
Agar: 20.0g
Distilled water: 1000ml
pH: 5.4

In order to inhibit the growth of bacteria 10% sterile lactic acid solution can be added to the molten medium just before pouring the Petri plates so as to bring down the pH to 3.5.

b. Composition of Glucose Yeast extract Peptone Agar

Glucose: 20.0g
Bacto peptone: 10.0g
Bacto yeast extract: 5.0g
Agar: 20.0g
Distilled water: 1000ml
pH is not adjusted

For isolation of Blue Green Algae

a. Composition of Pringsheim's Medium

KNO_3: 0.02%
$MgSO_4.7H_2O$: 0.001%
$(NH_4)_2HPO_4$: 0.002%
$CaCl_2.6H_2O$: 0.0005%
$FeCl_3$: 0.00005%

b. Composition of Chu's Medium No.10

Ca $NO_3)_2$: 0.004%
$MgSO_4.7H_2O$: 0.0.0025%
K_2HPO_4: 0.0005 to 0.001%
Na_2CO_3: 0.002%
Na_2SiO_3: 0.0025%
$FeCl_3$: 0.0008%

For isolation of Protozoa

Holozoic protozoa are isolated by using pure cultures of bacteria such as Aerobacter known to be edible by protozoa as the substrate. Silica gel plates are usually prepared where glass rings are embedded to contain different strains of bacteria so as to prevent the spreading of one to another. 0.5ml of diluted soil sample is added as inoculum into bacterial circles in the centre.

When protozoa grow on the bacterial circles, the bacterial colonies disappear indicating the presence of protozoa.

Experiment 3

Aim:

Isolation and enumeration of microorganisms from soil by plate count method.

Principle:

Soil acts as an excellent medium for the growth of microorganisms. These microorganisms include microflora (bacteria, fungi and actinomycetes) and microfauna (protozoa and nematodes). In terms of numbers and biological activity the microorganisms are predominant in soil. Bacteria are small (about 1 - 10 µm) and occur in three general shapes rod (bacillus), spherical (coccus) and spiral (spirilla). Bacilli and cocci are more common in soil. Fungi are filamentous and macroscopic in nature. The branched hyphae exhibit cell divisions and fungal mycelia (hyphal mass). Actinomycetes are also filamentous, branched and macroscopic.

In this method, soil is dispersed in an agar medium so that individual microbial cells, spores or mycelial fragments develop into macroscopic colonies. The procedure involves successive dilutions of soil. Depending upon the extent of dilution, plates may be represented with a huge number of colonies or very few. Enumeration of colony-forming units (CFUs) present on the agar should be in between 20 to 300.

Requirements:

Soil

Media: Nutrient Agar, Martin's Rose-bengal Agar and Starch-casein Agar

Petri plates

Test tubes

Pipettes

Autoclave

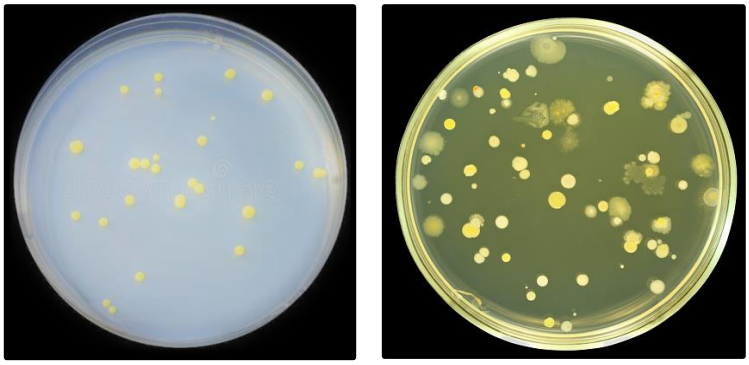
Procedure:

1. Take 1 flasks (250ml capacity), transfer 90 ml distilled water in flask, plug properly, and autoclave at 15 lb /inch^2 for 30 minutes. Similarly, take 10ml capacity test tubes containing 9ml distilled water each, sterilize and label 1-5.
2. Collect small amount of garden soil from a desired location.
3. Weigh 10 g soil and transfer into a flask containing 90 ml sterilized distilled water. It gives the stock solution.
4. Shake the flask gently for 10 minutes using magnetic stirrer or electric shaker to get homogenous suspension.
5. Transfer 1 ml suspension from stocksolution into Test tube 1 containing 9 ml sterilized distilled water to get dilution 10^{-1}. Mix the suspension gently.
6. Similarly, serially transfer 1 ml suspension from 10^{-1}dilution to Test tube 2 to get 10^{-2} and so on to test tube 5 containing 9 ml sterilized distilled water to get dilution the final dilution of 10^{-5}. Mix the suspension thoroughly and gently.
7. Aseptically pour 1 ml suspension from 10^{-3}dilution into Martin'sagar plates (supplemented with Streptomycin-30 mg/l) for isolation of fungi. Gently rotate the plates so as to spread the suspension on medium.

8. Similarly transfer 1 ml suspension from dilution 10^{-4} or 10^{-5} on Starch-casein agar plate for isolation of actinomycetes and 1 ml from dilution 10^{-5} or 10^{-6} onto Nutrient agar plate for isolation of bacteria.
9. Incubate the Nutrient Agar plates at 37± 1 ^{o}C for 24 hours. Martin's Agar plates at 25 ± 1^{o}C for 4-5 days, Starch casein agar plates at 30-35 ^{o}C for 7-10 days.

Observations:

Small, shiny bacterial colonies grow on Nutrient Agar plates. Fungal colonies of different size and colour grow on Sabouraud's agar, Potato dextrose and Martin's rose bengal Agar. Small radiating colonies of powdery appearance of actinomycetes grow on Starch casein Agar. Count the number of colonies on agar plates that contains from 30 to 300 colonies. Don't count from those plates that contain colonies larger than 2 cm diameter. Multiply by dilution, this gives the number of colony forming units (CFUs) per gram of soil.

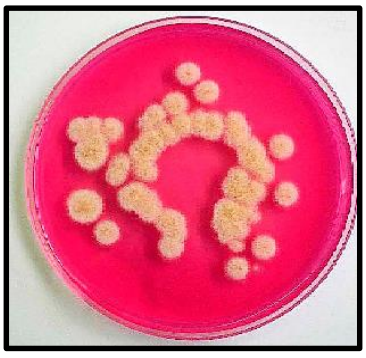


Bacterial colonies on Nutrient Agar

***Actinomycetes* on Starch Casein Agar**

Sabouraud's Agar

Potato Dextrose Agar



Yeast on Rose Bengal Agar

Fungal colonies on different Agar media

Chapter - 8
Isolation of Pure Cultures

In natural habitats, microorganisms usually grow in complex, mixed populations with many species. This presents a problem for microbiologists because a single type of microorganism cannot be studied adequately in a mixed culture. Therefore, one needs to obtain a **pure culture**, a population of cells arising from a single cell to characterize an individual species.

There are special techniques employed to obtain pure cultures of microorganisms. In few cases it is possible to secure pure culture by direct isolation or direct transfer. This can be done only in those situations in which pure culture occurs naturally. Kinds of specimens taken for culturing will depend on the nature and habitat of microbes.

Different pathogens can be isolated from body tissues and fluids such as blood, urine, sputum, pus, faeces, spinal fluid, bile, pleural fluids, stomach fluids etc.

In the bloodstream of a patient suffering with typhoid fever, the bacteria Salmonella typhi can be cultured. A pure culture of this bacterium may be obtained by drawing blood samples using a sterilized hypodermic syringe and treating the blood with anticoagulants such as heparin and potassium oxalate. The presence of the anticoagulant prevents the pathogenic microbe from entrapping in fibrin clot. The sample of the blood may be inoculated into a suitable medium.

Many different techniques are followed to obtain pure cultures of microorganisms, those are:

1. Streaking
2. Plating
3. Dilution
4. Enriched procedure
5. Single cell technique.

1. Streaking

This is the most widely used method of isolation. The technique consists of pouring a suitable sterile medium into a sterile petri plate and allowing the medium to solidify. By means of a sterile loop or inoculating needle, a small amount of growth preferably from a broth culture or bacterial suspension is streaked back and forth across the surface of agar until about one third of the diameter of the plate has been covered.

The inoculating needle is then flamed and streaking is done at right angles to and across the first streak. This serves to drag bacteria perpendicular in a long line from the initial streak. When this streaking is completed, the needle is again incinerated, cooled and streaking is done at right angles to the second streak and parallel to the first.

2. Plating:

It includes diluting a mixed culture of microorganisms until only a few hundred bacteria are left in each millilitre of the suspension. A very small amount of the dilution is then placed in a sterile petri plate by means of a sterile loop or pipette. The melted agar medium is cooled to

about 45 °C and is poured into a plate. The microorganism and agar are mixed homogeneously and allowed to cool. When the agar is solidified the individual bacterium will be held in place and will grow to a visible colony.

3. Dilution:

This method is used for the microorganisms which cannot be easily isolated by streaking or plating methods. Sometimes when several organisms are present in a mixture, with one organism predominating, the predominating form may be isolated by this method. For example, when raw milk is allowed to sour at room temperature it will, at the time of curdling, have a mixture of microorganisms with high percentage of *Streptococcus lactis*.

If 1 ml of the sour milk is taken into a tube containing 9 ml of sterile milk (in which no organisms are present) then 1 ml of this mixture is transferred with a sterile pipette into a second tube containing 9 ml of sterile milk and the procedure is repeated i.e., from second to third tube, third to fourth tube until a series of dilutions are prepared. By this serial dilution, there are high chances of obtaining pure culture of *Streptococcus lactis*.

4. Enrichment technique:

This procedure involves the use of media and conditions of cultivation which favour the growth of the desired species. For example, when a man suffers with typhoid, the intestinal discharge possesses few numbers of *Salmonella typhi* when compared with *E. coli* and other forms.

It is almost impossible to isolate the typhoid organisms because they represent only a fraction of a percent of the total microorganisms present. The media are therefore derived, which allow the rapid growth of the desired organisms, at the same time inhibiting the growth of other microorganisms. Enrichment and Selective media are often used for isolation of pure culture of these microorganisms.

5. Single cell isolation Technique:

This is one of the most ideal and difficult methods of securing pure culture. In this method a suspension of the pure culture is placed on the under-side of a sterile cover-glass mounted over a moist chamber on the stage of the microscope.

While looking through the microscope, a single cell is picked up with the help of sterile micropipette and transferred to a small drop of sterile medium on a sterile cover-glass and is mounted on a sterile hanging drop slide, which is then incubated at suitable temperature. If the single cell reproduces/germinates in this drop, few cells are transferred into a tube containing sterile culture medium which is placed in the incubator to obtain pure culture originated from single cell.

6. Other methods:

The isolation of anaerobic microorganisms is very difficult. There are certain special techniques by which these organisms are isolated.

Experiment 4

Aim:

Isolation of pure culture from mixed cultures by Streak Plate Method.

Principle:

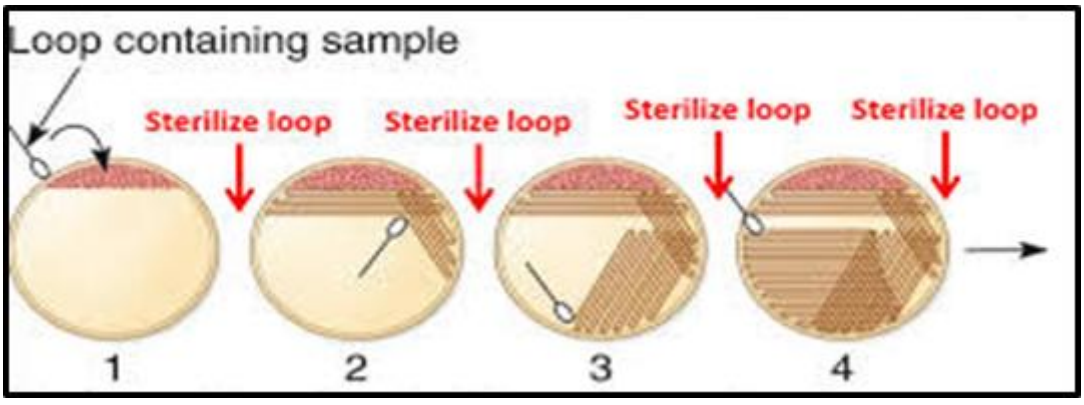
Microorganisms usually grow in mixed populations with other species in their natural habitat. If cells from mixed cultures can be spatially isolated from each other, each cell will give rise to a completely separate macroscopically visible colony on the agar. Because each colony arises from a single cell, each colony represents a pure culture. Therefore, pure cultures can be obtained by isolating individual cells using one of the methods i.e., streak plate method.

Requirements:

Nutrient Agar
Inoculating needle
Sterile petri plates
Mixed culture
Bunsen burner

Procedure:

1. Liquefy a nutrient agar and pour into the sterile Petri Plates, rotate the plate gently for uniform distribution of the medium and allow it to solidify.
2. Take a loopful of culture with the help of sterile inoculating needle and transfer it on the edge of the agar plate and then streak out over the surface of the agar. Streak the plate following 4 quadrants or radiant or T-streak or continuous streak.
3. In 4 quadrant procedures, after the first streak, the inoculating needle is sterilized and an inoculum for the second streak is obtained from the first streak sector.
4. A similar process is followed for streaking the third and fourth sector, except that the inoculum is obtained from the second and third streak sector respectively.
5. Keep the streaked plates in an inverted position at 37^0C for 24 hrs.
6. Place the petri plates in inverted position in an incubator to solve the problem of water condensation because if it drops down on the colonies, the organisms of one colony can spread on to the other colony.



Streak Plate Method: Steps in Quadrant Method

Observation:

Observe the petri plates for isolated bacterial colonies on the surface of the solidified nutrient agar.

Streak Plate method: *E. coli* on EMB Agar

Results:

The isolated colony of desired microorganism on the agar plates will be observed.

Experiment 5

Aim:

Isolation of microbial pure culture from mixed cultures by Pour Plate Method.

Principle:

Pour plate method is extensively used for bacteria, archaea, and fungi. The mixed culture is diluted several times to reduce the microbial population sufficiently to obtain separate colonies when plating. Then, an aliquot volume of diluted sample is mixed with liquid agar molten that has been cooled to about 45^0C and mixture is poured into sterile petri plates. This method has an advantage over the streak plate method as it does not require much skill, however it has disadvantages that the media and glassware requirement are more and is laborious.

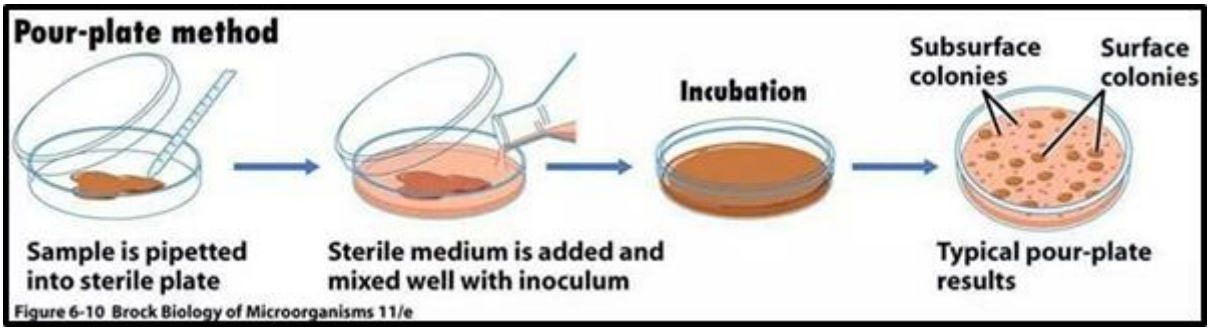
Requirements:

Mixed bacterial cultures
Nutrient Agar
Petri plates, Test tubes
Water bath
Pipettes
Glass marker

Procedure:

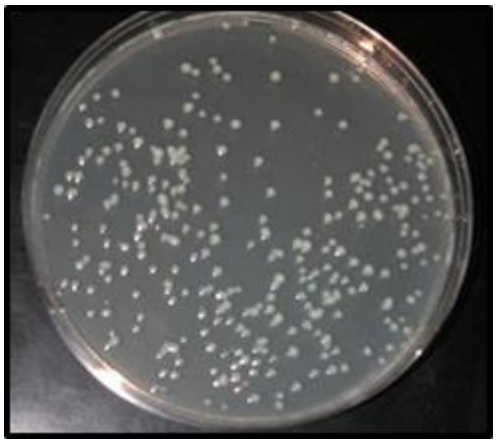
1. Take 5 sterilized test tubes containing 9ml sterilized distilled water and place them on a test tube stand, label them 1 to 5.
2. Prepare dilution by transferring 1ml of mixed bacterial suspension into tube 1(dilution 10^{-1}).
3. After thorough mixing, again transfer 1ml of mixed culture from tube 1 in tube 2 (dilution 10^{-2}).
4. Shake the tube gently between the palms and transfer 1ml of culture from tube 2 into tube 3 (dilution 10^{-3}).
5. Similarly prepare dilutions to the final dilution 10^{-5}.

6. Prepare Nutrient Agar and pour 10 to 12 ml in each 5 test tubes, autoclave at 121 ^{o}C at 15lbs pressure for 15 minutes.
7. Place all the test tubes containing liquefied nutrient agar on a water bath maintained at 45 ^{0}C.
8. Pour 1ml of dilutions 10^{-4}, 10^{-5} each into test tubes containing molten nutrient agar, gently mix and immediately pour onto the sterile petri plates.
9. After solidifying the medium, incubate at 37^{o}C for 24 hrs in inverted position in an incubator.



Observation:

Observe the petri plates for bacterial colonies. Each bacterial cell becomes fixed in place to form an individual colony after the agar is solidified and incubated. Therefore, you can observe colonies on the surface of agar as well embedded in the agar. The total number of colonies equals the number of viable microorganisms in the mixed culture capable of growing in the medium used.



Pour Plate method: *E. coli* (surface and embedded colonies)

Results:

The colonies of similar shape, size and colour will be visible. The isolated colonies can be counted or used to obtain pure cultures.

Experiment 6

Aim:

Isolation of microbial pure culture from mixed cultures by the Spread Plate Method.

Principle:

Spread plate and Pour plate techniques are similar in that they both dilute a sample or mixed cultures so as to separate cells spatially. They differ in that the spread plate spreads the cells on the surface of the agar, whereas the pour plate embeds the cells within the agar.

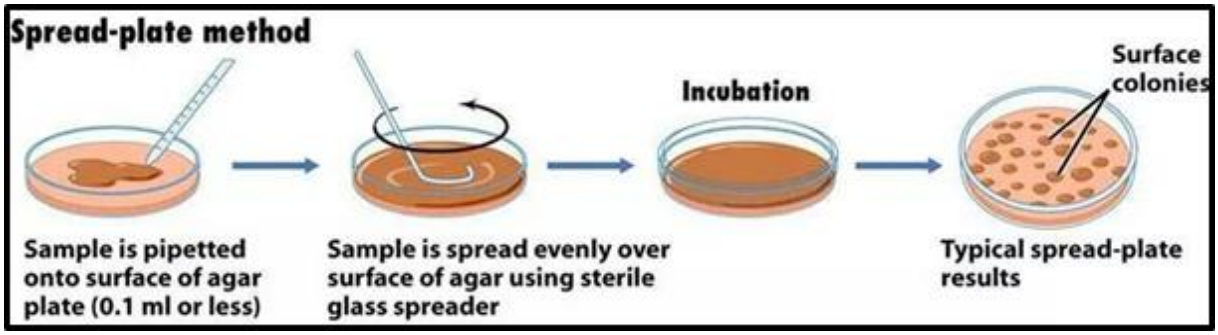
In this technique, the microorganisms in diluted samples are spread over solidified agar medium with the help of an L-shaped glass tube called spreader, when the petri plate is spinning on a turntable (revolving platform for reversing locomotive) or petri plate can be rotated manually.

Requirements:

Mixed culture of *S. aureus* and *E. coli*
Nutrient agar plates
Turntable
L-shaped glass spreader
Ethanol (95%)
Bunsen burner
Beaker
Incubator

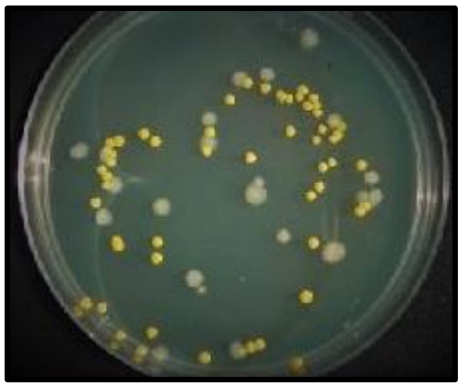
Procedure:

1. Take 5 sterilized test tubes containing 9ml sterilized distilled water and place them on a test tube stand, label them 1 to 5.
2. Prepare dilution by transferring 1ml of mixed bacterial suspension into tube 1(dilution 10^{-1}).
3. After thorough mixing, again transfer 1ml of mixed culture from tube 1 in tube 2 (dilution 10^{-2}).
4. Shake the tube gently between the palms and transfer 1ml of culture from tube 2 into tube 3 (dilution 10^{-3}).
5. Similarly prepare dilutions to the final dilution 10^{-5}.
6. Prepare Nutrient Agar plates.
7. 0.1ml of diluted mixed culture is then pipetted to the centre of solidified nutrient agar.
8. Place the petri plate on the turntable (revolving platform).
9. Sterilize the spreader by putting it first in ethanol (95%) in a beaker, then on the flame of Bunsen burner and cool the rod for 60 seconds.
10. Remove the lid of Petri Plate and spin the turntable, very gently touch the spreader on the surface of agar when the turntable is spinning or move it forth and back rotating the petri plate to spread bacterial cells on agar surface manually.
11. When the turntable stops running, put the lid over the lower half of the Petri Plate.
12. Sterilize the spreader as in step 9 and repeat steps 7 to 11 for petri plates 2 and 3.
13. Incubate all the plates at 37^{o}C for 24 hrs in inverted position in an incubator.



Observation:

Observe the plates for individual bacterial colonies on the surface of the solidified nutrient agar.



Streak Plate method: mixed culture on nutrient Agar

Results:

Few of the colonies grow individually and do not overlap the other colony. It may be picked up and purified through sub-culturing and maintained as pure culture.

Chapter - 9
Preparation, Maintenance and Preservation of Pure Cultures

The unknown cultures are often used for making many different kinds of slides and inoculations. Despite meticulous aseptic practice, the chance of contamination of these cultures increases with frequency of use. If you are allowed to make all your inoculations from the single tube given to you, it is very likely that somewhere along the way, contamination would surely result and spoil your pure culture.

Another problem is the aging of the culture. Two or three weeks are required for the performance of all tests. In this period of time, the organisms in the broth culture may die, particularly if the culture is kept very long at room temperature. To ensure against the hazards of contamination or death of your organisms, it is essential that you prepare stock cultures before any slides or routine inoculations are made.

Experiment 7

Aim:

To prepare working and reserve stock cultures

Principle:

Different types of organisms require different kinds of stock media, but for undergraduate students, performing microbiology experiments, nutrient agar slants will suffice. For each unknown, you will inoculate two slants. One of these will be your reserve stock and the other one will be your working stock.

The reserve stockculture will not be used for making slides or routine inoculations; instead, it will be stored in the refrigerator after incubation until some time later when a transfer may be made from it to another reserve stock or working stock culture.

The working stock culture will be used for making slides and routine inoculations. When it becomes too old to use or has been damaged in some way, replace it with a fresh culture that is made from the reserve stock.

Note that one slant will be incubated at 20° C and the other at 37° C. This will enable you to learn something about the optimum growth temperature of your unknown.

Inoculate two nutrient agar slants from each of your unknowns as follows:

Requirements:

Nutrient agar slants (screw-cap tubes)
Test Microorganisms (unknown)

Procedure:

1. Label two slants with the code number of the unknown and your initials. Use gummed labels. Also, mark one tube 20° C and the other 37° C.
2. With a loop, inoculate each slant with a straight streak from the bottom to the top. Since these slants will be used for your cultural study in Exercise 47, a straight streak is more useful than one that is spread over the entire surface.
3. Place the two slants in separate baskets on the demonstration table that are designated

with labels for the two temperatures (20° C and 37° C).

4. Although the 20° C temperature is thought of as "room temperature," it should be incubated in a biological incubator instead of leaving it out at laboratory room temperature. Laboratory temperatures are often quite variable in a 24-hour period.

Observation:

After 24 hours of incubation, evaluate the slants made from each of your unknowns, as follows:

1. Examine the slants to know the extent of growth. Some organisms require close examination to see the growth, especially if the growth is thin and translucent.
2. Determine which temperature seems to promote the best growth.
3. Record the presumed optimum temperature. (Obviously, this may not be the actual optimum growth temperature, but for all practical purposes, it will suffice.)
4. If there is no growth visible on either slant, there are several possible explanations:
    a. It may be that the culture you were issued was not viable.
    b. Another possibility might be that the organism grows too slowly to be visible at this time.
    c. Or, possibly, neither temperature was suitable!

    Think through these possibilities and decide what you should do to circumvent the problem.
5. Label the tube with the best growth reserve stock. Label the other tube working stock.
    a. If both tubes have good growth, place them in the refrigerator until needed.
    b. If one tube has very scanty growth, refrigerate the good one (reserve stock) and incubate the other one at the more desirable temperature for another 24 hours, then refrigerate.
6. Remember these points concerning your stock cultures:
    1. Most stock cultures will keep for 4 weeks in the refrigerator. Some fastidious pathogens will survive for only a few days. Although none of the organisms issued in this unit are of the extremely delicate type, don't wait 4 weeks to make a new reserve stock culture; instead, make fresh transfers every 10 days.
    2. Don't use your reserve stock culture for making slides or routine inoculations.
    3. Don't store either of your stock cultures in your desk drawer or a cupboard.

After the initial incubation period cultures must be refrigerated. After 2 or 3 days at room temperature, cultures begin to deteriorate. Some die out completely.

Preservation of Pure cultures

Once a microorganism has been isolated and grown in pure culture, it becomes necessary to maintain the viability and purity of the microorganism by keeping the pure culture free from contamination. Normally in laboratories, the pure cultures are transferred periodically onto or into a fresh medium (subculturing) to allow continuous growth and viability of microorganisms. The transfer is always subject to aseptic conditions to avoid contamination.

Since repeated subculturing is time-consuming, it becomes difficult to maintain a large number of pure cultures successfully for a long time. In addition, there is a risk of genetic

changes as well as contamination. Therefore, it is now being replaced by some modern methods that do not need frequent subculturing. These methods include refrigeration, paraffin method, cryopreservation, and lyophilization (freeze-drying).

A. Periodic Transfer to Fresh Media:

Strains can be maintained by periodically preparing a fresh culture from the previous stock culture. The culture medium, the storage temperature, and the time interval at which the transfers are made vary with the species and must be ascertained beforehand. The temperature and the type of medium chosen should support a slow rather than a rapid rate of growth so that the time interval between transfers can be as long as possible. Many of the more common heterotrophs remain viable for several weeks or months on a medium like nutrient agar.

The transfer method has the disadvantage of failing to prevent changes in the characteristics of a strain due to the development of variants and mutants.

B. Refrigeration:

Pure cultures can be successfully stored at 0-4 °C either in refrigerators or in cold-rooms. This method is applied for a short duration (2-3 weeks for bacteria and 3-4 months for fungi) because the metabolic activities of the microorganisms are greatly slowed down but not stopped. Thus their growth continues slowly, nutrients are utilized and waste products released in medium. This results in, finally, the death of the microbes after some time.

C. Paraffin Method/ preservation by overlaying cultures with mineral oil:

This is a simple and most economical method of maintaining pure cultures of bacteria and fungi. In this method, sterile liquid paraffin is poured over the slant (slope) of culture and stored upright at room temperature. The layer of paraffin ensures anaerobic conditions and prevents dehydration of the medium. This condition helps microorganisms or pure culture to remain in a dormant state and, therefore, the culture can be preserved from months to years

The advantage of this method is that we can remove some of the growth under the oil with a transfer needle, inoculate a fresh medium, and still preserve the original culture. The simplicity of the method makes it attractive, but changes in the characteristics of a strain can still occur.

D. Cryopreservation:

Cryopreservation (i.e., freezing in liquid nitrogen at -196 °C or in the gas phase above the liquid nitrogen at -150 °C) helps the survival of pure cultures for long storage times. In this method, the microorganisms of culture are rapidly frozen in liquid nitrogen at -196 °C in the presence of stabilizing agents such as glycerol or dimethyl sulfoxide (DMSO) that prevent cell damage due to the formation of ice crystals and promote cell survival.

This liquid nitrogen method has been successful with many species that cannot be preserved by lyophilization and most species can remain viable under these conditions for 10 to 30 years without undergoing a change in their characteristics, however, this method is expensive.

E. Lyophilization (Freeze-Drying):

Freeze-drying is a process where water and other solvents are removed from a frozen product **via sublimation.** Sublimation occurs when a frozen liquid goes directly to a gaseous state without entering a liquid phase.

Cryopreservation method

Lyophilization requires slow rates of cooling, as this will result in the formation of vertical ice crystal structures, thus allowing for more efficient water sublimation from the frozen product. Freeze-dried products are hygroscopic and must be protected from moisture during storage. Under these conditions, the microbial cells are dehydrated and their metabolic activities are stopped; the microbes go into a dormant state and retain viability for years. Lyophilized pure cultures are then sealed and stored in the dark at 4 °C in refrigerators.

Lyophilization or Freeze-drying method is the most frequently used technique by culture collection centers. Many species of bacteria preserved by this method have remained viable and unchanged in their characteristics for more than 30 years.

Advantage of Lyophilization:

1. Only minimal storage space is required; hundreds of lyophilized cultures can be stored in a small area.
2. Small vials can be sent conveniently through the mail to other microbiology laboratories when packaged in a special sealed mailing container.
3. Lyophilized cultures can be revived by opening the vials, adding the liquid medium, and transferring the rehydrated culture to a suitable growth medium.

Lyophilization of cultures

Chapter - 10
Microbial Growth in Liquid and on Solid Surfaces

A culture Medium is a solid or liquid preparation used to grow, transport, and maintain microorganisms. The medium must contain all the nutrients the microorganisms require for their growth. Specialized media are essential in the isolation and identification of microorganisms, testing of antibiotic sensitivities, water and food analysis, industrial microbiology and other activities. Media can also be specifically designed to facilitate the growth of one type of microbe present in their natural habitat or from a sample obtained.

Microbial growth is exhibited differently in Liquid and on solidified media. Growth is indicated as turbidity in the broth whereas colony development is observed on agar. Bacteria growing on the solid surface of agar can form quite complex and intricate colony shapes. These patterns vary with nutrient availability and the hardness of the agar surface. The size and shape of a colony depend on many factors. Nutrient diffusion and availability, bacterial chemotaxis, and presence of liquid on the surface, cell-cell communication, all of these play an important role in pattern formation of the colony.

Experiment 8

Aim:

To demonstrate microbial growth in Liquid and on Solid media

Principle:

Media can be in a liquid form (broth) or solidified by a gelling agent such as agar. A medium in which all the chemical components are known is a defined or synthetic medium and when some components of unknown chemical composition are used are complex media. Although both liquid and solidified media are routinely used, solidified media are particularly important because they can be used to isolate different microbes from each other for study of their morphological characteristics, their identification, classification, demonstrating relationship between a microbe and disease, and industrial importance.

Microbial growth is indicated as turbidity in the broth or development of colonies on the surface of agar. In the broth, a cell suspension looks cloudy (turbid) to the eye, because cells scatter light passing through the suspension. The more bacterial cells that are present, the more light is scattered, and hence the more turbid observed in the Broth.

Requirements:

Pure culture of *E. coli*
Soil sample
Nutrient Broth
Nutrient agar and PDA plates
Ethanol (95%)
Bunsen burner
Inoculating needle
Incubator

Composition of Nutrient Broth (Complex medium)

Peptone:	5g
Beef extract/yeast extract:	3g
NaCl:	5g
Distilled water:	1000ml

The pH is adjusted to neutral (7.4) at 25 °C.

Composition of Nutrient Broth (Synthetic medium for E. coli)

Glucose:	1.0g
Na_2HPO_4:	16.4g
KH_2PO_4:	1.5g
$(NH_4)_2SO_4$:	2.0g
$MgSO_4.7H_2O$:	200.0mg
$CaCl_2$:	10.0mg
$FeSO_4.7H_2O$	0.5mg

Final pH 6.8 -7.0

Composition of Nutrient Agar

Peptone:	5g
Beef extract/yeast extract:	3g
NaCl:	5g
Agar:	15g
Distilled water:	1000ml

The pH is adjusted to neutral (7.4) at 25 °C. The pH is adjusted to neutral (7.4) at 25 °C.

Composition of Mac Conkey's Agar

Pancreatic digest of gelatin:	17.0g
Pancreatic digest of casein:	1.5g
Peptic digest of animal tissue:	1.5g
Lactose:	10.0g
Bile Salts:	1.5g
NaCl:	5.0g
Neutral red:	0.03g
Crystal violet:	0.001g
Agar:	13.5g
Distilled water:	1000ml

Procedure:

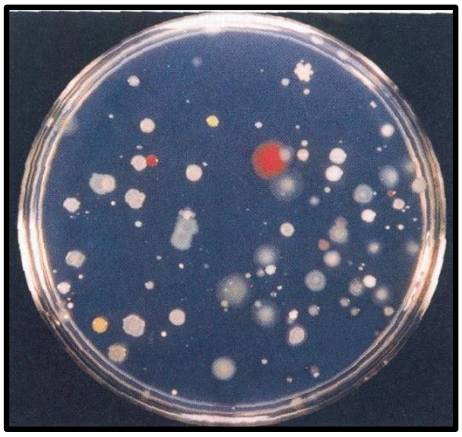
1. Prepare Nutrient (broth and Agar), Synthetic medium and Mac Conkey's agar in the Erlenmeyer Flasks, Label them and autoclave at 121^0C at 15 lbs for 15mins.
2. Take a loopful of pure culture of *E. coli* with the help of sterilized inoculating needle and inoculate the broth media 10-12 ml each filled in sterilized Test tubes with cotton plugs.
3. Similarly, perform four-way streak plate method on Nutrient and Mac Conkey's Agar Plates by taking a loopful of pure culture of *E. coli*.
4. Perform spread plate technique using soil dilution (10^{-5}) for colony characteristics on Nutrient and PDA plates.

5. Incubate the tubes by placing them upright in the beaker in an incubator at 37 ^{o}C for 24 hrs.
6. Incubate all the petri plates at 37 ^{o}C for 24 hrs in an inverted position in an incubator.

Observation:

Observe the tubes for turbidity and petri plates for individual bacterial colonies with different colony morphology on the surface of the solidified nutrient agar and PDA plates.

Turbidity observed in the tubes (right) and control (left)



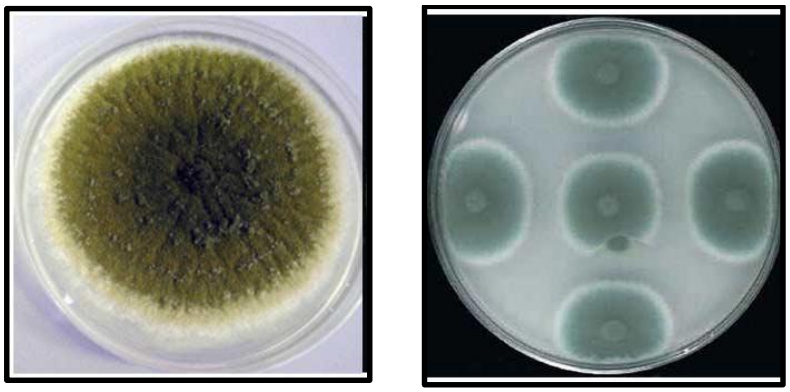
Colonies on Nutrient Agar

MARGIN	COLOUR	ELEVATION	TEXTURE	SHAPE
Curled	Orange	Raised	Slimy, moist	Round
Entire (smooth)	Red or pink	Umbonate	Matte, brittle	Punctiform
Filamentous	Black	Flat	Shiny, viscous	Rhizoid (root-like)
Undulate (wavy)	Brown	Convex	Dry, mucoid	Filamentous
Lobate	Opaque or white	Pulvinate (Cushion-shaped)	Translucent	Irregular
Erose (serrated)	**Milky**	**Growth into culture medium**	**Iridescent (changes colour in reflected light)**	**Spindle**

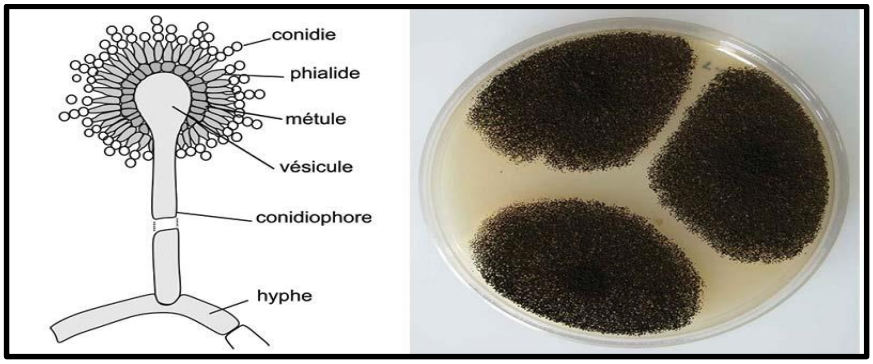
Source: MicroDok

Colony Characteristics of Bacterial colonies on Nutrient Agar

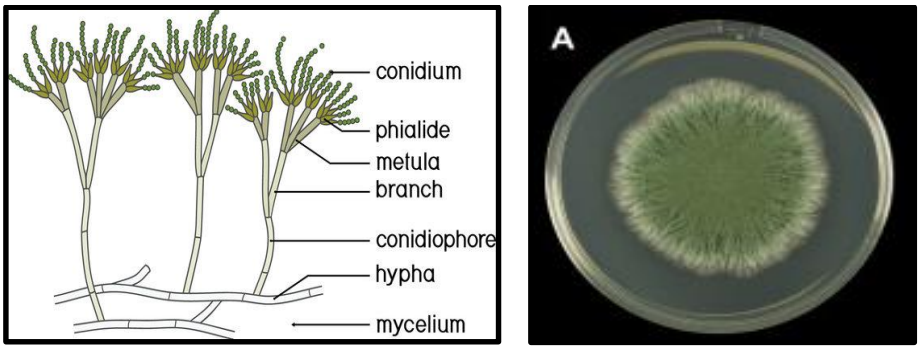
Fungal Growth: (Microscopic observation of Fungi)



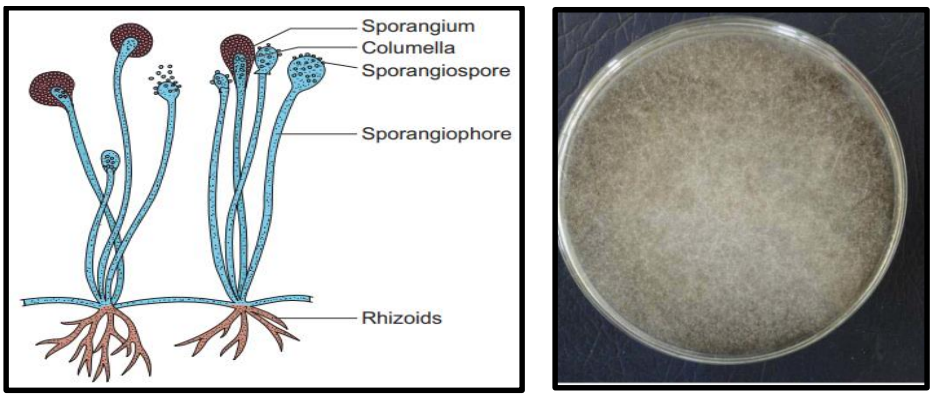
***Aspergillus flavus* and *Penicillium* on Potato Dextrose Agar**



Aspergillus niger



Penicillium roqueforte



Rhizopus oryzae

Chapter - 11
Microbial growth Curve

A characteristic growth pattern of bacteria is observed when fresh liquid medium is inoculated with a given number of bacteria and incubated for a sufficient period of time. If the bacterial population is measured periodically and the log of number of viable bacteria is plotted in a graph against time, it gives a characteristic growth curve which is known as growth curve.

Experiment 9

Aim:

To study the microbial growth curve

Principle:

Measuring the growth rate of bacteria is a fundamental microbiological technique, and has widespread use in basic research as well as in agricultural and industrial applications.

When bacteria are inoculated into a liquid medium and the cell population is counted at intervals, it is possible to plot a typical bacterial growth curve that shows the growth of cells over time. It shows four distinct phases of growth.

- **Lag phase:** Slow growth or lack of growth due to physiological adaptation of cells to culture conditions or dilution of exoenzymes due to initial low cell densities. It is the synthesis phase where DNA replication occurs and the synthesis of cellular components necessary for growth.
- **Log or exponential phase:** Optimal growth rates, during which cell numbers double at discrete time intervals known as the mean generation time.
- **Stationary phase:** Growth (cell division) and death of cells counterbalance each other resulting in no net increase in cell numbers. The reduced growth rate is usually due to a lack of nutrients and/or a buildup of toxic waste constituents.
- **Decline or death phase:** Death rate exceeds growth rate resulting in a net loss of viable cells.

Turbidimetric determination is useful for plotting growth curves of bacteria in broth or liquid media. It is one of the simplest methods used to analyze trends in growth because it uses a spectrophotometer to track changes in the optical density (OD) over time. In other words, as the number of cells in a sample increases, the transmission of light through the sample will decrease.

Requirements:

Bacterial culture (E. coli), Broth (Luria Bertani (LB) Broth, Nutrient Broth)
Conical flasks, Measuring cylinder
Sterile test tubes, Sterile Petri Plates
Incubator
Spectrophotometer
Micropipettes

Procedure:

Day 1:

1. Using a sterile loop, streak a loopful of bacterial culture onto the agar plate.
2. Incubate at 37 ºC for 18-24 hours.

Day 2:

1. Pick up a single colony of each strain from the agar plate and inoculate it into a test tube containing 10 ml of autoclaved broth.
2. Incubate the test tube overnight at 37 ºC.

Day 3:

1. Take 250 ml of autoclaved broth in a sterile 500 ml conical flask.
2. Inoculate 5 ml of the overnight grown culture in the above flask.
3. Take OD at zero hour. Incubate the flask at 37 ºC.
4. Aliquot 1 ml of the culture suspension at an interval of every 30 minutes and take the optical density (OD) at a wavelength of 600 nm using a spectrophotometer, till the reading becomes static.

Alternatively, 50-100 µl of formaldehyde can be added to all the 1 ml aliquots of culture suspension taken after every 30 minutes. Optical density of all the aliquots can be taken at the end of the experiment.

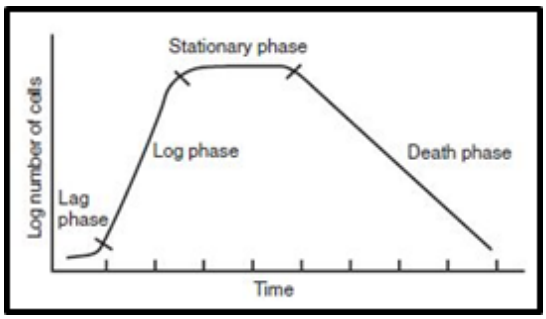
5. At the end of the experiment, plot a graph of time in minutes on X axis versus optical density at 600 nm on Y axis to obtain a growth curve of bacteria.

Observation:

Plot a graph and observe a growth curve of bacteria.

Result:

A logarithmic growth curve is obtained showing the changes in size of a bacterial population over time in the culture. The growth curve is hyperbolic due to the exponential bacterial growth pattern.



Bacterial Growth Curve

Chapter - 12
Staining Techniques

Bacteria are semi transparent and difficult to see in the unstained state. Stains are used to make them visible inorder

1. To view microscopic and semi transparent objects
2. To reveal their shape and size
3. To show the presence of various internal and external structures
4. To produce specific physical and chemical reactions.

Microorganisms also have the ability to produce inclusions and enzymes into the surrounding environment. The presence of certain structures, their staining reactions and enzyme specific reactions with the chemical reagents help in the identification and classification of microorganisms.

There are various staining procedures.

A. Simple staining
B. Differential staining
C. Structural staining

A 'smear' of bacteria or yeast is made on a microscope slide, fixed, stained, dried and, without using a coverslip, examined with the aid of a microscope. Aseptic technique using Laminar Air Flow must be observed when taking samples of a culture for making a smear. A culture on agar medium is much preferable to a liquid culture for making a smear.

A smear that is thin and even enables the shape and arrangement of cells to be clearly seen and ensures that the staining procedure is applied uniformly.

There are two broad types of staining methods:

1. A simple stain involves the application of one stain to show cell shape and arrangement and, sometimes, inclusions that do not stain, e.g. bacterial endospores;
2. A differential stain involves a sequential application of several stains, sometimes with heating, and includes a stage which differentiates between either different parts of a cell, e.g. areas of fat storage, or different groups, e.g. between Grampositive and Gram-negative bacteria.

The reaction of bacteria to Gram's staining method is a consequence of differences in the chemical structure of the bacterial cell wall and is a key feature in their identification. Yeast cells can be stained by Gram's method, but it is of no value in their identification and classification. The basis of Gram's staining method is the ability or otherwise of a cell stained with crystal violet to retain the colour when treated with a differentiating agent, usually alcohol (acetone). Bacteria that retain the violet/purple colour are called Gram-positive. Those that lose the colour, i.e. Gram-negative, are stained in the contrasting colour of a counterstain safranin, usually appearing pink/red.

Making a smear

1. Take a grease free microscope slide and thoroughly clean using lens tissue.

2. Label a microscope slide with a marker pen to record the culture being used, date and initials; this is also a useful reminder of which side of the slide is being used.
3. Flame a wire loop to ensure that no culture accidentally remains from a previous experimental work.
4. Transfer one or two loopfuls of sterile distilled water to the centre of the slide.
5. Flame loop and allow to cool.
6. Using aseptic technique, transfer a very small part of a single colony from a plate or slope of agar medium into the distilled water. If the amount of culture on the loop is easily visible you have taken too much!
7. Make a suspension of the culture in the distilled water on the slide and thoroughly but gently spread it evenly over an oval area of up to 2 cm length.
8. Flame the loop. If it is necessary to use a liquid culture or sample, the use of distilled water to prepare the smear will probably be unnecessary and may result in a smear with too few cells.
9. Dry the suspension by warming gently over a Bunsen burner flame and then 'fix' it by quickly passing it through the flame a few times.

This is called a **heat-fixed smear**; it should be visible to the naked eye as a whitish area when held in slant against the light. Fixing is necessary to ensure that cells adhere to the slide and to minimise any post-mortem changes before staining. The smear is now ready to be stained.

A simple stain:

1. Monochrome Staining:

The coloration of bacteria by applying a single staining solution to a fixed smear is termed as simple staining or monochrome staining (Greek Mono = single, Croma = colour).

Experiment 10

Aim:

To perform monochrome Staining of the bacterial culture

Principle:

When a stain is applied on the bacterial smear, the bacterial cell which is negatively charged combines with positively charged ions off the basic stain and appears coloured against a bright coloured background.

Mechanism:

The surface of the bacterial cell is negatively charged because of the large amount of carboxyl groups, ionization of carboxyl groups in parts negative charge to the bacterial cell surface.

$$\mathbf{COOH \rightarrow COO^- + H^+}$$

In nature hydrogen ions are replaced by other positively charged ions, for example Na+ or K+. Thus in the peripheral area of bacterial cell we see a bacterial cell negative in combination with positively charged ions for example

$$\mathbf{[Bacterial\ cell^-]\ [Na^+]}$$

When we apply basic dye such as methylene blue, it is available as its salt methylene blue chloride

$$[MB^+][Cl^-]$$

During staining the Ion exchange takes place and is represented by following equation in which Na^+ is replaced by MB^+

$$[\text{Bacterial cell}^-]\ [Na^+] + [MB^+][Cl^-] \rightarrow [\text{Bacterial cell}^-][MB^+] + NaCl$$

Suitable stains include basic dyes (i.e. salts with the colour-bearing ion, the chromophore, being the cation), such as carbol fuchsin, methylene blue, crystal violet and safranin.

Procedure:

1. Place the slide with the fixed smear uppermost on a staining rack over a sink or staining tray.
2. Thoroughly cover the smear with stain and leave for, usually, 30 seconds.
3. Hold the slide with forceps (optional but avoids stained fingers), at a 45° angle over the sink.
4. Rinse off the stain with tap/distilled water.
5. Blot-dry the smear with filter/fibre-free blotting paper using firm pressure, but not sideways movements that might remove the smear.
6. Examine under oil immersion.
7. When finished, dispose of the slides into a discard jar.

Observation:

Bacteria take the colour of stain methylene blue and appear blue against the light microscopic field.

***S. aureus* stained with methylene blue**

Uses:

The purpose of simple staining is to colour the bacteria so that they may be more easily seen and to reveal their size and shape.

2. Negative Staining Method:

This staining method employs a stain which does not stain the bacteria but the background is stained. Therefore bacteria appear colorless as bacteria stand out in relief. Thus negative staining is also called relief staining or indirect staining. Acidic stains such as India ink, Nigrosine and

Congo red are widely used for negative staining. Phosphotungstic acid is used in negative staining of viruses, which are studied with electron microscopy.

There are two methods of negative staining: Burr's India ink method and Fleming's Nigrosine method.

Experiment 11

Aim:

To perform negative Staining of the bacteria

Principle:

Negative staining requires the use of an acidic stain such as India ink or nigrosine. The acidic stain, with its negatively charged chromogen, will not penetrate the cells because of the negative charge on the surface of bacteria. The negative -negative charges repels each other. Therefore, the unstained cells are easily visible against the colored background.

Mechanism:

Bacterial cell surface is negatively charged, **[Bacterial cell$^-$]** when acidic stain is applied acidic stain carries negative charge so it repels. Acidic stains surround the cell and leaves the bacteria unstained but it stains the background which appears coloured **[Congo red$^-$]**.

[Bacterial cell$^-$] + [Congo red$^-$]→Bacteria unstained due repulsion of negative charges.

The practical application of negative staining is twofold. First, since heat fixation is not required and the cells are not subjected to the distorting effects of chemicals and heat, their natural size and shape can be seen. Second, it is possible to observe bacteria that are difficult to stain, such as some spirilla. Because heat fixation is not done during the staining process, and that the organisms are not killed, the slides should be handled with care.

Requirement:

24-hour agar slant cultures of *Micrococcus luteus and Bacillus cereus*

Nigrosine 10%

Bunsen burner, inoculating loop, staining tray,

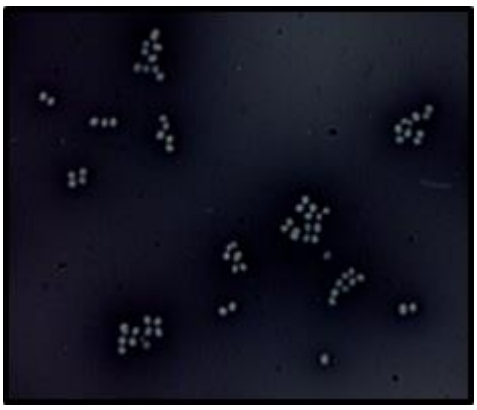
Glass slides, lens paper, and Compound microscope.

Procedure:

1. Place a small drop of 10% nigrosine close to one end of a clean slide.
2. Using aseptic technique, place a loopful of inoculum from the *M. luteus* culture in the drop of nigrosin and mix.
3. Place a slide against the drop of suspended organisms at a 45° angle and allow the drop to spread along the edge of the applied slide.
4. Push the slide away from the drop of suspended organisms to form a thin smear.
5. Air-dry. **Note:** Do not heat fix the slide.
6. Repeat Steps 1–4 for slide preparations of *B. cereus*.
7. Examine the slides under oil immersion, and record your observations.

Observation:

Observe under immersion objective. Bacteria are colourless against the coloured microscopic field.



Microorganism stained with nigrosin

Uses:

Negative staining is used to observe colorless microorganisms to reveal their size, shape and arrangement. It is also used to demonstrate spirochaetes, bacterial capsules and to view the motility of bacteria.

B. Differential stain:

1. ***Gram's staining method:***

Gram Staining is the common, important, and most used differential staining techniques in Microbiology, which was introduced by Danish Bacteriologist Hans Christian Gram in 1884. This test differentiate the bacteria into Gram Positive and Gram Negative Bacteria, which helps in the classification and differentiations of microorganisms.

Experiment 12

Aim:

To perform Gram staining technique of bacteria

Principle:

The bacterial smear when stained with basic stain Crystal Violet and treated with iodine solution as a mordant the CVI complex is formed. The Crystal Violet- iodine complex imparts purple black colour to the cells. In Gram Positive Bacteria, this Complex binds to the magnesium ribonucleic acid component of the cell wall, which is difficult to remove. Therefore, Gram Positive Bacteria retains the colour of Crystal Violet whereas when the bacterial smear is decolorized by 95% alcohol, alcohol serves as a lipid solvent and dehydrating agent for protein. Gram Negative bacteria possess high lipid content, therefore after decolourisation the CVI Complex comes out and when counter-stained with safranin, Gram Negative bacteria retain the colour of safranin.

Mechanism:

The Gram reaction is determined by interaction of Crystal Violet and Iodine and by the integrity and the structure of the cell wall (peptidoglycan layer) of the bacteria. The cell walls of

gram positive bacteria have a thick layer of protein-sugar complexes called peptidoglycan (40-90%) and lipid content (0-4%) is low. Decolorizing the cell causes this thick cell wall to dehydrate and shrink, which closes the pores in the cell wall and prevents the stain from exiting the cell. So the ethanol cannot remove the Crystal Violet-Iodine complex that is bound to the thick layer of peptidoglycan of gram positive bacteria and appears blue or purple in colour.

In case of gram negative bacteria, the cell wall also takes up the CV-Iodine complex but due to the thin layer of peptidoglycan (5-10%) and thick outer layer which is formed of lipopolysaccharides (50%) and lipids (10-20%), CV-Iodine complex gets washed off. When they are exposed to alcohol, decolorizer dissolves the lipids in the cell walls, which allows the crystal violet-iodine complex to leach out of the cells. Then, when again stained with the counterstain, safranin, they take the stain and appear red in color.

Requirements:

A. Alcohol: 95%

B. Crystal violet solution:

A. Crystal violet 2.0 g in
Absolute alcohol 100 ml
B. Ammonium oxalate 1.0 g in
Distilled/deionised water 100 ml
Add 25 ml A to 100 ml B

C. Lugol's iodine solution:

Iodine 1.0 g
Potassium iodide 2.0 g
Distilled/deionised water 300 ml

D. Safranin:

Safranin 0.5 % aqueous solution

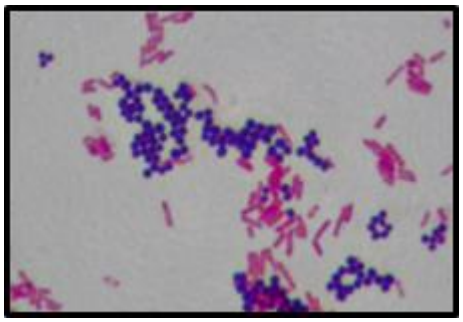
Procedure:

1. Prepare bacterial smear on a grease free glass slide and heat fix the smear.
2. Place the slide with the fixed smear uppermost on a staining rack over a sink or staining tray.
3. Thoroughly cover the smear with crystal violet solution and leave for 30 seconds.
4. Hold the slide with forceps (optional but avoids stained fingers), at a 45° angle over the sink.
5. Pour off the stain, and wash under tap water.
6. Put the slide back on the staining rack, cover the smear with iodine solution and leave for 1 minute. Iodine solution acts as a **'mordant'** (a component of a staining procedure that helps the stain to adhere to the specimen), a crystal violet–iodine complex is formed and the smear looks black.
7. Hold the slide with forceps at a 45° angle over the sink and wash off the iodine solution with 95% alcohol (not water) dropwise; continue treating with alcohol until the washings are pale violet.
8. Rinse immediately with distilled water.
9. Put the slide back on the staining rack.

10. Cover the smear with the counterstain, e.g. aqueous safranin solution, 0.5 % (w/v), for 1-2 minutes.
11. Rinse off the stain with distilled water.
12. Blot dry the smear with filter/fibre free blotting paper using firm pressure but not sideways movements that might remove the smear.
13. Examine under oil immersion.
14. When finished, dispose of the slides into a discard jar.

Observation:

Under oil immersion objective you will observe Gram Positive Bacteria which appears dark purple and Gram Negative bacteria appears red or pink in colour.



Gram staining: *S. aureus* and *E. coli*

Examples of typical Gram stain results are as follows.

Gram-positive bacteria	**Gram-negative bacteria**
Bacillus megaterium	*Escherichia coli*
Bacillus subtilis	*Pseudomonas fluorescens*
Lactobacillus spp.	*Salmonella, Shigella,*
Micrococcus luteus	*Helicobacter Staphylococcus*
albus	*Acetic acid bacteria,*
Streptococcus lactis	*Legionella*

Yeasts appear violet and red, but this has no taxonomic significance.

Uses:

Gram reaction is of great economic importance and is often the first step in the identification of unknown prokaryotic organisms.

Precautions:

Always use a young culture because older cultures of Gram-positive bacteria tend to lose the ability to retain the crystal violet–iodine complex and appear to be Gram-negative; but some bacteria are naturally only weakly Gram-positive.

The amount of alcohol treatment (the differential stage) must be judged carefully because over-treatment washes the crystal violet– iodine complex from Gram-positive bacteria and they will appear to be Gram-negative.

Take care to make a uniform thin smear otherwise alcohol will continue to wash the violet/purple colour from thick parts of the smear while thin parts are being over-decolorized.

At the end of the procedure, check that the labelling has not been washed off by the alcohol. Don't despair if the stained smear is not visible to the naked eye; this may happen with a Gram-negative reaction.

2. Acid fast Staining Method:

Acid fast staining is another widely used differential staining procedure in bacteriology. This staining procedure distinguishes acid fast bacteria (Mycobacteria and Nocardia) from non-acid fast bacteria.This staining method was demonstrated by Ziehl Neelson.

Experiment 13

Aim:

To perform Acid fast staining of the bacteria

Principle:

Acid fast bacteria contains mycolic acid in the cell envelope therefore, when the bacterial cell is stained with carbol fuchsin (with steam) and when decolorized by 20% Sulfuric acid or 3% hydrochloric acid, It doesn't get decolorized and appears red. The retention of red colour even after addition of acid or acid alcohol is due to the presence of mycolic acid.

Mechanism:

Acid fastness is attributed to the presence of high lipid content in the cell envelope of Mycobacteria and Nocardia species. The lipid content is as high as 60 % of the dry weight of the cell wall. The major lipids are mycolic acid, glycolipids and mycosides.

The stain carbol fuchsin is more soluble in phenol than in water or acid. Phenol inturn is more soluble in lipids that are present in the envelope of acid fast bacteria. During staining, basic carbol fuchsin enters the cell. Phenol and heat acts as intensifiers therefore, carbol fuchsin is retained by acid fast bacteria even after decolorization with acid. Non-acid fast bacteria lose carbol fuchsin after acid treatment because of low liquid content and take the colour of counterstain with methylene blue or malachite green.

Requirements:

Bacterial cultures (*Mycobacteria*, *Nocardia*)
carbol fuchsin (2%)
3% HCl or 20% H_2SO4
Malachite green (1%) or Methylene Blue (0.2%)

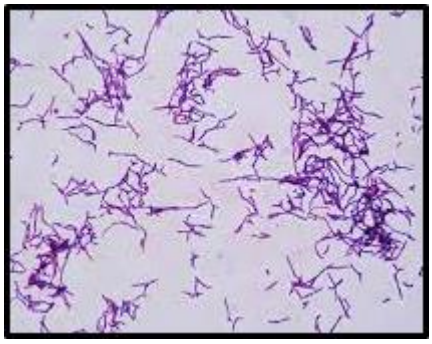
Procedure:

1. Prepare the smear on a grease free glass slide from the sputum sample and heat fix.
2. Place the slide with the fixed smear uppermost on a Tripod stand over a sink or staining tray.
3. Cover the slide with filtered Ziehl Neelson carbol fuchsin and heat till steam arises.
4. Allow the preparation to stain for 5 minutes with the heat being applied at intervals to keep the stain hot. **Note:** The stain must not be allowed to boil and evaporate or dry on the slide.
5. Wash the slide with distilled water.

6. Decolorize with 3% HCl or 20% H_2SO_4 and immediately wash with tap water. The stain should appear faintly pink.
7. Counter stain with malachite green for 20-30 seconds or methylene blue for 2 minutes.
8. Wash the slide with distilled water.
9. Air dry and observe the slide under oil immersion objective.

Observation:

Acid fast bacteria appear bright red while the tissue cells and other organisms (non acid fast) appear blue in colour.



Acid fast staining of *Mycobacteria*

Uses:

This staining procedure is used in the diagnosis of tuberculosis and leprosy.

C) Differential staining methods for visualization of Bacterial cell Structures:

Differential staining techniques for visualization of Bacterial cell Structures are carried out to view structural morphology, internal structures like endospores, metachromatic granules, volutin granules, nucleus etc.

***1. Cell wall staining method* (Chance staining method):**

This method was introduced by Chance in for the staining of cell walls of bacteria. This staining reveals the shape of the bacterial cell.

Experiment 14

Aim:

To perform cell wall staining method

Principle:

All true bacteria possess a rigid cell wall. The cell wall is the structure that immediately surrounds the cell membrane / cytoplasmic membrane. It comprises about 10 to 40% of the dry weight of the cell. A bacterium from which the cell wall has been completely removed, usually by enzymatic digestion is known as **protoplast**.

The enzyme lysozyme (egg white) selectively dissolves the cell wall of Gram Positive Bacteria. The chemical composition of the cell wall varies in different species of bacteria. The cell wall is composed of peptidoglycan and is differentially permeable. When the cell wall is stained by Congo red in presence of cetylpyridinium chloride it takes the colour red.

Mechanism:

Cetylpyridinium chloride dissociates in water to form positively charged cetylpyridinium and negatively charged chloride ions. The bacterial cells absorb cetylpyridinium cations and become positively charged and on subsequent treatment with acidic dye like Congo red, the cell wall gets stained while cytoplasm is stained with basic dye methylene blue.

Requirements:

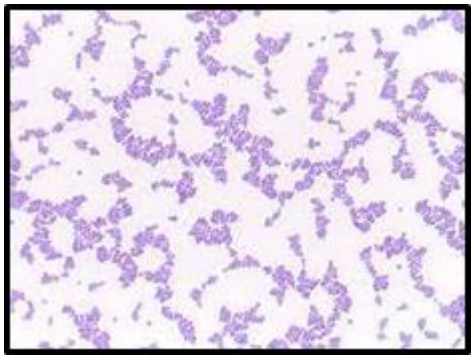
Bacterial culture (*Bacillus cereus*)
Cetyl pyridinium chloride (aqueous solution 0.34%)
Congo red (5%)
Loeffler's Methylene Blue (0.3%)

Procedure:

1. Prepare a smear of bacterial culture (*Bacillus cereus*)
2. Heatfix the smear.
3. Apply 3 drops of cetyl pyridinium chloride (0.34%) and one drop of Congo red.
4. Mix with a glass rod for 5 minutes.
5. Flood the smear with Congo red (5%)
6. Wash the slide with distilled water.
7. Air dry the smear
8. Counterstain with Loeffler's methylene blue (0.3%) solution for 1 to 2 minutes.
9. Wash the slide with distilled water.
10. Air dry the slide and observe under oil immersion objective.

Observation:

When observed under oil immersion objective, the cell wall of the bacteria appears red and the cytoplasm stains blue which is clearly distinguished.



Cell Wall staining of bacteria

Uses:

Cell wall staining method is used to stain cell walls and to reveal size and shape of bacteria.

***2. Capsule Staining method* (Duguid staining method):**

Many microorganisms contain a gelatinous covering called a capsule / slime layer. They are covering the cell wall of the bacterial cell. Capsules and Slime layers are composed of polysaccharides (glycoproteins, lipopolysaccharides).

All the capsules are not required for bacterial growth and reproduction in lab cultures, they determine the organism's **virulence**, the degree to which a pathogen can cause disease. for

example *Streptococcus pneumoniae*. When bacteria lack a capsule, it is easily destroyed and does not cause any disease.

Capsules contain a great deal of water and can protect bacteria against desiccation. The glycocalyx capsule also helps in bacterial attachments to the solid surfaces in aquatic environments or to tissue surfaces in plants and animal hosts.

Experiment 15

Aim:

To perform capsule staining method

Principle:

Capsule staining is more difficult than other types of staining procedure because capsules are soluble in water and may get removed during repeated washing. Negative staining method is applied using India ink to demonstrate the presence of the capsule.

In capsule staining, negative stains do not penetrate the capsule and thus they appear colourless.When counter stained with safranin, they provide a contrast as capsule appears colourless and the bacteria stain pink against dark background.

In capsule staining, the smear should not be heated. Detection of capsule and Slime layer is done by Duguid staining method.

***Requirements*:**

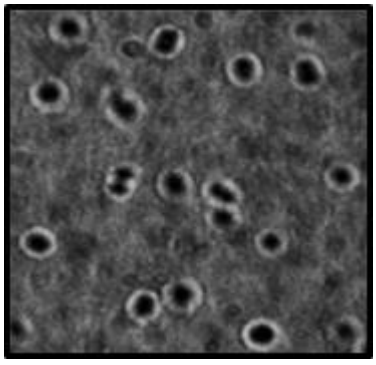
Bacterial culture (*Streptococcus pneumoniae, haemophilus influenzae*)
Safranin
India Ink

Procedure:

1. Mix loop ful of bacterial culture *Streptococcus pneumoniae* with small drop of India ink.
2. Spread bacterial suspension over the glass slide and air dry the slide.
3. Heat fix gently (not heated).
4. Stain with safranin for 1 to 2 minutes.
5. Wash the slide with water.
6. Finally press it with the help of a filter paper until the ink in sepia colour beneath the cover glass is pressed properly.
7. Observe the slide under oil immersion objective.

Observation:

Capsule appears halo surrounding each Saffron stained bacterial cell against a dark background.



Capsule staining of *S. pneumoniae*

Uses:

To demonstrate the presence of a capsule

To know the virulence of bacteria as in *Streptococcus pneumoniae*

3. Endospore Staining Method* (Schaeffer and Fulton Method)*:

A number of Gram Positive Bacteria can form a special resistant, dormant structure called an endospore. Endospores develop within vegetative bacterial cells of several gram positive genera *Bacillus, Clostridium* and *Sporosarcina* and two gram-negative genera *Sulphatomaculum* and *Oscillospira.*

Endospores protects bacteria from adverse environmental conditions such as heat, ultraviolet radiation, chemical disinfectants and desiccation. Some endospores can remain viable for well over 500 years and actinomycetes remain alive after burial in mud or soil for 7500 years!

Endospores are of great importance in Industrial and Medical Microbiology. Spores survive boiling for an hour or more so sterilization of media must be thoroughly efficient to kill the endospore, otherwise they will germinate and cause/ result in contamination of Industrial Products.

Experiment 16

Aim:

To perform endospore staining method

Principle:

Endospores cannot be stained by ordinary methods such as simple staining and Gram staining because the stains do not penetrate the wall of the endospore. Endospores can be examined with both light and electron microscopy. Because endospores are highly refractive, they can be detected under light microscope when unstained but they cannot be differentiated from inclusions of stored material without a special stain.

Endospore staining by Schaeffer and Fulton staining technique shows endospore in green colour within the red or pink cells. Endospores take the colour of malachite green when the heat fixed smear is steamed for about 5 minutes.

***Requirements*:**

Bacterial culture (*Bacillus cereus, Clostridium butyricum*)

Malachite green (0.5%)

Safranine (0.5%)

Procedure:

1. Prepare the smear (*Bacillus cereus or Clostridium butyricum*)and heat fix the slide.
2. Flood the slide with 0.5% Malachite green
3. Keep the slide on the Tripod stand and flame, steam by adding the stain solution for 5 minutes (do not boil the stain).
4. Wash the slide with water properly (to remove malachite green).
5. Counterstain the smear with safranin for 30 seconds.
6. Wash the slide with water and air dry.
7. Observe the slide under oil immersion objective

Observation:

Spores are stained green, while bacterial cells are stained red. The green colour dot is seen within the red bacterial cell.

Endospore staining of *Bacillus cereus*

Uses:

To detect the presence of endospores in bacteria.

To locate the spore position in the bacterial cells.

4. FlagellaStaining Method:

Majority of motile bacteria move by use of flagella, thread like locomotor appendages extending outward from the plasma membrane and cell wall. They are slender structures (20nm x 15-20μm long). Flagella are so thin that they cannot be directly observed under a bright field microscope unless stained by special stains.

Bacterial species often differ distinctively in their patterns of flagella distribution.

1. **Monotrichous:** bacteria have one flagellum at the polar end. Ex. *Vibrio cholera, Pseudomonas aeruginosa*
2. **Amphitrichous:** bacteria have a single flagellum at each pole. Ex. *Pseudomonas alcaligenes*
3. **Lophotrichous:** tuft of flagella at one or both ends. Ex. *Spirillum*
4. **Peritrichous:** flagella spread all over the body surface of bacterial cells. Ex. *Proteus vulgaris, E. coli, S. typhi.*

Experiment 17

Aim:

To perform flagella staining by Gray's and Patel, Kulkarni and Gaikwad's method

Principle:

A tedious and delicate staining procedure which uses a mordant (20% tannic acid) and carbol fuchsin stain to build the diameters of the flagella until they become visible under the light microscope.

Requirements: (Gray's method)

Nutrient broth

10% Formalin

20% Tannic acid

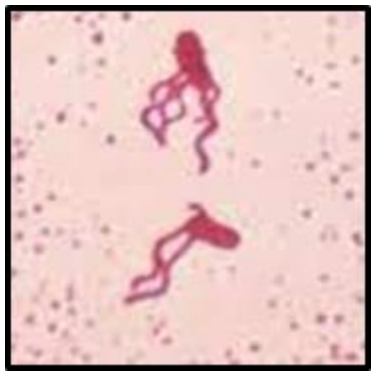
Carbol fuschin (0.3%)

Procedure:

1. Grow the flagellated bacteria *Pseudomonas* or *Rhizobium* in nutrient broth.
2. Centrifuge the broth to obtain bacterial pellets.
3. Resuspend the pellets in 10% Formalin to produce light, faint turbidity and for the prevention of flagella from breakage.
4. Allow a loopful of suspension to slide on the glass slide.
5. Flood the slide with 20% tannic acid for 6 minutes and wash the slide.
6. Flood the slide with carbol fuchsin (0.3%) for 3 minutes by placing a piece of blotting paper over the smear.
7. Remove the paper and wash the Smear gently.
8. Air dry and observe the slide under oil immersion objective.

Observation:

Flagella stains red when observed under oil immersion objective.



Flagella staining of *Pseudomonas*

Requirements: (Patel, Kulkarni and Gaikwad method)

A. Tannic acid solution

a. Tannic acid: 0.2g
b. Distilled water: 100ml

B. Iodine solution

a. Iodine crystal: 2.0g
b. 1N NaOH: 10ml

C. Basic Fuchsin solution

a. Basic fuchsin: 0.3g
b. Absolute alcohol: 10ml
c. Phenol: 5.0g
d. Distilled water: 75ml

D. Motility Agar

a. Peptone: 10g
b. NaCl: 5g
c. Agar: 4g
d. Distilled water: 1000ml
e. pH: 7.2

Autoclave at 121 ℃ for 15 minutes

Procedure:

1. Grow the Bacteria in Motility agar for 18-20 hr.
2. Prepare the smear delicately so as to maintain the flagella.
3. Treat the smear with 4 to 6 drops of **solution A,** immediately followed by equal drops of **solution B.**
4. Heat the smear from below gently for 1 to 2 minutes
5. Add 2-3 drops of **solution C** to smear and again heat for 3 minutes.
6. Wash the slide with water, air dry and examine under the oil immersion objective.

Observation:

Bacteria appear red and the flagella appear as a slender structure which is pink in colour.

5. Inclusion bodies in Prokaryotes

Within the cytoplasm of prokaryotic cells are several kinds of reserve deposits known as 'inclusions'. Some inclusions are common to a wide variety of bacteria, whereas others are limited to a small number of species and therefore they serve as a basis for identification.

Inclusions for example metachromatic granules, phosphate granules, volutin granules, poly beta hydroxybutyrate granules etc. are seen in in cytoplasm of cell so they are called '**cytoplasmic inclusions**'

A. Metachromatic granules:

Metachromatic granules are large inclusions and named accordingly because they show metachromatic effect that is they appear red or a different shade of blue when stained with blue dyes like methylene blue or toluidine blue.

Collectively they are known as volutin. Many bacteria, some fungi, algae and protozoa store inorganic phosphate as polyphosphate granules or volutin granules. Polyphosphate is a linear polymer of author phosphates joined by ester bonds. Thus volutin functions as a storage reservoir for phosphates, an important component of cell constituents such as nucleic acids. Polyphosphates is used in the synthesis of ATP (energy) in energy metabolism and cell division. These granules are found to be most prominent in old cultures before starvation. The clinical microbiologists are interested in them as these inclusions are found to occur in the genus *Corynebacterium diphtheriae*, the causative agent of diphtheria.

Experiment 18

Aim:

To perform metachromatic granules with Loeffler's methylene blue or toluidine blue (1%) method.

Principle:

Staining of metachromatic granules with Loeffler's methylene blue or toluidine blue (Albert stain) reveals its identity in the form of red colour granules in the cytoplasm.

Requirements:

Bacterial culture *Corynebacterium diphtheriae*

Loeffler's methylene blue (0.5%) or toluidine blue (1%)

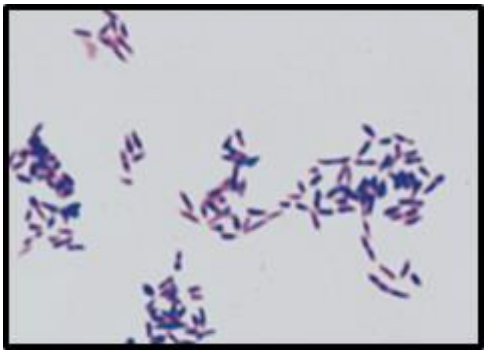
Lugol's iodine solution

Procedure:

1. Prepare the smear of *Corynebacterium diphtheriae* and heat fix the smear.
2. Stain the smear with Loeffler's methylene blue (0.5%) or toluidine blue (1%) for 10 to 30 seconds.
3. Drain the stain and rinse the slide with tap water.
4. Flood the slide with lugol's iodine solution and allow it to react for one minute
5. Wash gently with water and air dry the slide.
6. Observe under oil immersion objective

Observation:

Deep blue to violet spheres of metachromatic granules are visible under oil immersion microscope and the cytoplasm appears blue in colour.



Metachromatic granules staining of *Corynebacterium diphtheriae*

Uses:

It is majorly used to identify the metachromatic granules found in disease-causing microorganisms like *Corynebacterium diphtheriae*.

Being a differential stain, it helps distinguish *Corynebacterium diphtheriae* from other nonpathogenic diphtheroids that lack the metachromatic granules.

B. Poly beta hydroxybutyrate (PHB) granules:

PHB granules are organic inclusion bodies of poly beta hydroxybutyrate. PHB comprises Beta hydroxybutyrate molecules joined together by ester bonds between the carboxyl and hydroxyl groups of adjacent molecules. It is an internal reserve food material for providing energy and biosynthesis. Many bacteria including *Rhizobia* show presence of PHB granules. PHB accumulates in distinct bodies that are readily stained with Sudan black and is clearly visible in light microscopy.

Experiment 19

Aim:

To perform PHB granules staining using Sudan Black B stain.

Principle:

Sudan Black B is a slightly basic dye that combines with the acidic groups in the lipid compounds, hence staining the phospholipids, lipoproteins, and triglycerides found in the staining smears.

Requirements:

Bacterial culture (*Rhizobium or Alcaligenes* sp.)

Sudan black B solution (0.3% in 70% alcohol)

Xylene

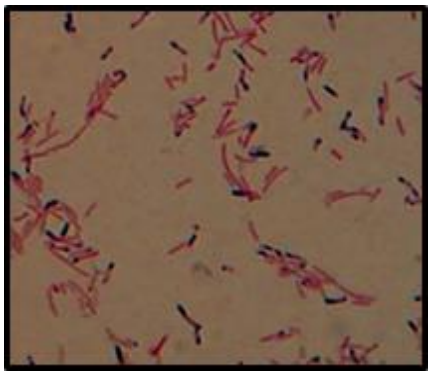
Safranin 0.5% aq.

Procedure:

1. Prepare bacterial smear of *Rhizobium or Alcaligenes* sp. on a slide.
2. Immerse the slide in Sudan black B solution for 5 to 15 minutes.
3. Drain and dry the slide and immerse in Xylene until completely decolorized.
4. Remove the slide and dry it.
5. Counterstain with 0.5% aqueous safranin for 10 seconds.
6. Wash the slide in distilled water, and dry.
7. Observe the slide under oil immersion objective.

Observation:

Blue droplets of PHB are observed while cytoplasm appears pink in colour.



PHB staining of *Rhizobium*

Uses:

Polyhydroxybutyrate (PHB) is used in versatile fields as it is a biodegradable, biocompatible, and ecologically safe thermoplastic.

Chapter - 13
Nutritional and physical requirements of microorganisms

Similar to all living organisms, microorganisms too require certain basic nutrients and physical factors for the sustenance of life. However, their particular requirements vary greatly. Understanding their nutritional and physical requirements is necessary for successful cultivation of microorganisms in the laboratory.

A. Nutritional Requirements:

Nutritional needs of microbial cells are supplied in the laboratory through a variety of media. The following are the nutritional requirements among microbes.

1. Carbon: The most essential and central atom common to all cellular structures and functions. Among microbial cells, two carbon dependent types are found:

a. Autotrophs: These organisms can be cultivated in a medium consisting solely of inorganic compounds; specifically, they use inorganic carbon in the form of carbon dioxide.

b. Heterotrophs: These organisms cannot be cultivated in a medium consisting solely of inorganic compounds; they must be supplied with organic nutrients, primarily glucose or dextrose.

2. Nitrogen: This is also an essential atom in many cellular macromolecules, particularly proteins and nucleic acids. Proteins serve as the structural molecules forming the peptidoglycan layer of the cell and as functional molecules, enzymes, that are responsible for the metabolic activities of the cell. Nucleic acids include DNA, the genetic basis of cell life, and RNA, which plays an active role in protein synthesis within the cell. Some microbes use atmospheric nitrogen, others rely on inorganic compounds such as ammonium or nitrate salts, and still others require nitrogen-containing organic compounds such as amino acids.

3. Nonmetallic elements: Two major nonmetallic ions are used for cellular nutrition:

a. Sulfur: Sulfur is an essential component to some amino acids and is therefore a component of proteins. Sources include organic compounds such as sulfur-containing amino acids, inorganic compounds such as sulfates, and elementary sulfur.

b. Phosphorus: Phosphorus is necessary for the formation of the nucleic acids DNA and RNA and also for synthesis of the high-energy organic compound adenosine triphosphate (ATP). Phosphorus is supplied in the form of phosphate salts for use by all microbial cells.

4. Metallic elements: Ca^{2+}, Zn^{2+}, Na^{+}, K^{+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, and Fe^{3+} are some of the metallic ions necessary for efficient performance of various cellular activities. Some of these activities are osmoregulation, regulation of enzyme activity, and electron transport during biooxidation. However, these ions are micronutrients and are required in trace concentrations only. Inorganic salts supply these materials.

5. Vitamins: These organic substances contribute to cellular growth and are essential in minute concentrations for cell activities. They are also sources of coenzymes, which are required for the formation of active enzyme systems. Some microbes require vitamins to be supplied in a preformed state for normal metabolic activities. Some possess extensive vitamin synthesizing

pathways, whereas others can synthesize only a limited number from other compounds present in the medium.

6. Water: All cells require distilled water in the medium so that the low-molecular-weight nutrients can cross the cell membrane.

7. Energy: Active transport, biosynthesis, and biodegradation of macromolecules are the metabolic activities of cellular life. These activities can be sustained only if there is a constant availability of energy within the cell. Two bioenergetic types of microorganisms exist:

a. Phototrophs: These microorganisms use radiant energy as their sole energy source.

b. Chemotrophs: These microorganisms depend on oxidation of chemical compounds as their energy source. Some microbes use organic molecules such as glucose; others utilize inorganic compounds such as H_2S or $NaNO_2$.

In **Chapter 7** and **9** we have already covered different types of culture media, their preparation and cultivation of microorganisms. There are numerous special-purpose media available for functions including the following:

1. Isolation of bacterial types from a mixed population of organisms.
2. Differentiation among closely related groups of bacteria on the basis of macroscopic appearance of the colonies and biochemical reactions within the medium.
3. Enumeration of bacteria in microbiology, such as in water and sewage, and also in food and dairy products.
4. Assay of naturally occurring substances such as antibiotics, vitamins, and products of industrial fermentation.
5. Characterization and identification of bacteria by their abilities to produce chemical changes in different media.

In addition to nutrients necessary for the growth of all bacteria, special-purpose media contain both nutrients and chemical compounds important for specific metabolic pathways in different types of bacteria. Three types of media which are routinely used in Microbiology Laboratory are.

A. Selective Media:

These media are used to select (isolate) specific groups of bacteria. They incorporate chemical substances that inhibit the growth of one type of bacteria while permitting growth of another, thus facilitating bacterial isolation.

1. ***Crystal violet agar:*** This medium is selective for most gram-negative microorganisms. Crystal violet dye exerts an inhibitory effect on most gram-positive organisms.
2. ***7.5% sodium chloride agar:*** This medium is inhibitory to most organisms other than halophilic (salt-loving) microorganisms. It is most useful in the detection of *Staphylococcus*.

B. Differential / Selective Media:

These media can distinguish among morphologically and biochemically related groups of organisms. They incorporate chemical compounds that, following inoculation and incubation, produce a characteristic change in the appearance of bacterial growth and/or the medium surrounding the colonies, which permits differentiation. Sometimes differential and selective characteristics are combined in a single medium. MacConkey agar is a good example of this because it contains bile salts and crystal violet, which inhibit gram-positive organisms and allow gram negatives to grow. In addition, it contains the substrate lactose and the pH indicator neutral

red, which differentiates the red lactose-fermenting colonies from the translucent nonfermenting colonies.

The following media are examples of this type of media:

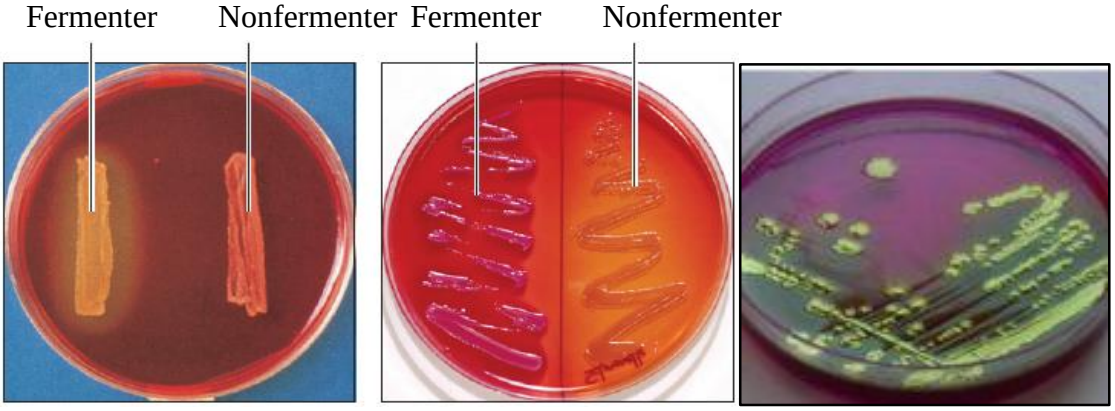
1. ***Mannitol salt agar:*** This medium contains a high salt concentration, 7.5% NaCl, which is inhibitory to the growth of most, but not all, bacteria other than the *Staphylococci*. The medium also performs a differential function: It contains the carbohydrate mannitol, which some *Staphylococci* are capable of fermenting, and phenol red, a pH indicator for detecting acid produced by mannitol-fermenting *Staphylococci*. These *Staphylococci* exhibit a yellow zone surrounding their growth; *Staphylococci* that do not ferment mannitol will not produce a change in coloration.
2. ***Mac Conkey's agar:*** The inhibitory action of crystal violet on the growth of gram positive organisms allows the isolation of gram-negative bacteria. Incorporation of the carbohydrate lactose, bile salts, and the pH indicator neutral red permits differentiation of enteric bacteria on the basis of their ability to ferment lactose. On this basis, enteric bacteria are separated into two groups:

a. Coliform bacilli produce acid as a result of lactose fermentation. The bacteria exhibit a red coloration on their surface. Escherichia coli produce greater quantities of acid from lactose than other coliform species. When this occurs, the medium surrounding the growth also becomes pink because of the action of the acid that precipitates the bile salts, followed by absorption of the neutral red.

b. Dysentery, typhoid, and paratyphoid bacilli are not lactose fermenters and therefore do not produce acid. The colonies appear tan and transparent.

3. Eosin methylene blue agar:

Lactose and the dyes eosin and methylene blue permit differentiation between enteric lactose fermenters and non fermenters as well as identification of the colon bacillus, E. coli. The E. coli colonies are blue-black with a metallic green sheen caused by the large quantity of acid that is produced and that precipitates the dyes onto the growth's surface. Other coliform bacteria, such as Enterobacter aerogenes, produce thick, mucoid, pink colonies on this medium. Enteric bacteria that do not ferment lactose produce colorless colonies, which, because of their transparency, appear to take on the purple color of the medium. This medium is also partially inhibitory to the growth of gram-positive organisms, and thus gram-negative growth is more abundant.



Mannitol Agar **Mac Conkey Agar** **Eosin methylene blue Agar**

C. Enriched Media:

Enriched media are media that have been supplemented with highly nutritious materials, such as blood, serum, or yeast extract, for the purpose of cultivating fastidious organisms. For example, in blood agar, the blood incorporated into the medium is an enrichment ingredient for the cultivation of fastidious organisms such as the Streptococcus spp. The blood also permits demonstration of the hemolytic properties of some microorganisms, particularly the streptococci, whose hemolytic activities are classified as follows:

1. ***Gamma hemolysis:*** no lysis of red blood cells results in any significant change in the appearance of the medium surrounding the colonies.
2. ***Alpha hemolysis:*** Incomplete lysis of red blood cells, with reduction of hemoglobin to methemoglobin, results in a greenish halo around the bacterial growth.
3. ***Beta hemolysis:*** Lysis of red blood cells with complete destruction and use of hemoglobin by the organism results in a clear zone surrounding the colonies. This hemolysis is produced by two types of beta hemolysins, namely streptolysin O, an antigenic, oxygen labile enzyme, and streptolysin S, a nonantigenic, oxygen-stable lysin. The hemolytic reaction is enhanced when blood agar plates are streaked and simultaneously stabbed to show subsurface hemolysis by streptolysin O in an environment with reduced oxygen tension. Based on the hemolytic patterns on blood agar, the pathogenic beta-hemolytic streptococci may be differentiated from other streptococci.

Haemolysis on Blood agar

B. Physical Factors:

Three of the most important physical factors that influence the growth and survival of cells are temperature, pH, and the gaseous environment. Thus, understanding their needs play an important role in cell metabolism.

1. Temperature: It influences the rate of chemical reactions through its action on cellular enzymes. Bacteria, as a group of organisms, exist over a wide range of temperatures. However, individual species can exist only within a narrower spectrum of temperatures. Low temperatures slow down or inhibit enzyme activity, thereby slowing down or inhibiting cell metabolism and, consequently, cell growth. High temperatures cause coagulation and thus irreversibly denatured

thermolabile enzymes. Although enzymes differ in their degree of heat sensitivity, generally temperatures in the range of 70°C destroy most essential enzymes and cause cell death.

2. pH: The pH of the extracellular environment greatly affects enzymatic activities of the microorganisms. Most commonly, the optimum pH for cell metabolism is in the neutral range of 7. An increase in the hydrogen ion concentration resulting in an acidic pH (below 7) or a decrease in the hydrogen ion concentration resulting in an alkaline pH (above 7) is often detrimental. Either increase or decrease will slow down the rate of chemical reactions because of the destruction of cellular enzymes, thereby affecting the rate of growth and, ultimately, survival.

3. The gaseous requirement: The gaseous requirement in most cells is atmospheric oxygen, which is necessary for the bio oxidative process of respiration. Atmospheric oxygen plays an important role in ATP formation and the availability of energy in a utilizable form for cell activities. Other bacteria, however, lack the enzyme systems for respiration in the presence of oxygen and therefore they use an anaerobic form of respiration or fermentation.

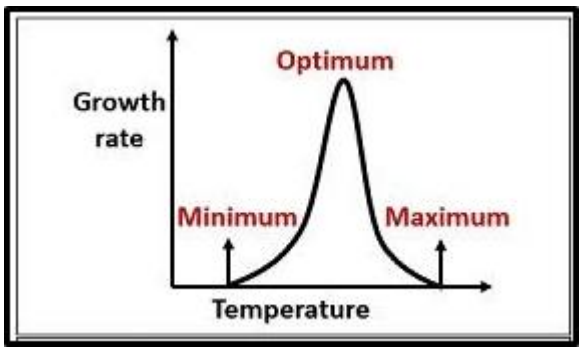
1. Physical Factors: Temperature

Microbial growth is directly dependent on how temperature affects cellular enzymes. With increasing temperatures (10 °C rise), enzyme activity increases until the three - dimensional configuration of these molecules is lost because of denaturation of their protein structure. On the other hand, as the temperature is lowered toward the freezing point, enzyme inactivation occurs and cellular metabolism gradually diminishes. At 0 °C, biochemical reactions cease in most cells. Bacteria, as a group of living organisms, are capable of growth within an overall temperature range of minus 5 °C to 80 °C. Each species, however, requires a narrower range that is determined by the heat sensitivity of its enzyme systems. Specific temperature ranges consist of the following cardinal (significant) temperature points:

1. Minimum growth temperature: The lowest temperature at which growth will occur. Below this temperature, enzyme activity is inhibited and the cells are metabolically inactive so that growth is negligible or absent.

2. Maximum growth temperature: The highest temperature at which growth will occur. Above this temperature, most cell enzymes are destroyed and the organism dies.

3. Optimum growth temperature: The temperature at which the rate of reproduction is most rapid; however, it is not necessarily optimum or ideal for all enzymatic activities of the cell.



The cardinal temperature points of microbial growth

All bacteria can be classified into one of three major groups, depending on their temperature requirements:

A. Psychrophiles: Bacterial species that will grow within a temperature range of −5°C to 20°C. The distinguishing characteristic of all psychrophiles is that they will grow between 0 °C and 5°C.

B. Mesophiles: Bacterial species that will grow within a temperature range of 20°C to 45 °C. The distinguishing characteristics of all mesophiles are their ability to grow at human body temperature (37°C) and their inability to grow at temperatures above 45°C. Included among the mesophiles are two distinct groups:

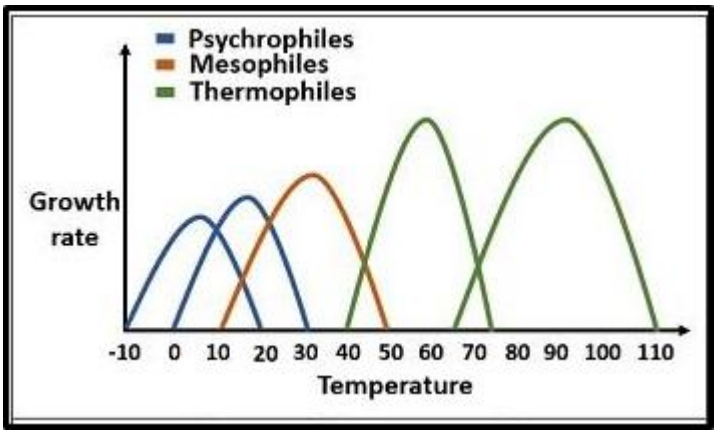
a. Those whose optimum growth temperature is in the range of 20°C to 30°C are plant saprophytes.

b. Those whose optimum growth temperature is in the range of 35°C to 40°C are organisms that prefer to grow in the bodies of warm-blooded hosts.

C. Thermophiles: Bacterial species that will grow at 35°C and above. Two groups of thermophiles exist:

a. Facultative thermophiles: Organisms that will grow at 37°C, with an optimum growth temperature of 45°C to 60°C.

b. Obligate thermophiles: Organisms that will grow only at temperatures above 50°C, with optimum growth temperatures above 60°C. The ideal temperature for specific enzymatic activities may not coincide with the optimum growth temperature for a given organism.



The effect of temperature on the growth of microorganisms

Experiment 20

Aim:

To study the effect of temperature on the growth of microorganisms

Principle:

Bacteria, as a group of organisms, have different temperature requirements. Accordingly, bacteria grow at a wide range of temperatures. Some bacteria are psychrophiles and grow between −5 °C to 20 °C. Mesophiles grow within a temperature range of 20 °C to 45 °C. Bacterial species that will grow at 35 °C and above belong to thermophiles. Few species of Thermophiles can also grow at a temperature above 60 °C.

Requirements:

24 to 48 hour nutrient broth cultures of *Escherichia coli*,

Bacillus stearothermophilus, Pseudomonas syringae, Serratia marcescens
Yeast *Saccharomyces cerevisiae*
Trypticase Soy Agar Plates (TSA), Sabouraud Broth tubes,
Bunsen burner, inoculating loop,
Refrigerator set at 4 °C
Two incubators set at 37 °C and 60 °C
Sterile Pipette, test tube rack, and glasswares
Glass marker pen

Composition of TSA

Pancreatic digest of casein:	17.0g
Papaic digest of soybean meal:	3.0g
Sodium chloride:	5.0g
Dextrose:	2.5g
Dibasic potassium phosphate:	2.5g
Distilled water:	1000ml
Agar powder:	15g
Final pH (at 25°C)	7.3±0.2

Trypticase Soy Broth (Soybean-Casein Digest Medium) is a general purpose liquid enrichment medium used in qualitative procedures for the sterility test and for the enrichment and cultivation of aerobic microorganisms that are not excessively fastidious. In clinical microbiology, it may be used for the suspension, enrichment and cultivation of strains isolated on other media. Trypticase Soy Broth is also used for cultivation of fungi.

Procedure:

1. Mark the underside of all plates into four quadrants with a glass marker. Label each section with the name of the test organism to be inoculated. While labeling each petri plate should include the temperature of incubation (4 °C, 20 °C, 37 °C, or 60 °C) and date and time of experiment
2. Aseptically inoculate each of the plates with *Escherichia coli, Bacillus stearothermophilus, Pseudomonas syringae, Serratia marcescens* by means of a single line of inoculation of each organism in its appropriately labeled section.
3. Appropriately label the four Sabouraud broth tubes, including the temperatures of incubation as indicated above.
4. Gently shake the *S. cerevisiae* culture to suspend the organisms. Using a sterile pipette, aseptically add one drop of the culture into each of the four tubes of broth media.
5. Incubate all plates in an inverted position and the broth cultures at each of the four experimental temperatures (4 °C, 20 °C, 37 °C, or 60 °C) for 24 to 48 hours.

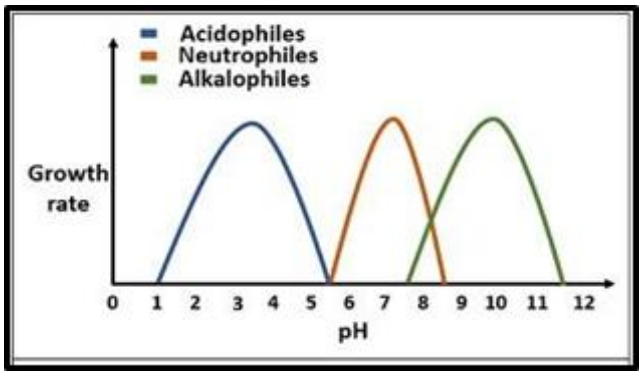
Observation:

Observe all the cultures tubes of *S. cerevisiae* for the presence of growth. Evaluate the amount of growth in the S. cerevisiae cultures by noting the degree of developed turbidity. Record and classify the bacterial cultures as psychrophiles, mesophiles, facultative thermophiles, or obligate thermophiles as observed on Trypticase Soy agar Plates. Also observe the *S.*

marcescens growth on all the plate cultures for the presence or absence of orange to red pigmentation.

2. Physical Factors: pH

Growth and survival of microorganisms are greatly influenced by the pH of the environment, and all bacteria and other microorganisms differ as to their requirements. Based on their pH optima, microorganisms may be classified as acidophiles, neutrophiles, or alkalophiles. Each species has the ability to grow within a specific pH range, which may be broad or limited, with the most rapid growth occurring within a narrow optimum range. These specific pH needs reflect the organisms adaptations to their natural environment. For example, enteric bacteria are capable of survival within a broad pH range, which is characteristic of their natural habitat, the digestive system. Bacterial blood parasites, on the other hand, can tolerate only a narrow range; the pH of the circulatory system remains fairly constant at approximately 7.4 pH. Despite this diversity and the fact that certain organisms can grow at extremes of the pH scale, generalities can be made. The specific range for bacteria is between 4 and 9, with the optimum being 6.5 to 7.5. Fungi (molds and yeasts) prefer an acidic environment, with optimum activities at a pH of 4 to 6.



The effect of pH on the growth of microorganisms

Metabolic activities of the microorganism will result in the production of wastes, such as acids from carbohydrate degradation and alkali from protein breakdown, and these will cause shifts in pH that can be detrimental to growth. To retard this shift, chemical substances that act as buffers are frequently incorporated when the medium is prepared. A commonly used buffering system involves the addition of equimolar concentrations of K_2HPO_4, a salt of a weak base, and KH_2PO_4, a salt of a weak acid. In a medium that has become acidic, the K_2HPO_4 absorbs excess H+ to form a weakly acidic salt and a potassium salt with the anion of the strong acid.

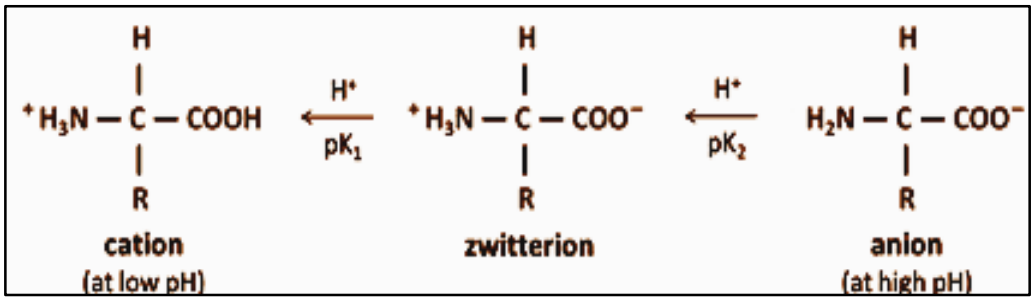
K_2HPO_4	+	HCL	→	KH_2PO_4	+ KCL
Salt of a Weak base		Strong acid		Salt of a weak acid	Potassium chloride salt

In a medium that has become alkaline, KH_2PO_4 releases H^+, which combines with the excess OH^- to form water, and the remaining anionic portion of the weakly acidic salt combines with the cation of the alkali.

KH_2PO_4	+	KOH	→	K_2HPO_4	+ H_2O
Salt of a		Strong		Salt of a	Water

Weak acid base weak base

Most media contain amino acids, peptones, and proteins, which can act as natural buffers because of their amphoteric nature. For example, amino acids are zwitterions, molecules in which the amino group and the carboxyl group ionize to form dipolar ions. These behave in the following manner:



Experiment 21

Aim:

To study the effect of pH on the growth of microorganisms

Principle:

Bacteria, as a group of organisms, have different pH requirements. Pathogenic bacteria normally grow at a pH of 6.5 to 7.5. Fungi grow at a pH of 4 to 6. Because a neutral or nearly neutral environment is generally advantageous to the growth of microorganisms, the pH of the laboratory medium is frequently adjusted to approximately 7.

Requirements:

24-hour nutrient broth cultures of *Alcaligenes faecalis,*
Escherichia coli, and yeast *Saccharomyces cerevisiae.*
Trypticase soy broth (TSB) tubes (3 test tubes each, pH designations: 3, 6, 7, and 9).
The pH has been adjusted with 1N NaOH or 1N HCl.
Bunsen burner, sterile 1-ml pipettes,
Test tube rack, and glass marker.

Composition of Trypticase Soy Broth:

Pancreatic digest of casein:	17.0g
Papaic digest of soybean meal:	3.0g
Sodium chloride:	5.0g
Dextrose:	2.5g
Dibasic potassium phosphate:	2.5g
Distilled water:	1000ml
Final pH (at 25 °C)	7.3±0.2

Procedure:

1. Using a sterile pipette, inoculate a series of the test tubes containing TSB, labelled with pH values of 3, 6, 7, and 9, with *E. coli* by adding 0.1 ml of the broth culture to each.
2. Repeat **Step 1** for the inoculation of *A. faecalis* and *S. cerevisiae*, using a new sterile pipette each time.

3. Incubate the *A. faecalis* and *E. coli* cultures for 24 to 48 hours at 37 °C and the *S. cerevisiae* cultures for 48 to 72 hours at 25 °C.

Observation:

Observe all the test tubes of *A. faecalis* and *E. coli* cultures and *S. cerevisiae* for the presence of growth and turbidity at varied pH values.

3. Physical Factors: Oxygen concentrations

Microorganisms exhibit great diversity in their ability to use free oxygen (O_2) for cellular respiration. These variations in O_2 requirements reflect the differences in bio oxidative enzyme systems present in the various species. Microorganisms can be classified into one of five major groups according to their O_2 needs:

1. Aerobes require the presence of atmospheric oxygen for growth. Their enzyme system necessitates use of O_2 as the final hydrogen (electron) acceptor in the complete oxidative degradation of high-energy molecules such as glucose.
2. Microaerophiles require limited amounts of atmospheric oxygen for growth. Oxygen in excess of the required amount appears to block the activities of their oxidative enzymes and results in death.
3. Obligate anaerobes require the absence of free oxygen for growth because their oxidative enzyme system requires the presence of molecules other than O_2 to act as the final hydrogen (electron) acceptor. In these organisms, as in aerobes, the presence of atmospheric oxygen results in the formation of toxic metabolic end products, such as superoxide, O_2, a free radical of oxygen. However, these organisms lack the enzymes superoxide dismutase and catalase, whose function is to degrade the superoxide to water and oxygen as follows:

$$2O_2^- + 2H^+ \xrightarrow{\text{Superoxide dismutase}} H_2O_2 + O_2$$

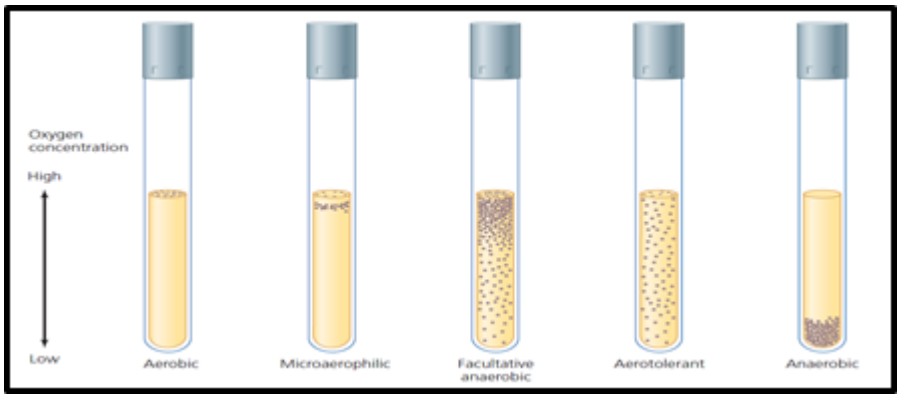
$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

In the absence of these enzymes, small amounts of atmospheric oxygen are lethal, and these organisms are justifiably called obligate anaerobes.

4. Aerotolerant anaerobes are fermentative organisms, and therefore they do not use O_2 as a final electron acceptor. Unlike the obligate anaerobes, they produce catalase and/or superoxide dismutase, and thus they are not killed by the presence of O_2. Hence, these organisms are anaerobes that are termed aerotolerant.
5. Facultative anaerobes can grow in the presence or absence of free oxygen. They preferentially use oxygen for aerobic respiration. However, in an oxygen-poor environment, cellular respiration may occur anaerobically, utilizing such compounds as nitrates (NO_3-) or sulfates ($SO_4$2-) as final hydrogen acceptors, or via a fermentative pathway

   The oxygen needs of microorganisms can be determined by noting their growth distributions following a shake-tube inoculation. This procedure requires introduction of the inoculum into a melted agar medium, shaking of the test tube to disperse the microorganisms throughout the agar, and rapid solidification of the medium to ensure that the cells remain dispersed. Following incubation, the growth distribution indicates the organisms' oxygen requirements. Aerobes exhibit surface growth, whereas anaerobic growth is limited to the bottom of the

deep tube. Facultative anaerobes, because of their indifference to the presence or absence of oxygen, exhibit growth throughout the medium. Microaerophiles grow in a zone slightly below the surface. Figure illustrates the shake-tube inoculation procedure and the distribution of growth following an appropriate incubation period.



The growth rate and distribution of growth at different oxygen concentrations

Experiment 22

Aim:

To study the effect of atmospheric oxygen on the growth of microorganisms.

Principle:

The bacterial species that use molecular oxygen (O_2) produce more energy from nutrients than anaerobes. Oxygen functions as a terminal electron acceptor for an electron transport chain during aerobic respiration. Based on oxygen concentration, the microorganisms can be categorized into the following types:

1. **Obligate aerobes** include microorganisms like *Pseudomonas sp,* common nosocomial pathogens, etc., which require oxygen to survive.
2. **Facultative anaerobes** include *E. coli*, *Staphylococcus sp,* etc., which uses oxygen and can also grow without its presence.
3. **Obligate anaerobes** include *Clostridium sp, Pasteurianum sp* etc., that cannot use oxygen, and they are harmed by the presence of toxic forms of oxygen.
4. **Aerotolerant anaerobes** include *Lactobacillus sp, Enterococcus faecalis* etc., that can use oxygen but can tolerate its presence.
5. **Microaerophiles** include *Campylobacter jejuni* that require oxygen but at low concentration and are sensitive to toxic forms of oxygen.

Requirements:

24-hour nutrient broth cultures of *Staphylococcus aureus, Escherichia coli, Pseudomonas sp., Enterococcus faecalis, Clostridium sp., Campylobacter jejuni*

Six Brain heart infusion agar deep tubes

Bunsen burner,

Water bath, iced water bath,

Thermometer,

Sterile pipettes,

Test tube rack

Glass marker

Composition of BHI Agar (Brain Heart Infusion Agar):
Hi Media M211

HM infusion powder:	12.5g
BHI powder:	5.0g
Proteose peptone:	10.0g
Dextrose (Glucose):	2.0g
Sodium chloride:	5.0g
Disodium phosphate:	2.5g
Agar:	15.0g
Distilled water:	1000ml
Final pH (at 25 °C):	7.4±0.2

Suspend 52.0 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 minutes. Cool to 45-50 °C.

Procedure:

1. Liquefy the sterile Brain Heart Infusion Agar by boiling in a water bath at 100 °C.
2. Cool molten agar to 45 °C; check temperature with a thermometer inserted into the water bath.
3. Using aseptic technique, inoculate each experimental organism by introducing two drops of the culture by a sterile Pasteur pipette into the appropriately labeled tubes of molten agar.
4. Vigorously rotate the freshly inoculated molten infusion agar between the palms of the hands to distribute the organisms.
5. Place inoculated test tubes in an upright position in the iced water bath to solidify the medium rapidly.
6. Incubate all the test tubes for 24 to 48 hours at 37°C.

Observation:

Observe each of the bacterial cultures for the distribution of growth in each tube. Record your observations and determine the oxygen requirements for each of the bacterial species.

4. Physical Factors: Carbon Dioxide

The group of bacteria, which utilizes a higher amount of CO_2 for their growth, refers to **capnophilic bacteria** (*Haemophilus influenzae, Brucella abortus*, etc.). For their optimum growth, they require the presence of 5-10% CO_2 and 15% O_2. In a candle jar, 3% CO_2 can be achieved. Capnophiles are usually found as the normal flora of some ruminants.

5. Physical Factors:Light

It is another factor that affects the bacterial growth, and those bacteria which makes the use of light source can be classified as:

Phototrophs: It refers to a group of bacteria, which derives energy by capturing photons mainly from the **sunlight**. Phototrophs can be either classified into autotrophs (fix carbon) or heterotrophs (utilizes carbon). Examples: *Rhodobacter capsulatus*, *Chromatium*, *Chlorobium* etc.

6. Cultivation of Anaerobic microorganisms:

Microorganisms differ in their abilities to use oxygen for cellular respiration. Respiration involves the oxidation of substrates for energy necessary to life. A substrate is oxidized when it loses a hydrogen ion and its electron (H^+e-). Since the H^+e- cannot remain free in the cell, it must immediately be picked up by an electron acceptor, which becomes reduced. Therefore reduction means gaining the H^+e-. These are termed oxidation-reduction (redox) reactions. Some microorganisms have enzyme systems in which oxygen can serve as an electron acceptor, thereby being reduced to water. These cells have high oxidation-reduction potentials; others have low potentials and must use other substances as electron acceptors.

The regulation of bacterial metabolism is accomplished through changes in the redox potential. The redox potential is a factor determining the growth of anaerobic bacteria, which is regulated by oxidizers and reducers. Under anaerobic conditions, bacteria are sensitive to changes in the redox potential and have redox taxis.

Experiment 23

Aim:

Cultivation of anaerobic microorganisms.

Principle:

Oxygen is ubiquitous in the air so special methods are needed to culture anaerobic microorganisms. A number of procedures are available for reducing the O_2 content of cultures; some simple but suitable mainly for less sensitive organisms, others more complex but necessary for growth of strict anaerobes. Bottles or tubes filled completely to the top with culture medium and provided with tightly fitting stoppers are suitable for organisms which are not too sensitive to small amounts of oxygen. For cultivation of anaerobic microorganisms, a reducing agent is added that reacts with oxygen and reduces it to water e.g., Thioglycolate in thioglycolate broth. Thioglycolate reacts with oxygen deep into the tube, oxygen can penetrate only near the top of the tube where the medium contacts air.

Anaerobic bacteria have special nutritional requirements for vitamin K, haemin and yeast extract, and all primary isolation media for anaerobes should contain these three ingredients.

A. Pyrogallic acid-NaOH Method :

Pyrogallic acid and NaOH absorbs O_2 from the tube and creates an anaerobic atmosphere for the growth of the organism.

Requirements:

24- 48 hour cultures of *Clostridium sporogenes* and *Escherichia coli*.
Two Nutrient agar deep tubes
Pyrogallic acid crystals
4% NaOH
5. Rubber stopper

6. Pasteur pipettes
7. Glass rod
8. Inoculating loop
9. Bunsen Burner
10. Glass marker
11. Incubator

Procedure:

1. Inoculate both bacterial cultures in Nutri-ent agar deep tubes respectively.
2. Ignite the cotton plug before replacing it by passing over the Bunsen flame.
3. With the glass rod push the burning cot-ton plug into the tube until it just touches the slant.
4. Place pyrogallic acid crystals to fill the space above the cotton plug.
5. Add 2 ml of 4% NaOH on top and im-mediately place the rubber stopper tightly on each test tube.
6. Incubate the tubes in an inverted posi-tion at 37 °C for 24 - 48 hours.

Observation:

Anaerobic *Clostridium* will grow whereas *E.coli* will not grow since there was no free oxygen for the growth of the latter.

B. Fluid Thioglycollate medium (pH 7.2):Hi Media M009

This medium contains sodium thioglycollate, which binds to O_2 , thus acting as a reducing compound. Also present is a redox potential indicator, such as resazurin, that produces a pink coloration in an oxidized environment.

Requirements:

24 hour culture of *Escherichia coli*.
48-hour thioglycollate broth culture of *Clostridium sporogenes*
Screw-cap tubes of Fluid thioglycollate Broth
Bunsen burner, inoculating loop
Test tube rack
Glass marker

Composition of Fluid Thioglycollate medium:

Tryptone:	15.0g
Yeast extract	5.0g
Dextrose (Glucose)	5.50g
Sodium chloride	2.5g
L-Cystine	0.5g
Sodium thioglycollate	0.5g
Resazurin sodium	0.001g
Agar	0.75g
Final pH (at 25°C)	7.1±0.2

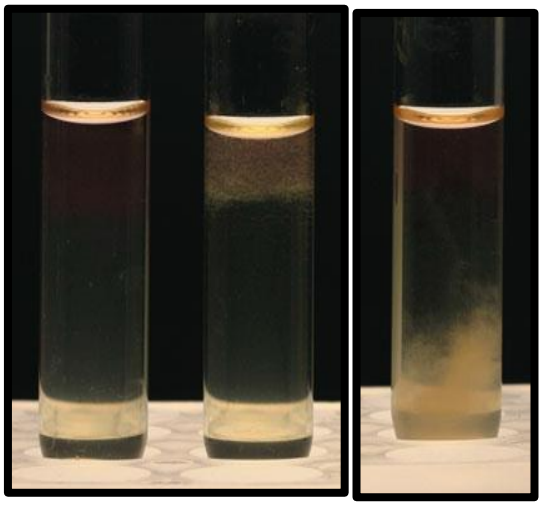
Suspend 29.75 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 minutes.

***Procedure**:*

1. For the performance of this procedure, the fluid thioglycollate medium must be fresh. Freshness is indicated by the absence of a pink color in the upper one-third of the medium. If this coloration is present, loosen the screw caps and place the tubes in a boiling water bath for 10 minutes to drive off the dissolved O_2 from the medium. Cool the tubes to 45 °C before inoculation.
2. Aseptically inoculate the appropriately labeled tubes of thioglycollate broth with the respective test organisms by means of inoculation loop to the depths of the media.
3. Incubate the cultures for 24 to 48 hours at 37 °C.

Observation:

Observe the growth of organisms according to their gaseous requirements in thioglycollate medium. *Clostridium* will grow in the deeper regions while *E. coli* will grow on the surface.



The growth is indicated as: Tube 1 is uninoculated, Tube 2 *E. coli* (surface), Tube *3 Clostridium sporogenes* (bottom)

Chapter - 14
Biochemical Characteristics of Microorganisms

Although morphological and cultural characteristics are essential in identification upto the genus level, species determination requires a good deal more information. The biochemical characteristics such as sugar fermentation, oxidative reactions, IMViC test, enzyme studies make species identification possible.

The chemical reactions that occur within the cells of all living organisms are referred to as **metabolism**. These reactions are catalyzed by protein molecules called enzymes. The majority of enzymes function within the cell and are called **endoenzymes**. Many bacteria also produce **exoenzymes**, which are released by the cell to catalyze reactions outside of the cell. The sum of all these chemical reactions is defined as cellular metabolism, and the biochemical transformations that occur both outside and inside the cell are governed by biological catalysts called enzymes.

In deriving energy from food, bacteria may be either oxidative or fermentative. Oxidative bacteria utilize oxygen to yield carbon dioxide and water. These bacteria have a cytochrome enzyme system. By utilizing organic compounds as electron donors, with oxygen as the ultimate electron (and hydrogen) acceptor, they produce CO_2 and water as end products. Fermentative bacteria, on the other hand, also utilize organic compounds for energy, but they lack a cytochrome system. Instead of only CO_2 and water, they produce complex end products, such as acids, aldehydes, and alcohols. Various gases, such as carbon dioxide, hydrogen, and methane, are also produced. In fermentative bacteria, the organic compounds act both as electron donors and electron acceptors.

Sugars, particularly glucose, are the compounds most widely used by fermenting organisms. Other substances such as organic acids, amino acids, purines, and pyrimidines also can be fermented by some bacteria. The end-products of a particular fermentation are determined by the nature of the organism, the characteristics of the substrate, and environmental conditions such as temperature and pH.

Extracellular Enzymes (Exoenzymes)

Exoenzymes act on substances outside of the cell. Most high-molecular-weight substances are not able to pass through cell membranes, and therefore foods such as polysaccharides, lipids, and proteins must be degraded to low-molecular-weight materials i.e. nutrients before they can be transported into the cell. Because of the reactions involved, exoenzymes are mainly hydrolytic enzymes that reduce high molecular- weight materials into their building blocks by introducing water into the molecule. This liberates smaller molecules, which may then be transported into the cell and assimilated.

A. Starch Hydrolysis

Starch is a high-molecular-weight, branching polymer composed of glucose units; Amylose is linear and Amylopectin is a branched polysaccharide. In these molecules, shorter chains of glucose units linked by α-1,4 are also joined to each other by α-1,6 linkages. The degradation of this macromolecule first requires the presence of the extracellular enzyme

amylase for its hydrolysis into shorter polysaccharides, namely dextrins, and ultimately into maltose molecules. The final hydrolysis of this disaccharide, which is catalyzed by maltase, yields low-molecular-weight, soluble glucose molecules that can be transported into the cell and used for energy production through the process of glycolysis.

Experiment 24

Aim:

To study microorganisms capable of hydrolyzing the polysaccharide Starch by producing exoenzyme α amylases.

Principle:

This test is used to identify bacteria that can hydrolyze starch (amylose and amylopectin) using the enzymes α-amylase and oligo-1,6-glucosidase. It is often used to differentiate species from the genera Clostridium and Bacillus. Because of the large size of amylose and amylopectin molecules, these molecules can not pass through the bacterial cell wall. In order to use starch as a carbon source, bacteria must secrete α-amylase and oligo-1,6-glucosidase into the extracellular space. These enzymes break the starch molecules into smaller glucose subunits which can then enter directly into the glycolytic pathway. In order to interpret the results of the starch hydrolysis test, iodine must be added to the agar. The iodine reacts with the starch to form a blue-black color. Thus, hydrolysis of the starch will create a clear zone around the bacterial growth.

Requirements:

24 hour cultures of *Bacillus subtilis and Clostridium*

Starch agar plates (1% Starch added to Nutrient Agar)

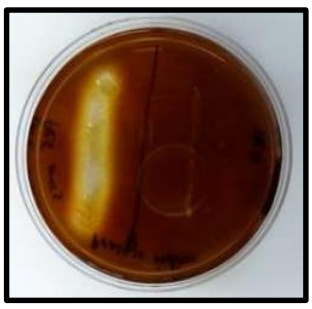
Iodine Solution

Procedure:

1. Using a sterile technique, make a single streak inoculation of the organism to be tested into the centre of the labeled plate.
2. Incubate the bacteria inoculated plates for 24- 48 hours at 37°C.
3. Following incubation, flood the surface of the plates with iodine solution with a dropper for 30 seconds.
4. Pour off the excess iodine.
5. Examine for the clear zone around the line of streaking.

Observation:

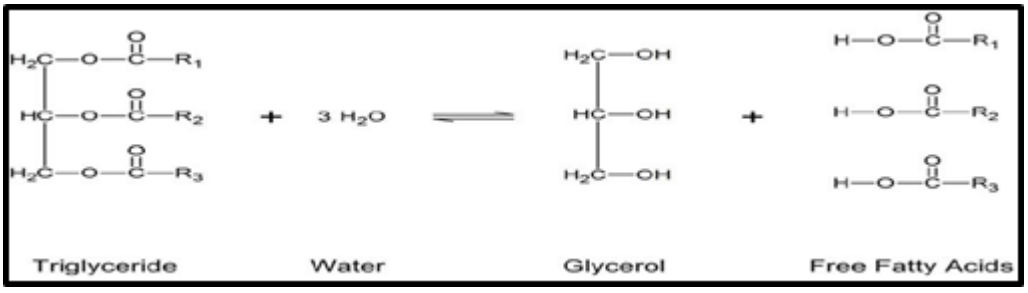
Bacillus subtilis is positive for starch hydrolysis. The organism shown on the right is negative for starch hydrolysis.



Starch hydrolysis: Left streak *B. subtilis* (positive) and Right *E. coli* (negative)

B. Lipid Hydrolysis

Lipids are high-molecular-weight compounds possessing large amounts of energy. The degradation of lipids such as triglycerides is accomplished by extracellular hydrolyzing enzymes, called lipases (esterases), that cleave the ester bonds in this molecule by the addition of water to form the building blocks glycerol (an alcohol) and fatty acids.



Once assimilated into the cell, these basic components can be further metabolized through aerobic respiration to produce cellular energy, adenosine triphosphate (ATP). The components may also enter other metabolic pathways for the synthesis of other cellular protoplasmic requirements.

Experiment 25

Aim:

To study microorganisms capable of hydrolyzing the Lipid by producing exoenzymes Lipases

Principle:

In this experimental procedure, tributyrin agar is used to demonstrate the hydrolytic activities of the exoenzyme lipase. The medium is composed of nutrient agar supplemented with the triglyceride tributyrin as the lipid substrate. Tributyrin forms an emulsion when dispersed in the agar, producing an opaque medium that is necessary for observing exo enzymatic activity. Following inoculation and incubation of the agar plate cultures, organisms secreting lipase will show a zone of lipolysis, which is demonstrated by a clear area surrounding the bacterial growth.

The loss of opacity is the result of the hydrolytic reaction yielding soluble glycerol and fatty acids and represents a positive reaction for lipid hydrolysis. In the absence of lipolytic enzymes, the medium retains its opacity.

Requirements:

24 hour cultures of *E. coli and Pseudomonas aeruginosa*

Tributyrin agar Agar plates

Composition of Tributyrin agar:

Peptic digest of animal tissue:	5.0 g
Yeast extract:	3.0 g
Agar:	15.0 g
Final pH (at 25°C):	7.5±0.2

Suspend 23 grams in 990 ml distilled water. Add 10 ml of Tributyrin (HiMedia FD081). Mix and heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 minutes. Shake the flask and individual plate so as to maintain uniform turbidity

Procedure:

1. Using aseptic technique, streak tributyrin agar plate by making a single-line streak inoculation of each test organism on the agar surface.
2. Incubate all plates in an inverted position for 24 to 48 hours at 37 °C.
3. Observe the clear zone around the bacterial growth.

Observation:

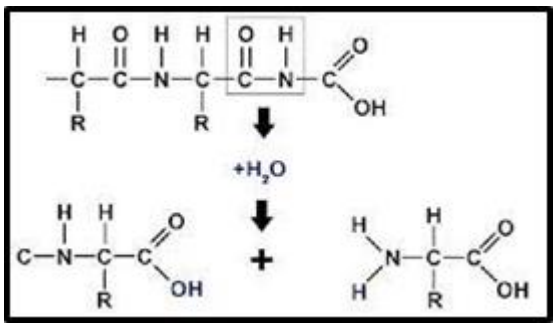
P. aeruginosa is positive for lipid hydrolysis whereas *E. coli* shows negative results.

Lipid hydrolysis: Left streak *E. coli* (negative) and Right *P. aeruginosa* (positive)

C. Casein Hydrolysis

Casein, the major milk protein, is a macromolecule composed of amino acid subunits linked together by peptide bonds (CO-NH). Before their assimilation into the cell, proteins must undergo step-by-step degradation into peptones, polypeptides, dipeptides, and ultimately into their building blocks, amino acids. This process is called peptonization, or proteolysis, and it is mediated by extracellular enzymes called proteases. The function of these proteases is to cleave the peptide bond CO-NH by introducing water into the molecule.

The reaction then liberates the amino acids, as illustrated in Figure below.



Hydrolysis of Protein

The low-molecular-weight soluble amino acids can now be transported through the cell membrane into the intracellular amino acid pool for use in the synthesis of structural and functional cellular proteins.

Experiment 26

Aim:

To study microorganisms capable of hydrolyzing the Casein by producing exoenzymes proteases.

Principle:

In this experimental procedure, milk agar is used to demonstrate the hydrolytic activity of these exoenzymes. The medium is composed of nutrient agar supplemented with milk that contains the protein substrate casein. Similar to other proteins, milk protein is a colloidal suspension that gives the medium its color and opacity because it deflects light rays rather than transmitting them. Following inoculation and incubation of the agar plate cultures, organisms secreting proteases will exhibit a zone of proteolysis, which is demonstrated by a clear area surrounding the bacterial growth.

The loss of opacity is the result of a hydrolytic reaction yielding soluble, non colloidal amino acids, and it represents a positive reaction. In the absence of protease activity, the medium surrounding the growth of the organism remains opaque, which is a negative reaction.

Requirements:

24 hour cultures of *E. coli and Bacillus subtilis*
Skim Milk Agar Plates

Composition of Skim Milk Agar:

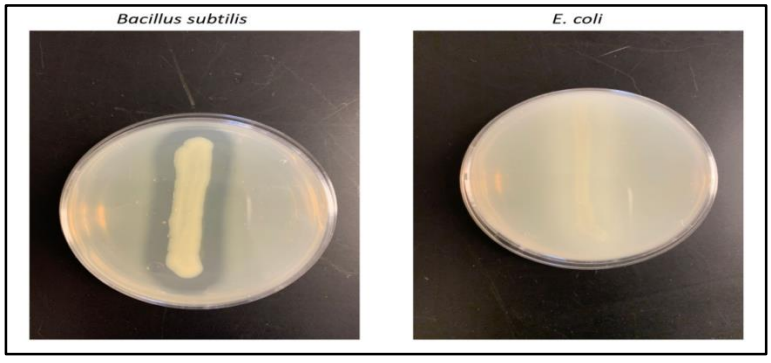
Skim Milk powder:	28.0g
Tryptone:	5.0g
Yeast extract:	2.5g
Dextrose (Glucose):	1.0g
Agar:	15.0g
Distilled Water:	1000ml
Final pH (at 25°C):	7.0±0.2

Procedure:

1. Using aseptic technique, streak Skim Milk Agar plate by making a single-line streak inoculation of each test organism on the agar surface.
2. Incubate all plates in an inverted position for 24 to 48 hours at 37 °C.
3. Observe the clear zone around the bacterial growth.

Observation:

B. subtilis is positive for casein hydrolysis whereas *E. coli* shows negative results.



Casein hydrolysis: *B. subtilis* (positive) and *E. coli* (negative)

D. Gelatin Hydrolysis

Although gelatin is not a nutritional source of microorganisms (it is an incomplete protein, lacking the essential amino acid tryptophan), it is very important in identifying bacterial species. Gelatin is a protein produced by hydrolysis of collagen, a major component of connective tissue and tendons in humans and other animals. Below temperatures of 25 °C, gelatin will maintain its gel properties and exist as a solid; at temperatures above 25 °C, gelatin is liquid. Liquefaction is carried out by some microorganisms capable of producing a proteolytic extracellular enzyme called gelatinase, which acts to hydrolyze this protein to amino acids. After degradation, even very low temperatures of 4 °C will not restore its gelling property.

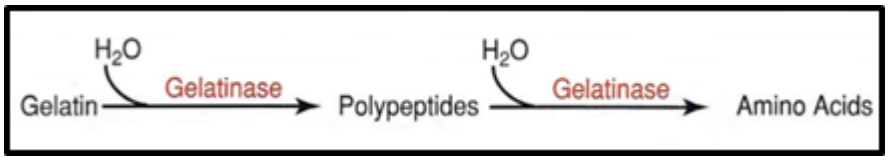
Experiment 27

Aim:

To study microorganisms capable of gelatin liquefaction by producing exoenzymes gelatinases.

Principle:

This test is used to determine the ability of an organism to produce extracellular proteolytic enzymes (gelatinases) that liquefy gelatin, a component of vertebrate connective tissue. The medium consists of nutrient broth supplemented with 12% gelatin. The high gelatin concentration results in a gelled medium and also serves as the substrate for the gelatinase activity. Gelatinases hydrolyze gelatin into polypeptides and then polypeptides are further converted into amino acids. The amino acid is taken up by the cell and used for metabolic purposes.



Following inoculation and incubation for 48 hours, the cultures are placed in a refrigerator at 4 °C for 30 minutes. Cultures that remain liquefied produce gelatinase and demonstrate rapid gelatin hydrolysis. Re-incubate all solidified cultures for an additional 5 days. Refrigerate at 4 °C for 30 minutes and observe for liquefaction. Cultures that remain liquefied are indicative of slow gelatin hydrolysis.

Requirements:

24 hour cultures of *Staphylococcus aureus, Proteus vulgaris* and *E. coli*

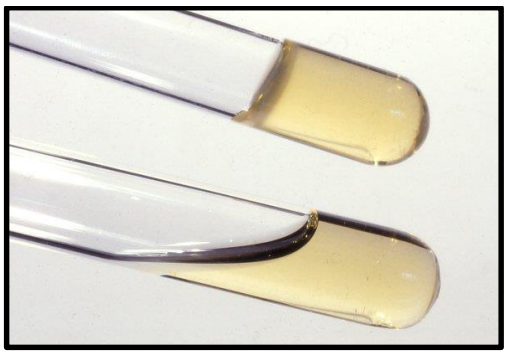
Gelatin Agar deep tubes (12% gelatin)

Procedure:

1. Using aseptic technique, inoculate the gelatin agar deep tubes using a 24-hour-old colony by stabbing four or five times, 0.5 inch into the medium.
2. Incubate the gelatin agar deep tubes at 37 °C for 24-48 hrs.
3. Remove the gelatin tube from the incubator and place at 4 °C to check for liquefaction of gelatin.

Observation:

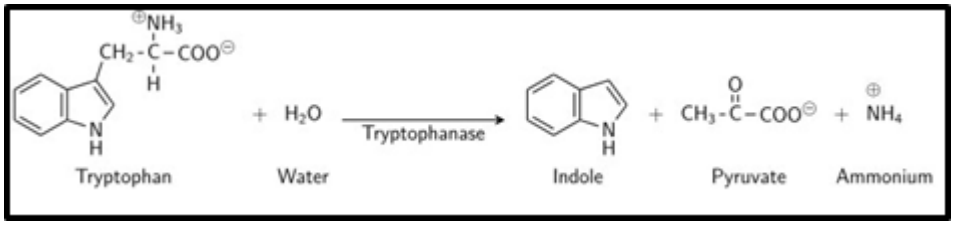
Observe the tubes for liquefaction of gelatin. *Staphylococcus aureus* and *Proteus vulgaris* liquefies gelatin whereas *E. coli* is gelatinase negative.



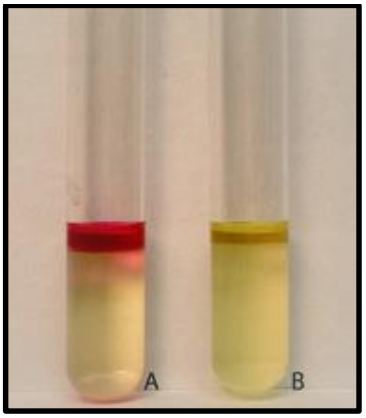
Gelatin liquefaction: Tube below 1 is *Proteus* (positive), Tube above 2 *E. coli* (negative)

E. Tryptophan Hydrolysis

Certain bacteria, such as E. coli, have the ability to split the amino acid tryptophan into indole and pyruvic acid. The enzyme that causes this hydrolysis is tryptophanase. Indole can be easily detected with Kovacs' reagent.



It is particularly useful in differentiating E. coli from some closely related enteric bacteria. This test is studied in more detail in the IMViC Test.

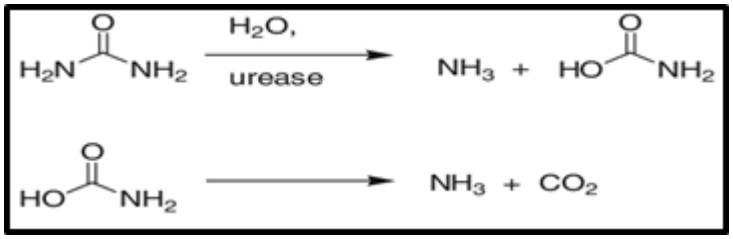


Tryptophan hydrolysis: Tube A *E. coli* (positive),Tube B (negative)

F. Urea Hydrolysis

Urea Agar was developed by Christensen in 1946 for the differentiation of enteric bacilli. The urease test is used to determine the ability of an organism to split urea, through the production of the enzyme urease.

Urea is the product of decarboxylation of amino acids. Hydrolysis of urea produces ammonia and CO2.



The formation of ammonia alkalinizes the medium, and the pH shift is detected by the color change of phenol red from light orange at pH 6.8 to magenta (pink) at pH 8.1. Rapid urease-positive organisms turn the entire medium pink within 24 hours.

Experiment 28

Aim:

To study microorganisms capable of hydrolyzing urea by producing exoenzymes ureases.

Principle:

When urease is produced by an organism in this medium, the ammonia that is released raises the pH. As the pH becomes higher, the phenol red changes from a yellow color (pH 6.8) to a red or cerise color (pH 8.1 or more).

Urea broth is a buffered solution of yeast extract and urea. It also contains phenol red as a pH indicator. Since urea is unstable and breaks down in the autoclave at 15 psi steam pressure, it is usually sterilized by filtration. It is tubed in small amounts to hasten the visibility of the reaction.

Requirements:

24 hour cultures of *Proteus vulgaris* and *E. coli*

UREA BROTH AND CHRISTENSEN'S UREA AGAR SLANTS

Composition of Christensen's Urea Agar:

Urea:	20.0 g
Sodium Chloride:	5.0 g
Monopotassium Phosphate:	2.0 g
Peptone:	1.0 g
Dextrose:	1.0 g
Phenol Red:	0.012 g
Agar:	15.0 g
Distilled water:	1000ml

Final pH 6.7 ± 0.2 at 25°C.

Dissolve all the ingredients (except urea) in 950 ml of distilled water, boil to dissolve completely. Autoclave at 121°C and 15 lbs for 15 minutes. Cool the agar to 50 to 55 °C.

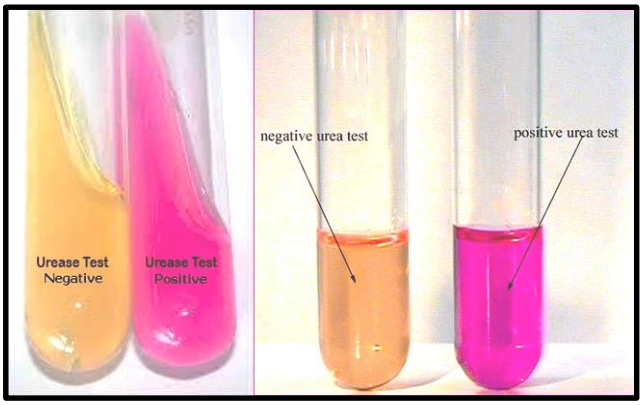
Aseptically add 50 ml of filter-sterilized urea (40% solution) to the cooled agar solution and mix thoroughly. Prepare the agar slants.

Procedure:

1. Streak the surface of a urea agar slant with a portion of a well-isolated colony or inoculate slant with 1 to 2 drops from an overnight brain-heart infusion broth culture.
2. Incubate the tubes at 35 °C-37 °C in the incubator for 24-48 hours.
3. Examine the development of a pink color.

Observation:

Examine your tubes of urea broth and Christensen's Urea Agar slantsthat were inoculated with *Proteus vulgaris*.



Urea hydrolysis: *E. coli* (negative), *P. vulgaris* (positive)

Sugar fermentation

Microorganisms obtain their energy through a series of enzymatic reactions leading to the biooxidation of a substrate, frequently a carbohydrate. Organisms use different carbohydrates depending on their enzyme systems. Some organisms are capable of fermenting sugars such as glucose anaerobically, while others use the aerobic pathway. Still others, facultative anaerobes, are enzymatically competent to use both aerobic and anaerobic pathways, and some organisms lack the ability to oxidize glucose by either. They utilize EMP, HMP, ED pathways depending on the types of organism.

Experiment 29

Aim:

To study microorganisms capable of fermenting different carbohydrate sources.

Principle:

In fermentation, substrates such as carbohydrates and alcohols undergo anaerobic dissimilation and produce an organic acid (lactic, formic, or acetic acid) that may be accompanied by gases such as hydrogen or carbon dioxide. Facultative anaerobes are usually called as fermenters of carbohydrates.

Fermentative degradation under anaerobic conditions is carried out in a fermentation broth tube containing a Durham tube, an inverted inner vial for the detection of gas production. A typical carbohydrate fermentation medium contains:

1. Nutrient broth ingredients for the support of the growth of all organisms.
2. A specific carbohydrate that serves as the substrate for determining the organism's fermentative capabilities.

3. The pH indicator is phenol red, which is red at a neutral pH 7 and changes to yellow at a slightly acidic pH of 6.8, indicating that slight amounts of acid will cause a color change.

Following incubation, carbohydrates that have been fermented with the production of acidic wastes will cause the phenol red to turn yellow, thereby indicating a positive reaction. In some cases, acid production is accompanied by the evolution of a gas (CO_2) that will be visible as a bubble in the inverted tube. Cultures that are not capable of fermenting a carbohydrate substrate will not change the indicator, a negative reaction.

The lack of carbohydrate fermentation by some organisms should not be considered as absence of growth. These organisms use other nutrients in the medium as energy sources such as peptones present in nutrient broth. Peptones can be degraded by microbial enzymes to amino acids that are in turn enzymatically converted by oxidative deamination to keto amino acids. These are then metabolized through the Krebs cycle for energy production. These reactions liberate ammonia, which accumulates in the medium, forming ammonium hydroxide (NH_4OH) and producing an alkaline environment. When this occurs, the phenol red turns to a deep red in the now basic medium.

Requirements:

24 hour cultures of *S. aureus, Pseudomonas aeruginosa* and *E. coli*

CARBOHYDRATE FERMENTATION BROTH TUBES

Bunsen burner

Inoculating loop

Glassware marker

Incubator

Composition of Phenol Red Carbohydrate Broth:

Trypticase or protease peptone:	10 g
Sodium chloride:	5 g
Beef extract:	1 g
Phenol red (7.2 ml of 0.25% phenol red solution):	0.018 g
Carbohydrate source:	10 g

Glucose, sucrose, lactose, mannitol, maltose, sorbitol are the Carbohydrate sources.

PREPARATION OF THE MEDIA:

1. Prepare broth media by mixing all ingredients in 1000 mL of distilled/deionized water and heating gently to dissolve it *(Note: Use a single carbohydrate source based on your requirements).*
2. Fill 13 x 100 mm test tubes with 4-5 ml of phenol red carbohydrate broth.
3. Insert a fully filled Durham tube with broth to detect gas production.
4. Autoclave the prepared test media (at 121°C for 15 minutes) to sterilize.
5. Prepare specific carbohydrate solution separately, filter the solution using membrane filter (pore size: 0.45 μm).
6. Add 0.5% to 1% of the desired carbohydrate into all flasks and mix it gently.

Procedure:

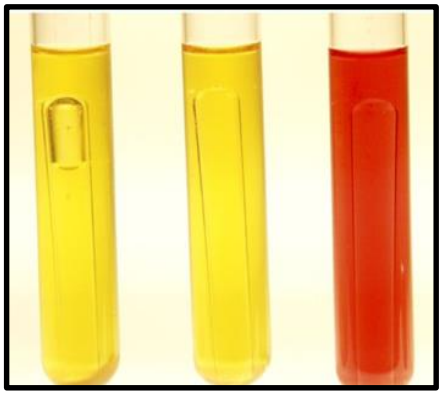
1. Inoculate each tube with 1 drop of an 18 hour or 24-hour cultural broth in aseptic condition (keep uninoculated tubes as control tubes).

2. Incubate the tubes at 18-24 hours at 37 °C.
3. Examine the tubes for acid and gas production.

No color change or a result indicating alkalinity may occur if the organism deaminates the peptone, masking the carbohydrate fermentation evidence.

Observation:

Examine your tubes for Acid and Gas production.



Sugar fermentation: *E. coli* (A/G +ve); *S. aureus* (A+ve); *P. aeruginosa* (A/G-ve)

Triple Sugar Iron Test

Most bacteria have the ability to ferment carbohydrates, particularly sugars. Among them, each bacteria can ferment only some of the sugars, while it cannot ferment the others. Thus, the sugars, which a bacteria can ferment and the sugars, which it cannot, is the characteristic of the bacteria and thus an important criterion for its identification.

The Triple Sugar Iron (TSI) test is a microbiological test to test a microorganism's ability to ferment sugars and to produce hydrogen sulfide. An agar slant of a special medium with multiple sugars constituting a pH-sensitive dye (phenol red), 1% lactose, 1% sucrose, 0.1% glucose, as well as sodium thiosulfate and ferrous sulfate or ferrous ammonium sulfate is used for carrying out this test. All these ingredients when mixed together and allowed solidification at an angle result in an agar test tube at a slanted angle. The slanted shape of this medium provides an array of surfaces that are either exposed to oxygen-containing air in varying degrees (an aerobic environment) or not exposed to air (an anaerobic environment), the butt region under which fermentation patterns of organisms are determined.

Experiment 30

Aim:

To determine the ability of an organism to ferment glucose, lactose, and sucrose, and their ability to produce hydrogen sulfide.

Principle:

The triple sugar- iron agar test employing Triple Sugar Iron Agar is designed to differentiate among organisms based on the differences in carbohydrate fermentation patterns and hydrogen sulfide production. Carbohydrate fermentation is indicated by the production of gas and a change in the colour of the pH indicator from red to yellow.

TSI Agar contains three fermentative sugars, lactose and sucrose in 1% concentrations and glucose in 0.1% concentration. Due to the production of acid during fermentation, the pH falls. The acid base indicator phenol red is incorporated for detecting carbohydrate fermentation that is indicated by the change in color of the carbohydrate medium from orange red to yellow in the presence of acids. In case of oxidative decarboxylation of peptone, alkaline products are accumulated and the pH rises. This is indicated by the change in colour of the medium from orange red to deep red. Sodium thiosulfate and ferrous ammonium sulfate present in the medium detects the production of hydrogen sulfide and is indicated by the black color in the butt of the tube.

To facilitate the detection of organisms that only ferment glucose, the glucose concentration is one-tenth the concentration of lactose or sucrose. The small amount of acid production in the slant of the tube during glucose fermentation oxidizes rapidly, causing the medium to remain orange red or revert to an alkaline pH. In contrast, the acid reaction (yellow) is maintained in the butt of the tube since it is under lower oxygen tension.

After depletion of the limited glucose, organisms able to do so will begin to utilize the lactose or sucrose. To enhance the alkaline condition of the slant, free exchange of air must be permitted by closing the tube cap loosely.

Requirements:

24 hour cultures of *Pseudomonas aeruginosa, E. coli, Salmonella typhimurium* and *Shigella boydii*

TSI Agar tubes

Bunsen burner

Inoculating loop

Glassware marker

Incubator

Composition of TSI Agar:

Enzymatic digest of casein:	5g
Enzymatic digest of animal tissue:	5g
Yeast enriched peptone:	10g
Dextrose:	1g
Lactose:	10g
Sucrose:	10g
Ferric ammonium citrate:	0.2g
NaCl:	5g
Sodium thiosulfate:	0.3g
Phenol red:	0.025g
Agar:	13.5g
Distilled water:	1000 mL
Final pH:	7.3.

Procedure:

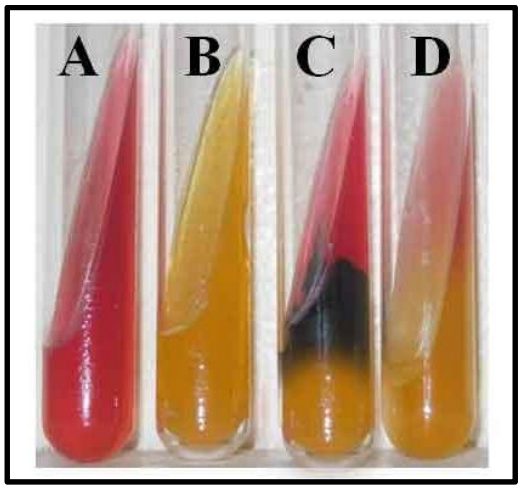
1. With a straight inoculation needle, touch the top of a well-isolated colony.

2. Inoculate TSI by first stabbing through the center of the medium to the bottom of the tube and then streaking the surface of the agar slant using an inoculation loop.
3. Leave the cap on loosely and incubate the tube at 35 °C-37 °C in the incubator for 18 to 24 hours.
4. Examine the reaction of the medium.

Observation and expected Results:

Observe the TSI Agar tubes, its slant and Butt region for change in colour, production of acid and hydrogen sulphide gas.

1. **An alkaline/acid (red slant/yellow butt) reaction:** It is indicative of glucose fermentation only.
2. **An acid/acid (yellow slant/yellow butt) reaction:** It indicates the fermentation of glucose, lactose and/or sucrose.
3. **An alkaline/alkaline (red slant, red butt) reaction:** Absence of carbohydrate fermentation results.
4. **Blackening of the medium:** Occurs in the presence of H_2S
5. **Gas production:** Bubbles or cracks in the agar indicate the production of gas (formation of CO_2 and H_2).



TSI Test: A) *P. aeruginosa:* Glu(-), Lac/Suc(-), H_2S(-);
B) *E. coli:* Glu(+), Lac/Suc(+), H_2S(-);
C) *S. typhimurium:* Glu(+), Lac/Suc(-), H_2S(+);
D) *Shigella boydii:* Glu(+), Lac/Suc(-), H_2S(-).

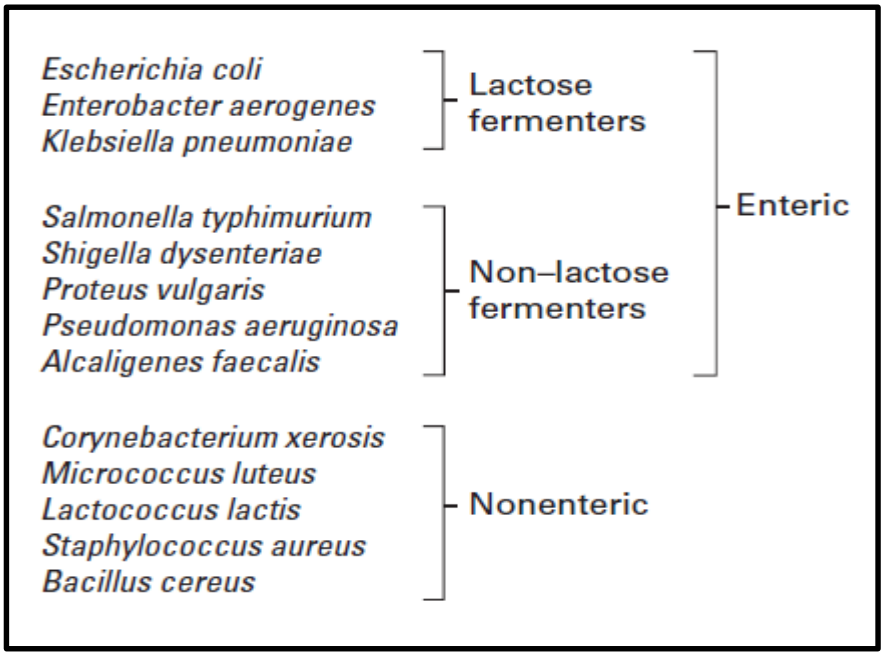
IMViC Test

Identification of enteric bacteria is very important in controlling intestinal infections, thereby controlling contamination of food and water supplies. The groups of bacteria that are commonly found in the intestinal tract of humans and lower mammals belong to the family Enterobacteriaceae. They are short, gram negative, non–spore-forming bacilli. Following are included in this family:

1. Pathogens such as members of the genera *Salmonella* and *Shigella*.
2. Occasional pathogens such as members of the genera *Proteus* and *Klebsiella*.

3. Normal intestinal flora such as members of the genera *Escherichia* and *Enterobacter*, saprophytic inhabitants of the intestinal tract.

Differentiation of these Enterobacteriaceae groups can be accomplished on the basis of their biochemical properties and enzymatic reactions in the presence of specific substrates. The IMViC series of tests (Indole, Methyl Red, Voges-Proskauer, and Citrate utilization) are used to differentiate those enteric groups on the basis of carbohydrate fermentation with acid and gas productions and other stable end products which are characteristics of specific organisms. The enteric organisms are subdivided as lactose fermenters and non–lactose fermenters.



A. Indole Production Test

Indole is generated by reductive deamination from tryptophan via the intermediate molecule indole pyruvic acid. Tryptophanase catalyzes the deamination reaction, during which the amine (NH_2) group of the tryptophan molecule is removed. Final products of the reaction are indole, pyruvic acid, ammonium (NH_4^+) and energy. Pyridoxal phosphate is required as a coenzyme.

Experiment 31

Aim:

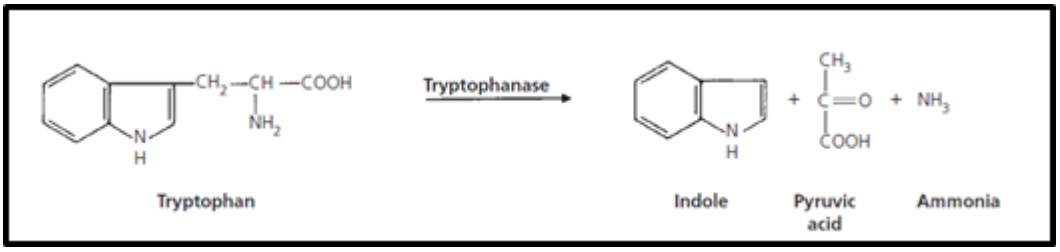
To determine the ability of microorganisms to degrade the amino acid tryptophan.

Principle:

Tryptophan is an essential amino acid that can undergo oxidation by some bacteria. Conversion of tryptophan into metabolic products is mediated by the enzyme tryptophanase. This ability to hydrolyze tryptophan with the production of indole is not a characteristic of all microorganisms and therefore serves as a differentiating factor.

SIM agar, which contains the substrate tryptophan, is used for Indole production. SIM medium is a combination differential medium that tests three different parameters, which are represented by the three letters in the name:

Sulfur Reduction
Indole Production

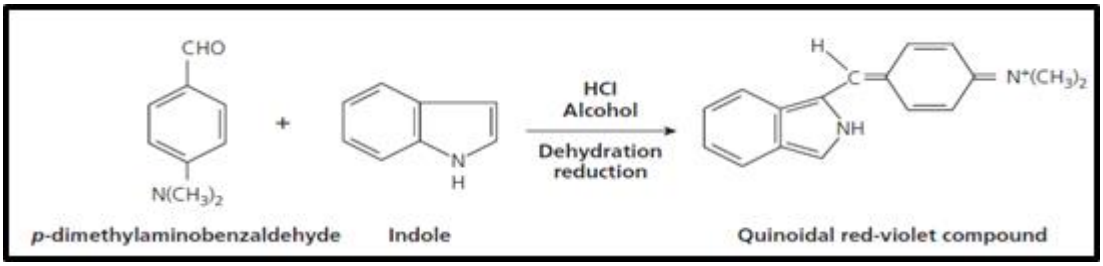


Tryptophan degradation by tryptophanase with production of Indole

Motility

The sulfur reduction test is useful in differentiating enteric organisms. The indole test is a component of the IMViC series of tests, which is used for differentiating the *Enterobacteriaceae*. The motility test is useful for testing motility in a wide variety of organisms. As a whole, the SIM test is primarily useful for differentiating *Salmonella* and *Shigella*. SIM medium contains nutrients, iron, and sodium thiosulfate. One of the nutrients is peptone, which contains amino acids, including tryptophan. If an organism can reduce sulfur to hydrogen sulfide, the hydrogen sulfide will combine with the iron to form ferric sulfide, which is a black precipitate. If there is any blackening of the medium, it indicates the reduction of sulfur and is a positive result.

The presence of indole is detectable by adding Kovac's reagent, which produces a cherry red/ crimson pink reagent layer. This color is produced by the reagent, which is composed of p-dimethylaminobenzaldehyde, butanol, and hydrochloric acid. Indole is extracted from the medium into the reagent layer by the acidified butyl alcohol component and forms a complex with the p-dimethylaminobenzaldehyde, yielding the cherry red color. The chemical reaction is illustrated below.



Indole reaction with Kovac's reagent

Requirements:

24 hour culture of *E. coli, P. vulgaris, and E. aerogenes*
SIM agar deep tubes
Kovac's reagent
Bunsen burner
Inoculating needle
Test tube rack
Glass marker

Composition of SIM agar:

Peptone:	30.0g
Beef extract:	3.0g
Ferrous ammonium sulfate:	0.2g
Sodium thiosulfate:	0.025g
Agar:	3.0g
Distilled water:	1000ml
pH	7.3

Kovac's reagent:

p-Dimethylaminobenzaldehyde:	5.0 g
Amyl alcohol:	75.0 ml
Hydrochloric acid (concentrated):	25.0 ml

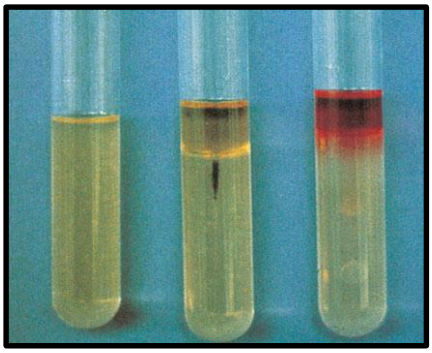
Dissolve the p-dimethylaminobenzaldehyde in the amyl alcohol. Add the hydrochloric acid.

Procedure:

1. Using aseptic technique, inoculate each test organism into its appropriately labeled
2. deep tube by means of a stab inoculation. The uninoculated tube will serve as a control.
3. Incubate tubes for 24 to 48 hours at 37 °C.
4. Add 10 drops of Kovac's reagent to all deep tube cultures and agitate the cultures gently.
5. Examine the color of the reagent layer in each culture tube.
6. Observe the tubes whether or not each organism was capable of hydrolyzing the tryptophan.

Observation:

Cultures producing a red reagent layer following addition of Kovac's reagent are indole-positive. The absence of red coloration demonstrates that the substrate tryptophan was not hydrolyzed and indicates an indole-negative reaction.



Indole Production Tube 1: uninoculated; Tube 2: Negative;Tube 3: Positive

Methyl Red Test

The hexose monosaccharide glucose is the major substrate utilized by all enteric organisms for energy production. The end products of this process vary depending on the specific enzymatic pathways present in the bacteria. In this test the pH indicator methyl red detects the presence of large concentrations of acid end products.

Experiment 32

Aim:

Determine the ability of microorganisms to ferment glucose with the production of acid end products.

Principle:

Although most enteric microorganisms ferment glucose with the production of organic acids, this test is of value in the separation of *E. coli* and *E. aerogenes*. Both of these organisms initially produce organic acid end products during the early incubation period. The low acidic pH 4 is stabilized and maintained by *E. coli* at the end of incubation. During the later incubation period, *E. aerogenes* enzymatically converts these acids to non acidic end products such as 2,3-butanediol and acetoin (acetylmethylcarbinol), resulting in an elevated pH of approximately 6. The glucose fermentation reaction generated by *E. coli* is illustrated below.

Glucose + H_2O → [Lactic acid, Acetic acid, Formic acid]+ CO_2 + H_2 (pH 4.0)→ Methyl red indicator turns red color.

The methyl red indicator in the pH range of 4 turns red, which is indicative of a positive test. At a pH of 6, still indicating the presence of acid but with a lower hydrogen ion concentration, the indicator turns yellow and is a negative test. Production and detection of the non acidic end products from glucose fermentation by *E. aerogenes* is described in the Voges-Proskauer test, which is performed simultaneously with the methyl red test.

Clark and Lubs developed MR-VP Broth which allowed both the MR and VP tests to be performed from the same inoculated medium by aliquoting portions to different tubes.

Requirements:

24- to 48-hour culture of *E. coli* and *E. aerogenes*
MR-VP broth tubes
Methyl red indicator
Bunsen burner
Inoculating loop
Test tubes
Glass marker
Incubator

Composition of MR VP Broth:

Peptone:	7.0g
Dextrose:	5.0g
Potassium phosphate:	5.0g
Distilled water:	1000ml
Final pH:	6.9

Methyl red solution:

Methyl red:	0.1 g
Ethyl alcohol:	300.0 ml
Distilled water:	200.0 ml

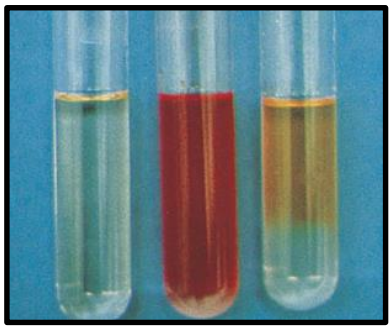
Dissolve the methyl red in the 95% ethyl alcohol. Dilute to 500 ml with distilled water.

Procedure:

1. Using aseptic conditions, inoculate the MR medium with test organisms taken from an 18-24 hour pure culture.
2. Incubate aerobically at 37 °C for 24 hours.
3. Following 24 hours of incubation, 1ml of the broth is taken in a clean test tube.
4. Reincubate the remaining broth for an additional 24 hours.
5. Add 2 to 3 drops of methyl red indicator to 1 ml broth.
6. Observe the red color immediately.

Observation:

Observe the tube for distinct red coloration after addition of methyl red indicator. *E. coli* shows positive results for MR test whereas *E. aerogenes* shows negative results.



Methyl Red Test Tube 1: uninoculated; Tube 2: *E. coli* (positive); Tube 3: *E. aerogenes* (negative)

Voges-Proskauer Test

Voges and Proskauer, in 1898, first observed the production of a red color after the addition of potassium hydroxide to cultures grown on specific media. Harden later revealed that the development of the red color was a result of acetyl-methyl carbinol production. In 1936 Barrit made the test more sensitive by adding alpha-naphthol to the medium before adding potassium hydroxide. The Voges-Proskauer (VP) test is used to determine if an organism produces acetyl methyl carbinol from glucose fermentation. If present, acetylmethylcarbinol is converted to diacetyl in the presence of ∝- naphthol, strong alkali (40% KOH), and atmospheric oxygen.

Experiment 33

Aim:

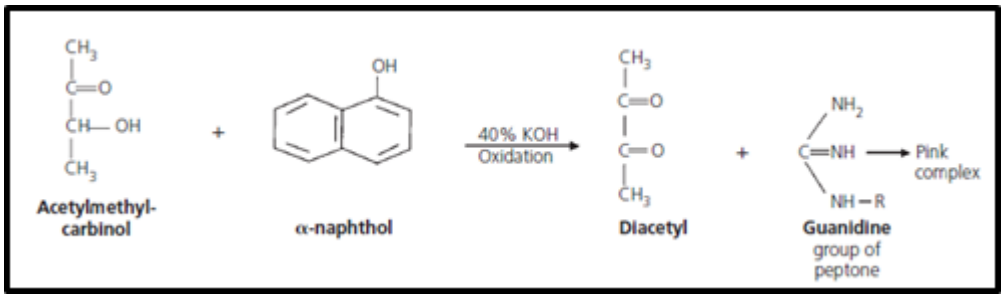
To determine if an organism produces acetyl methyl carbinol from glucose fermentation and further conversion of acetyl methyl carbinol to diacetyl and to differentiate among enteric organisms such as *E. coli*, *E. aerogenes*, and *K. pneumoniae*.

Principle:

The Voges-Proskauer test determines the capability of some organisms to produce non acidic or neutral end products, such as acetylmethylcarbinol, from the organic acids that result from glucose metabolism. This glucose fermentation, which is characteristic of E. aerogenes, is illustrated below.

Glucose + O_2 →Acetic acid → [2,3-butanediol, acetylmethylcarbinol] +CO_2+ H_2(pH 6.0)

The Barritt's reagent is used for this test. It consists of a mixture of alcoholic α-naphthol and 40% potassium hydroxide solution. Detection of acetylmethylcarbinol requires this end product to be oxidized to a diacetyl compound. This reaction will occur in the presence of the α-naphthol catalyst and a guanidine group that is present in the peptone of the MR-VP medium. As a result, a pink red complex is formed, imparting a crimson pink to the medium. The chemical reaction of Barritt's reagent with acetylmethylcarbinol is illustrated below.



Development of a pink red color in the culture 15 minutes following the addition of Barritt's reagent is indicative of the presence of acetylmethylcarbinol and represents a positive result. The absence of pink red coloration is a negative result.

Requirements:

24- to 48-hour culture of *E. coli* and *E. aerogenes*
MR-VP broth tubes
Barritt's reagent
Bunsen burner
Inoculating loop
Test tubes
Glass marker
Incubator

Composition of MR VP Broth:

Peptone: 7.0g
Dextrose: 5.0g
Potassium phosphate: 5.0g
Distilled water: 1000ml
Final pH: 6.9

Barritt's Reagent:

Solution A

Alpha-naphthol: 5.0 g
Ethanol (absolute): 95.0 ml
Dissolve the alpha-naphthol in the ethanol with constant stirring.

Solution B

Potassium hydroxide: 40.0 g
Creatine: 0.3 g
Distilled water: 100.0 ml

Dissolve the potassium hydroxide in 75 ml of distilled water. The solution will become warm. Allow to cool to room temperature. Add the creatine and stir to dissolve. Add the remaining water. Store in a refrigerator.

Procedure:

1. Using aseptic conditions, inoculate the VP medium with test organisms taken from an 18-24 hour pure culture.
2. Incubate aerobically at 37 °C for 24 hours.
3. Following 24 hours of incubation, 2ml of the broth is taken in a clean test tube.
4. Reincubate the remaining broth for an additional 24 hours.
5. Add 6 drops of 5% alpha-naphthol, and mix gently.
6. Add 2 drops of 40% potassium hydroxide, and mix gently.
7. Observe a pink-red color at the surface within 30 min. Shake the tube vigorously during the 30-min period.

Observation:

Observe the tube for distinct pink red coloration after addition of Barritt's reagent. *E. aerogenes* shows positive results whereas *E. coli* shows negative results for the VP test .

Voges Proskauer Test Tube 1: *E. aerogenes* (positive); Tube 2: *E.coli* (negative)

Citrate Utilization

Citrate agar is used to test an organism's ability to utilize citrate as a source of energy. The medium contains citrate as the sole carbon source and inorganic ammonium salts ($NH_4H_2PO_4$) as the sole source of nitrogen. Bacteria that can grow on this medium produce an enzyme, **citrate-permease**, capable of converting citrate to pyruvate. Pyruvate then enters the organism's metabolic cycle for the production of energy. Growth is indicative of utilization of citrate, an intermediate metabolite in the Krebs cycle.

When the bacteria metabolize citrate, the ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turns the bromothymol blue indicator in the medium from green to blue above pH 7.6.

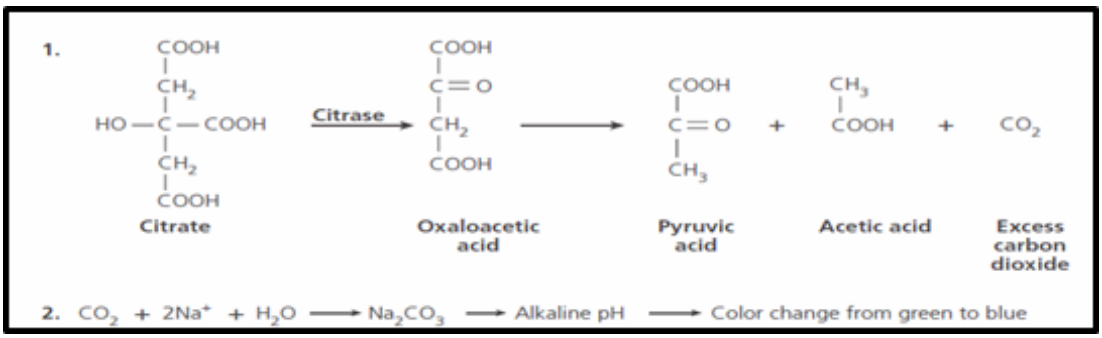
Experiment 34

Aim:

To study enteric organisms on the basis of their ability to utilize citrate as a sole source of carbon.

Principle:

In the absence of fermentable glucose or lactose, some microorganisms are capable of using citrate as a carbon source for their energy. This ability depends on the presence of a citrate permease that facilitates the transport of citrate in the cell. Citrate is the first major intermediate in the Krebs cycle and is produced by the condensation of active acetyl with oxaloacetic acid. Citrate is acted on by the enzyme citrase, which produces oxaloacetic acid and acetate. These products are then enzymatically converted to pyruvic acid and carbon dioxide. During this reaction the medium becomes alkaline—the carbon dioxide that is generated combines with sodium and water to form sodium carbonate, an alkaline product. The presence of sodium carbonate changes the bromothymol blue indicator incorporated into the medium from green to deep blue. The chemical reaction is illustrated below.



Requirements:

24- to 48-hour culture of *E. coli* and *E. aerogenes*
Simmons citrate agar slants
Bunsen burner
Inoculating loop
Test tubes
Glass marker

Composition of Simmons citrate agar:

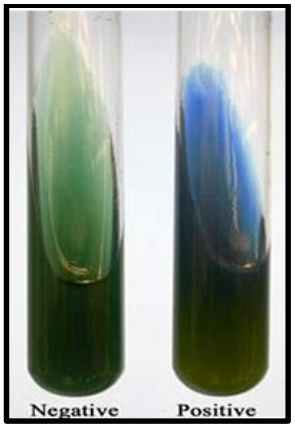
Ammonium dihydrogen phosphate:	1.0g
Dipotassium phosphate:	1.0g
Sodium chloride:	5.0g
Sodium citrate:	2.0g
Magnesium sulfate:	0.2g
Agar:	15.0g
Bromothymol blue:	0.08g
Distilled water:	1000ml
Final pH:	6.9

Procedure:

1. Using aseptic technique, inoculate each test organism into its appropriately labeled tube by means of streak inoculation. The uninoculated tube will serve as a control.
2. Incubate all culture tubes at 37 °C for 24 to 48 hours.

Observation:

Observe all agar slant cultures for the presence or absence of growth and coloration of the medium.



Citrate Utilization Tube 1: *E.coli* (negative); Tube 2: *E. aerogenes* (positive)

Other Biochemical Test for Identification of Bacteria

Catalase test

The catalase test is used for the biochemical differentiation of catalase positive, *Staphylococci* and catalase negative *Streptococci*, as well as members of the Enterobacteriaceae. With the increasing methicillin-resistant strains of *Staphylococcus* in hospital environments, the catalase test is a quick and easy way to differentiate *S. aureus* (MRSA), from other *Staphylococcus* methicillin sensitive strains.

Experiment 35

Aim:

To determine the ability of some microorganisms to degrade hydrogen peroxide by producing the enzyme catalase.

Principle:

During aerobic respiration, microorganisms produce hydrogen peroxide and, in some cases, an extremely toxic superoxide. Accumulation of these substances will result in death of the organism unless they are enzymatically degraded. These substances are produced when aerobes, facultative anaerobes, and microaerophiles use the aerobic respiratory pathway, in which oxygen is the final electron acceptor, during degradation of carbohydrates for energy production. Organisms capable of producing catalase rapidly degrade hydrogen peroxide as illustrated:

$$\mathbf{2H_2O_2 \rightarrow 2H_2O + O_2 \uparrow}$$

Aerobic organisms that lack catalase can degrade especially toxic superoxides using the enzyme superoxide dismutase; the end product of a superoxide dismutase is H_2O_2, but this is less toxic to the bacterial cells than are the superoxides. The inability of strict anaerobes to synthesize

catalase, peroxidase, or superoxide dismutase may explain why oxygen is poisonous to these microorganisms.

In the absence of these enzymes, the toxic concentration of H_2O_2 cannot be degraded when these organisms are cultivated in the presence of oxygen. Catalase production can be determined by adding the substrate H_2O_2 to an appropriately incubated Trypticase soy agar culture tube. If catalase is present, the chemical reaction mentioned is indicated by bubbles of free oxygen gas (O2↑). This is a positive catalase test; the absence of bubble formation is a negative catalase test.

Requirements:

24- to 48-hour culture of *Lactococcus lactis* and *Staphylococcus aureus*
Trypticase Soy Agar tubes
3% hydrogen peroxide
Bunsen burner
Inoculating loop
Test tubes
Glass marker
Incubator

Procedure:

Tube Method:

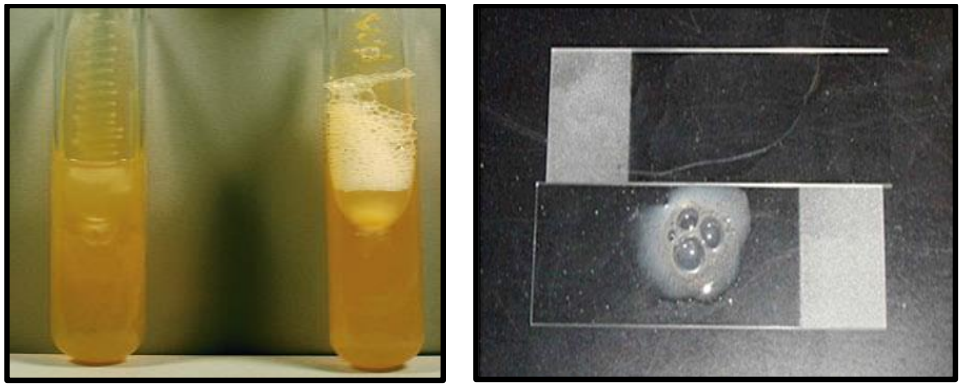
1. Using aseptic technique, inoculate each test organism into its appropriately labeled tube by means of a streak inoculation. The uninoculated tube will serve as a control.
2. Incubate all culture tubes at 37 °C for 24 to 48 hours.

Slide Method:

1. Label slides with the names of the organisms.
2. Using a sterile loop, take a culture of the first organism from the culture tube and transfer it to the appropriately labeled slide.
3. Place the slide in the Petri dish.
4. Place one drop of 3% hydrogen peroxide on the culture. Do not mix. Place the cover on the Petri dish to contain any aerosols.
5. Observe the immediate presence of bubble formation.
6. Repeat Steps 2 through 5 for the remaining for other test organisms.

Observation:

Observe the TSA culture tubes immediately after addition of 3% hydrogen peroxide for oxygen evolution. Also the slide for bubble formation.



Catalase test Tube 1: *Lactococcus lactis* (negative); Tube 2: *S.aureus* (positive)

Oxidase Test

The Enterobacteriaceae are cytochrome oxidase negative, while the genera *Neisseria* and *Pseudomonas* are cytochrome oxidase positive. The oxidase test is an important tool in the identification of *Neisseria meningitidis*, the causative agent of bacterial meningitis, which has a significant morbidity and mortality rate. In addition, yeast of medical importance such as Candida can be separated from Saccharomyces and Torulopsis by this test.

Experiment 36

Aim:

To distinguish Enterobacteriaceae from Non-Enterobacteriaceae among groups of bacteria on the basis of cytochrome oxidase activity.

Principle:

Oxidase enzymes play an important role in the electron transport system during aerobic respiration. Cytochrome oxidase catalyzes the oxidation of a reduced cytochrome by molecular oxygen (O_2), resulting in the formation of H_2O or H_2O_2. Aerobic bacteria, as well as some facultative anaerobes and microaerophiles, exhibit oxidase activity. The oxidase test aids in differentiation among members of the genera *Neisseria* and *Pseudomonas*, which are oxidase positive, and Enterobacteriaceae, which are oxidase-negative. The ability of bacteria to produce cytochrome oxidase can be determined by the addition of the test reagent p-aminodimethylaniline oxalate to colonies grown on a plate medium.

This light pink reagent serves as an artificial substrate, donating electrons and thereby becoming oxidized to a blackish compound in the presence of the oxidase and free oxygen. Following the addition of the test reagent, the development of pink, then maroon, and finally dark purple coloration on the surface of the colonies is indicative of cytochrome oxidase production and represents a positive test. No color change, or a light pink coloration on the colonies, is indicative of the absence of oxidase activity and is a negative test. The filter paper method may also be used.

Requirements:

24- to 48-hour culture of *N. gonorrhoeae* and *E. coli*
Trypticase Soy Agar plates
p-Aminodimethylaniline oxalate

Bunsen burner
Inoculating loop
Test tubes
Glass marker
Incubator

Composition of TSA:

Pancreatic digest of casein:	17.0g
Papaic digest of soybean meal:	3.0g
Sodium chloride:	5.0g
Dextrose:	2.5g
Dibasic potassium phosphate:	2.5g
Distilled water:	1000ml
Agar powder:	15g
Final pH (at 25 °C)	7.3±0.2

Trypticase Soy Broth (Soybean-Casein Digest Medium) is a general purpose liquid enrichment medium used in qualitative procedures for the sterility test and for the enrichment and cultivation of aerobic microorganisms that are not excessively fastidious.

Reagent to detect oxidase activity:

p-Aminodimethylaniline oxalate:	0.5 g
Distilled water:	50.0 ml

Note: To dissolve fully, gently warm the solution.

Procedure:

1. Prepare the Trypticase soy agar plate for inoculation as follows:
2. With a glass marker, divide the bottom of a Petri dish into two sections and label each section with the name of the test organism to be inoculated.
3. Using aseptic technique, make a single-line streak inoculation of each test organism on the agar surface of its appropriate section of the plate.
4. Incubate the plate in an inverted position at 37 °C for 24 to 48 hours.
5. Add two or three drops of the p-aminodimethylaniline oxalate to the surface of the growth of each test organism.
6. Observe for the presence or absence of a color change from pink, to maroon, and finally to purple.

Catalase test: *N. gonorrhoeae* (Positive)

Decarboxylase Test

Some organisms were found to be capable of decarboxylation of amino acids, providing a way to differentiate between the enteric genera and species. For instance, lysine decarboxylase is capable of differentiating between *Salmonella* and *Citrobacter*. Ornithine decarboxylase separates *Enterobacter* from *Klebsiella*. Decarboxylase enzymes are numerous, and each is specific for a particular substrate. It is now evident that decarboxylases and deaminases play an important role in the utilization of amino acids and the metabolism of nitrogen compounds.

Experiment 37

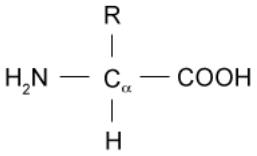
Aim:

To identify and differentiate organisms based on their ability to enzymatically degrade amino acid substrates.

Principle:

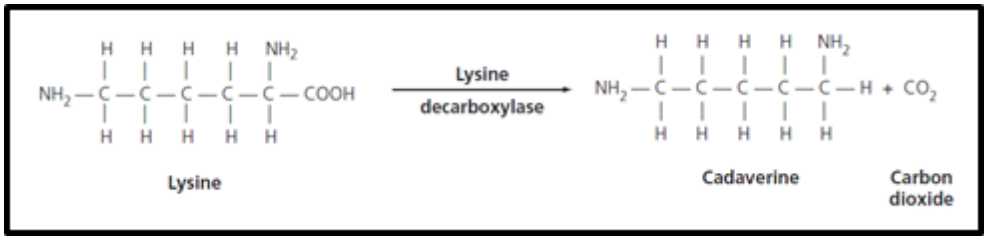
Every biologically active protein is composed of the 20 essential amino acids. Structurally, amino acids are composed of an alpha carbon (-C-), an amino group (-NH2), a carboxyl group (-COOH), and a hydrogen atom (-H). Also attached to the alpha carbon is a side group or an atom designated by an (-R), which differs in each of the amino acids.

Decarboxylation is a process whereby some microorganisms that possess decarboxylase enzymes are capable of removing the carboxyl group to yield end products consisting of an amine or diamine plus carbon dioxide. Decarboxylated amino acids play an essential role in cellular metabolism since the amines produced may serve as end products for the synthesis of other molecules required by the cell. Decarboxylase enzymes are designated as adaptive (or induced) enzymes and are produced in the presence of specific amino acid substrates upon which they act. These amino acid substrates must possess at least one chemical group other than an amine (-NH2) or a carboxyl group (-COOH).



In the process of decarboxylation, organisms are cultivated in an acid environment and in the presence of a specific substrate. The decarboxylation end product (amines) results in a shift to a more alkaline pH. In the clinical or diagnostic microbiology laboratory, three decarboxylase enzymes are used to differentiate members of the Enterobacteriaceae: lysine, ornithine, and arginine. Decarboxylase activity is determined by cultivating the organism in a nutrient medium containing glucose, the specific amino acid substrate, and bromothymol blue (the pH indicator). If decarboxylation occurs, the pH of the medium becomes alkaline despite the fermentation of glucose since the end products (amines or diamines) are alkaline. The function of the glucose in the medium is to ensure good microbial growth and thus more reliable results in the presence of the pH indicator.

The presence of each decarboxylase enzyme can be tested for by supplementing decarboxylase broth with the specific amino acid substrate, namely lysine, arginine, and ornithine. For example, lysine decarboxylase degrades L-lysine, forming the diamine end product cadaverine plus carbon dioxide as illustrated below.



In the experiment that follows, the decarboxylation of L-lysine will be studied. It should be noted that decarboxylation reactions occur under anaerobic conditions that are satisfied by sealing the culture tubes with sterile mineral oil. In the sealed tubes, all of the unbound oxygen is utilized during the organisms' initial growth phase, and the pH of the medium becomes alkaline as carbon dioxide (CO_2) is produced in the culture tube. A pH indicator such as bromcresol purple is usually incorporated into the medium for the easy detection of pH changes. The production of acid end products will cause the bromcresol purple to change color from purple to yellow, indicating that acid has formed, the medium has been acidified, and the decarboxylase enzymes have been activated. The activated enzyme responds with the production of the alkalinizing diamine (cadaverine) and carbon dioxide, which will produce a final color change from yellow back to purple, thereby indicating that L-lysine has been decarboxylated. The development of a turbid purple color verifies a positive test for amino acid decarboxylation. The absence of a purple color indicates a negative result.

Requirements:

24-hour nutrient broth cultures of *Escherichia coli*, *Proteus mirabilis*, and *Citrobacter freundii.*
Moeller's decarboxylase broth supplemented with L-lysine (10 gm/l) (labeled LD+)and without lysine (labeled LD-).
Bunsen burner
Glass marker
Inoculating loop and needle
Sterile Pasteur pipettes, Rubber bulbs
Test tube rack
Sterile mineral oil
Incubator

Composition of Moeller's decarboxylase broth HiMedia M687

Peptic digest of animal tissue:	5.0g
Beef extract:	5.0g
Dextrose:	0.5g
Bromocresol purple:	0.010g
Cresol red:	0.005g
Pyridoxal:	0.005g

L-Lysine hydrochloride: 10.0g
Final pH (at 25°C): 6.0±0.2

Suspend 20.52 grams in 1000 ml distilled water. Heat if necessary, to dissolve the medium completely. Dispense in 5 ml amount in screw-capped tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 10 minutes. Cool the tubed medium in an upright position. Inoculate the tubes and overlay with 2-3 ml of sterile mineral oil.

Procedure:

1. With a glassware marking pencil, label three tubes of the LD+ medium with the name of the organism to be inoculated. Similarly label three tubes of LD- medium.
2. Using aseptic technique, inoculate each experimental organism into its appropriately labeled tube using a loop inoculation.
3. Place a rubber bulb onto a sterile Pasteur pipette and overlay the surface of the inoculated culture tubes with 1 ml of sterile mineral oil.
4. Hold the tubes in a slanted position while adding the mineral oil. **Note:** Do not let the tip of the pipette touch the inoculated medium or the sides of the test tube walls.
5. Repeat the above procedure for the remaining test cultures.
6. Incubate all tubes at 37°C for 24 to 48 hours.

Observation:

Examine each culture tube for the presence of a color change.

Lysine decarboxylase test: A) Control; B)*Citrobacter freundii* (negative); C) *E. coli* (positive); D) *Proteus mirabilis* (negative)

Phenylalanine Deaminase Test

The phenylalanine deaminase test uses the differential medium phenylalanine agar to detect bacteria containing the enzyme phenylalanine deaminase, and is used to differentiate the genera *Proteus, Morganella*, and *Providencia* from other gram negative intestinal bacilli. These genera of enteric and environmental bacteria are known to cause UTIs and gastroenteritis. It is clinically important to distinguish them from other enteric bacteria due to their high level of antibiotic resistance.

Experiment 38

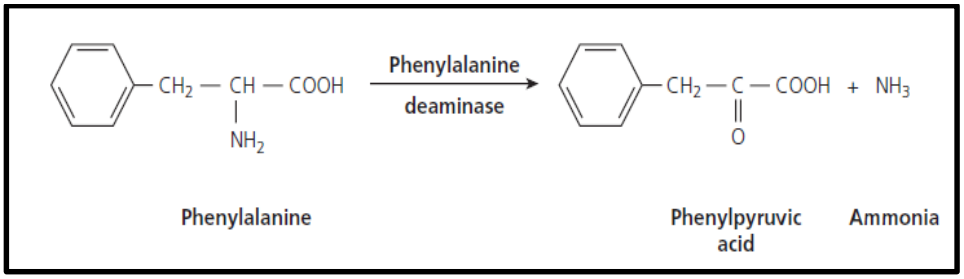
Aim:

Demonstrate the ability of some organisms to remove the amino group (-NH2) from amino acids.

Principle:

Microorganisms that contain deaminase enzymes are capable of removing the amino group (-NH2) from amino acids and other NH2-containing chemical compounds. During this process the amino acid, under the auspices of its specific deaminase, will produce keto acids and ammonia as end products.

In the experiment to follow, the amino acid phenylalanine will be deaminated by phenylalanine deaminase and converted to the keto acid phenylpyruvic acid and ammonia. The organisms are cultured on a medium incorporating phenylalanine as the substrate. This chemical reaction is illustrated below.



If the organism possesses phenylalanine deaminase, phenylpyruvic acid will be released into the medium and can be detected by the addition of a 10 to 12% ferric chloride solution to the surface of the medium. If a green color develops, the enzymatic deamination of the substrate has occurred and is indicative of a positive result. The absence of any color change indicates a negative result. The resultant green color produced upon the addition of ferric chloride (FeCl3) is due to the formation of a keto acid (phenylpyruvic acid). It has been shown that α- and β-keto acids give a positive color reaction with either alcoholic or aqueous solutions of FeCl3. Phenylpyruvic acid is an α-keto acid. The results should be read immediately following the addition of the reagent since the color produced fades quickly. When not in use, the ferric chloride reagent should be refrigerated and kept in a dark bottle to avoid exposure to light. The stability of this reagent varies and should be checked weekly with known positive cultures.

Requirements:

24-hour nutrient broth cultures of *Escherichia coli* and *Proteus vulgaris*
Phenylalanine agar slants
10 to 12% ferric chloride solution.
Bunsen burner
Glass marker
Pasteur pipettes, rubber bulbs
Test tube racks
Inoculating loop
Incubator

Composition of Phenylalanine agar:

Yeast extract:	3.0g
Dipotassium phosphate:	1.0g
Sodium chloride:	5.0g
DL-phenylalanine:	2.0g
Bacto agar:	12.0g
Distilled water:	1000.0 ml
pH at 25 ^{o}C:	7.3

Completely dissolve ingredients in boiling water. Dispense in tubes, autoclave, and cool in slanted position.

Procedure:

1. Using aseptic technique, inoculate each test organism into its appropriately labeled tube using a streak inoculation.
2. Incubate cultures at 37 °C for 24 to 48 hours.
3. Add 5 to 10 drops of the ferric chloride solution to each agar slant and mix gently.
4. Ferric chloride is a chelating agent and binds to the phenylpyruvic acid to produce a green color on the slant.

Observation:

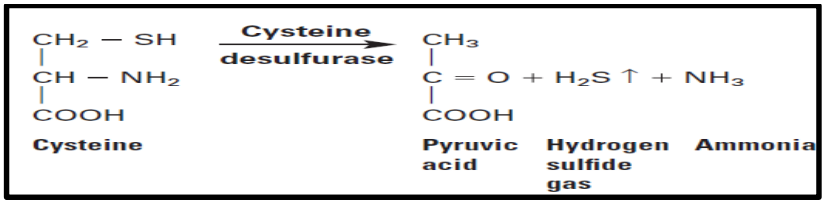
Observe the slants for the change in color. Determine whether the organism was capable of amino acid deamination.

Phenylalanine deaminase test: A) *Proteus vulgaris* (positive) B) *E. coli* (negative)

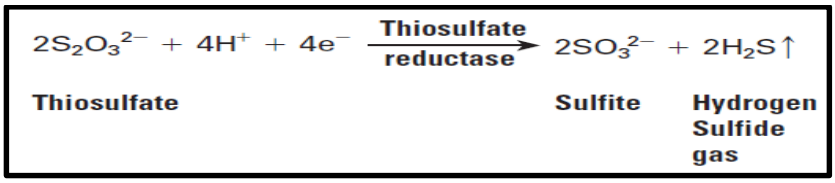
Hydrogen Sulfide Production

The ability of microorganisms to produce hydrogen sulfide from substances such as sulfur-containing amino acids or inorganic sulfur compounds is well documented. There are two major fermentative pathways by which some microorganisms are able to produce hydrogen sulfide (H_2S).

Pathway 1: Gaseous H_2S may be produced by the reduction (hydrogenation) of organic sulfur present in the amino acid cysteine, which is a component of peptones contained in the medium. These peptones are degraded by microbial enzymes to amino acids, including the sulfur containing amino acid cysteine. This amino acid in the presence of a cysteine desulfurase loses the sulfur atom, which is then reduced by the addition of hydrogen from water to form bubbles of hydrogen sulfide gas ($H_2S \uparrow$) as illustrated:



Pathway 2: Gaseous H2S may also be produced by the reduction of inorganic sulfur compounds such as the thiosulfates ($S2O_3^{2-}$), sulfates (SO_4^{2-}), or sulfites (SO_3^{2-}). The medium contains sodium thiosulfate, which certain microorganisms are capable of reducing to sulfite with the liberation of hydrogen sulfide. The sulfur atoms act as hydrogen acceptors during oxidation of the inorganic compound as illustrated in the following:



Experiment 39

Aim:

To demonstrate the ability of microorganisms to produce hydrogen sulfide from substances such as the sulfur-containing amino acids or inorganic sulfur compounds.

Principle:

The SIM medium is used which contains peptone and sodium thiosulfate as the sulfur substrates; ferrous sulfate ($FeSO_4$), which behaves as the H_2S indicator; and sufficient agar to make the medium semisolid and thus enhance anaerobic respiration.

Regardless of which pathway is used, the hydrogen sulfide gas is colorless and therefore not visible. Ferrous ammonium sulfate in the medium serves as an indicator by combining with the gas, forming an insoluble black ferrous sulfide precipitate that is seen along the line of the stab inoculation and is indicative of H_2S production. Absence of the precipitate is evidence of a negative reaction.

SIM agar may also be used to detect motile organisms. Motility is recognized when culture growth (turbidity) of flagellated organisms is not restricted to the line of inoculation. Growth of nonmotile organisms is confined to the line of inoculation.

Requirements:

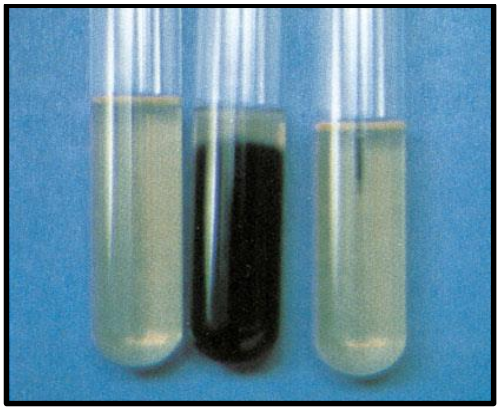
24- to 48-hour broth cultures of *Enterobacter aerogenes, Shigella dysenteriae, Proteus vulgaris,* and *Salmonella typhimurium*
SIM agar deep tubes
Bunsen burner
Inoculating needle
Test tube rack
Glass marker
Incubator

Procedure:

1. Aseptically inoculate each test organism into its appropriately labeled tube by means of stab inoculation. The uninoculated tube will serve as a control.
2. Incubate all cultures at 37 °C for 24 to 48 hours.
3. Observe all SIM cultures for the presence or absence of black coloration along the line of the stab inoculation.
4. Based on your observations, determine whether or not the test organism was capable of producing hydrogen sulfide.
5. Observe all cultures for the presence (+) or absence (-) of motility.

Observation:

Observe the SIM agar deep tubes for the change in color due to hydrogen sulfide production.



Hydrogen Sulfide production test: A) Control; B) *Proteus vulgaris* (positive); c) *E. aerogenes* (negative)

Nitrate Reduction

Anaerobic metabolism requires an electron acceptor other than atmospheric oxygen (O_2). Many gram-negative bacteria use nitrate as the final electron acceptor. Nitrate reduction test is a test that determines the production of an enzyme called nitrate reductase, which results in the reduction of nitrate (NO_3). Bacterial species may be differentiated on the basis of their ability to reduce nitrate to nitrite or nitrogen gases. Some Gram-negative bacteria (most Enterobacteriaceae) possess the enzyme nitrate reductase, a molybdenum- containing membrane-integrated enzyme that catalyzes the one-step reduction of nitrate to nitrite. Other microorganisms have the ability to further reduce nitrite to nitrogenous gases, such as NO, N_2O, and N_2. This process is known as denitrification and is widely used in the sewage treatment to stimulate algal growth. Denitrification is also of global significance, as it converts fixed nitrogen (nitrate) to environmentally significant gaseous nitrogen compounds. Alternatively, some bacteria may convert nitrite to ammonia through a dissimilative process.

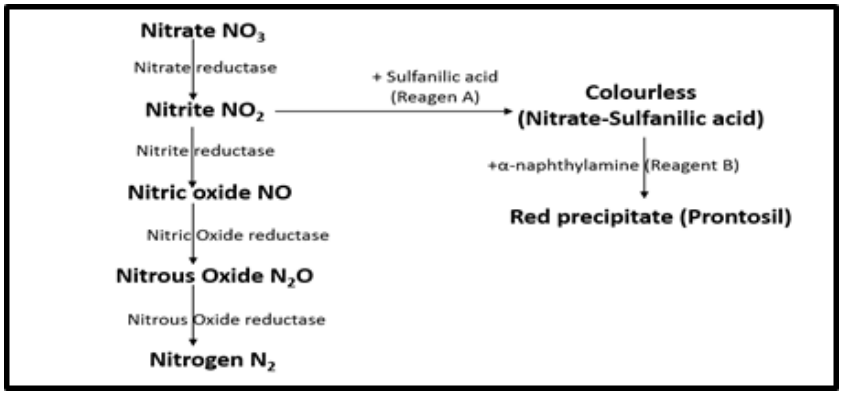
Experiment 40

Aim:

To determine the ability of some microorganisms to reduce nitrates (NO_3^-) to nitrites (NO_2^-) or beyond the nitrite stage.

Principle:

The reduction of nitrates by some aerobic and facultative anaerobic microorganisms occurs in the absence of molecular oxygen, an anaerobic process. In these organisms anaerobic respiration is an oxidative process whereby the cell uses inorganic substances such as nitrates (NO_3^-) or sulfates (SO_4^{2-}) to supply oxygen that is subsequently utilized as a final hydrogen acceptor during energy formation. Some organisms possess the enzymatic capacity to act further on nitrites to reduce them to ammonia (NH_3^+) or molecular nitrogen (N_2). These reactions may be described as follows: Nitrate reduction can be determined by cultivating organisms in a nitrate broth medium. The medium is basically a nutrient broth supplemented with 0.1% Potassium nitrate (KNO_3) as the nitrate substrate. In addition, the medium is made into a semisolid by the addition of 0.1% agar. The semi solidity impedes the diffusion of oxygen into the medium, thereby favoring the anaerobic requirement necessary for nitrate reduction.



Following incubation of the cultures, an organism's ability to reduce nitrates to nitrites is determined by the addition of two reagents: Solution A, which is sulfanilic acid, followed by Solution B, which is α-naphthylamine. Following reduction, the addition of Solutions A and B will produce an immediate cherry red color. Cultures not producing a color change suggest one of two possibilities: (1) nitrates were not reduced by the organism, or (2) the organism possessed such potent nitrate reductase enzymes that nitrates were rapidly reduced beyond nitrites to ammonia or even molecular nitrogen.

To determine whether or not nitrates were reduced past the nitrite stage, a small amount of zinc powder is added to the basically colorless cultures already containing Solutions A and B. Zinc reduces nitrates to nitrites. The development of red color therefore verifies that nitrates were not reduced to nitrites by the organism. If nitrates were not reduced, a negative nitrate reduction reaction has occurred. If the addition of zinc does not produce a color change, the nitrates in the medium were reduced beyond nitrites to ammonia or nitrogen gas.

Requirements:

24- to 48-hour broth cultures of *Alcaligenes faecalis, Proteus vulgaris*
and*Pseudomonas aeruginosa*
Trypticase nitrate broth tubes
Solution A: sulfanilic acid
Solution B: α-naphthylamine
Zinc powder

Composition of Trypticase nitrate broth:

Trypticase:	20.0g
Disodium phosphate:	2.0g
Dextrose:	1.0g
Agar:	1.0g
Potassium nitrate:	1.0g
Final pH	7.2

Nitrate test solution for detection of nitrites:

Solution A

Sulfanilic acid:	8.0 g
Acetic acid, 5 N:	1 part glacial acetic acid to 2.5 parts distilled water 1000.0 ml

Solution B

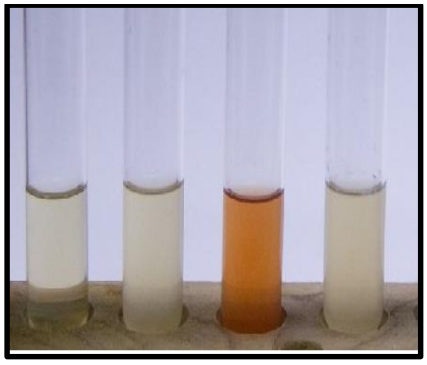
Alpha-naphthylamine:	5.0 g
Acetic acid, 5 N:	1000.0 ml

Procedure:

1. Using aseptic technique, inoculate each test organism into its appropriately labeled tube by means of a loop inoculation. The uninoculated tube will serve as a control.
2. Incubate all cultures at 37 °C for 24 to 48 hours.
3. Following incubation, add five drops of **Solution A** and then five drops of **Solution B** to all nitrate broth cultures.
4. Observe whether or not a red coloration develops in each of the culture tubes.
5. Add a minute quantity of zinc to the cultures in which no red color developed.
6. Observe and record whether or not red coloration develops in each of the cultures.

Observation:

Observe whether red coloration is developed in each of the culture tubes after addition of Solution A and B. Record your results in a Lab notebook. Now add a minute quantity of zinc to the cultures in which no red color is developed and again see for the color change and note the results.



After adding reagents A and B Tube 1: Control; Tube 2: *A. faecalis*; Tube 3: *P. vulgaris* (positive); Tube 4: *P. aeruginosa*

After adding Zinc to Tube 1,2 and 4: Tube 1: Control; Tube 2: *A. faecalis* (negative);Tube 3: *P. vulgaris* (positive); Tube 4: *P. aeruginosa* (positive Nitrate reduction and beyond the nitrite stage)

The litmus milk reaction

Milk is an excellent medium for the growth of microorganisms because it contains the milk protein casein, the sugar lactose, vitamins, minerals and water. The Litmus Milk Medium is used to differentiate bacteria on the basis of lactose fermentation, casein hydrolysis and coagulation, gas production, and reduction of litmus. It is especially useful in species differentiation within the genus *Clostridium* and to differentiate *Streptococcus bovis* (no growth) from *Streptococcus equinus* (growth). It is also used to cultivate lactic acid bacteria associated with dairy products because litmus is a reliable indicator of action upon milk. The litmus milk test differentiates members of the Enterobacteriaceae from other gram-negative bacilli based on the enterics' ability to reduce litmus.

Experiment 41

Aim:

Differentiate among microorganisms that enzymatically transform different milk substrates into varied metabolic end products.

Principle:

Litmus is both an indicator of pH and of the oxidation-reduction (Eh) potential of the medium. Milk provides lactose, casein, lactalbumin, and lactoglobulin. In an acid solution litmus is red and under alkaline conditions it is blue. When an organism ferments lactose, lactic acid is produced and the medium changes to a pink-red color. Some bacteria act on the nitrogenous substrates in the milk releasing ammonia and yielding a purplish-blue color. Organisms that reduce litmus cause the oxygen to be removed, leaving a leuco (white) base. Proteolytic enzymes produced by certain organisms, hydrolyze milk proteins and result in clot formation. Casease production results in peptonization which causes digestion of the clot, exhibited by a watery clearing of the medium. The end result of lactose fermentation may be gas production (CO_2 and H_2). An abundance of gas breaks up an acid clot causing a reaction referred to as stormy fermentation. This may occur with certain anaerobic *Clostridium* species.

The major milk substrates capable of transformation are the milk sugar lactose and the milk proteins casein, lactalbumin, and lactoglobulin. To distinguish among the metabolic changes produced in milk, a pH indicator, the oxidation reduction indicator litmus, is incorporated into the medium. Litmus milk now forms an excellent differential medium in which microorganisms can metabolize milk substrates depending on their enzymatic complement. A variety of different biochemical changes result, as follows:

Lactose Fermentation:

Organisms capable of using lactose as a carbon source for energy production utilize the inducible enzyme β-galactosidase and degrade lactose as follows:

The presence of lactic acid is easily detected because litmus is purple at a neutral pH and turns pink when the medium is acidified to an approximate pH of 4.

Gas Formation:

The end products of the microbial fermentation of lactose are likely to include the gases $CO_2\uparrow + H_2\uparrow$. The presence of gas may be seen as separations of the curd or by the development of cracks or fissures within the curd as gas rises to the surface.

Litmus Reduction:

Fermentation is an anaerobic process involving bio oxidations that occur in the absence of molecular oxygen. These oxidations may be visualized as the removal of hydrogen (dehydrogenation) from a substrate. Since hydrogen ions cannot exist in the free state, there must be an immediate and concomitant electron acceptor available to bind these hydrogen ions, or else oxidation-reduction reactions are not possible and cells cannot manufacture energy. In the litmus milk test, litmus acts as such an acceptor. While in the oxidized state, the litmus is purple; when it accepts hydrogen from a substrate, it will become reduced and turn white or milk-colored. This oxidation of lactose, which produces lactic acid, butyric acid, $CO_2\uparrow + H_2\uparrow$ is as follows:

The excess hydrogen is now accepted by the hydrogen acceptor litmus, which turns white and is said to be reduced.

Curd Formation:

The biochemical activities of different microorganisms grown in litmus milk may result in the production of two distinct types of curds (clots). Curds are designated as either acid or rennet, depending on the biochemical mechanism responsible for their formation.

a. Acid curd:

Lactic acid or other organic acids cause precipitation of the milk protein casein as calcium caseinate to form an insoluble clot. The clot is hard and will not retract from the walls of the test tube. An acid curd is easily identified if the tube is inverted and the clot remains immobile.

b. Rennin curd:

Some organisms produce rennin, an enzyme that acts on casein to form paracasein, which in the presence of calcium ions is converted to calcium paracaseinate and forms an insoluble clot. Unlike the acid curd, this is a soft semi solid clot that will flow slowly when the tube is tilted.

Proteolysis (Peptonization):

The inability of some microorganisms to obtain their energy by way of lactose fermentation means they must use other nutritional sources such as proteins. By means of proteolytic enzymes, these organisms hydrolyze the milk proteins, primarily casein, into their basic building blocks, namely amino acids. This digestion of proteins is accompanied by the evolution of large quantities of ammonia, resulting in an alkaline pH in the medium. The litmus turns deep purple in the upper portion of the tube, while the medium begins to lose body and produces a translucent, brown, wheylike appearance as the protein is hydrolyzed to amino acids.

Alkaline Reaction

An alkaline reaction is evident when the color of the medium remains unchanged or changes to a deeper blue. This reaction is indicative of the partial degradation of casein into shorter polypeptide chains, with the simultaneous release of alkaline end products that are responsible for the observable color change.

Requirements:

24- to 48-hour broth cultures of *Escherichia coli, Alcaligenes faecalis,*
Lactococcus lactis, and *Pseudomonas aeruginosa*
Litmus milk broth
Bunsen burner

Inoculating loop
Test tube rack
Glass marker

Composition of Litmus milk broth:

Skim milk powder: 100.0
Litmus: 0.075
Distilled water: 1000ml
Final pH: 6.8

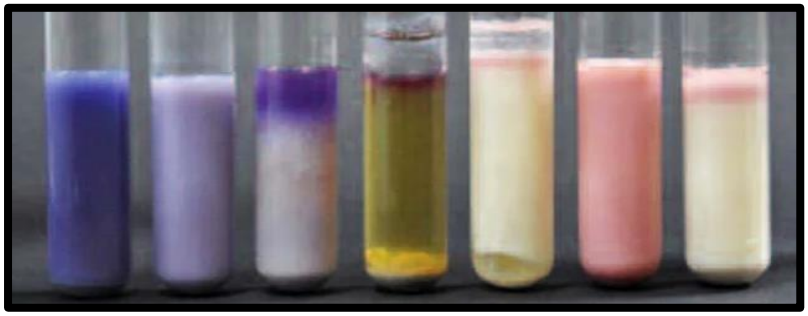
Autoclave at 12 lb pressure for 15 minutes

Procedure:

1. Using aseptic technique, inoculate each test organism into its appropriately labeled tube by means of a loop inoculation. The uninoculated tube will serve as a control.
2. Incubate all cultures at 37 °C for 24 to 48 hours.
3. Examine all the litmus milk cultures for color and consistency of the medium.
4. If *Clostridium* is suspected or anaerobiosis is desired, add sterile reduced iron (e.g., iron powder) to the tube or pour a layer of sterile mineral oil over the medium surface immediately after inoculation. Incubate in the appropriate atmosphere at 35-37 °C for 18-24 hours. Longer periods up to 14 days may be necessary.

Observation:

Based on your observations, determine and record the type(s) of reaction(s) that have taken place in each culture tube.



Interpretation of Results:

(ALK) Blue: Alkaline reaction, no fermentation, organisms attack nitrogenous substances in the medium
(A) Pinkish-red: Acid reaction, lactose fermentation.
(C) Clot or curd formation: Milk protein coagulation
(D) Digestion (peptonization): Milk protein digested, clearing of medium
(G) Gas production: CO2 and H2 Bubbles in medium, clot may be broken up
(NF) Purplish-blue: No fermentation, no change of indicator
(RED) White: Reduction of litmus to a white leuco base by enzyme reductase
(SF) Stormy Fermentation: Acid clot disrupted by an abundance of gas production

Chapter - 15
Identification of Unknown Bacteria

One of the most interesting experiences in Introductory Microbiology is to attempt to identify an unknown microorganism that has been assigned to you by your mentor as a laboratory problem. You have to use previously studied staining, cultural characteristics, and biochemical procedures for independent genus identification of an unknown bacterial culture. Physiological characteristics will be determined with a series of biochemical tests that you will perform on the organisms. Although correctly identifying the unknowns that are given to you is very important, it is just as important that you thoroughly understand the chemistry of the tests that you perform on the organisms.

The first step in the identification procedure is to accumulate information that pertains to the organisms' morphological, cultural, and physiological (biochemical) characteristics. This involves making different kinds of slides for cellular studies and the inoculation of various types of media to note the growth characteristics and types of enzymes produced.

After sufficient information has been recorded, the next step is to consult a taxonomic key, which enables one to identify the organism. For this final step, Bergey's Manual of Systematic Bacteriology will be used. Copies of volumes 1 to 4 of this book will be available in the library of your college/ institution.

Success in this endeavor will require meticulous techniques, intelligent interpretation, and careful recordkeeping. Your expertise in the handling of cultures and the performance of inoculations will show up clearly in your results. Contamination of your cultures with unwanted organisms will yield false results, making identification difficult. If you have reason to doubt the validity of the results of a specific test, please do repeat it; don't take a chance! As soon as you have made an observation or completed a test, record the information on the Descriptive Chart. Do not trust your memory- record data immediately.

Experiment 42

Aim:

Identification of unknown bacterial cultures at the level of Genus

Principle:

Identification of unknown bacterial cultures is one of the major responsibilities of the microbiologist. Samples of blood, tissue, food, water, and cosmetics are examined daily in laboratories throughout the world for the presence of contaminants. In addition, industrial organizations are constantly screening materials to isolate new antibiotic-producing organisms or organisms that will increase the yield of marketable products such as vitamins, solvents,organic acids, metabolites and enzymes. Once isolated, these unknown organisms must be identified and classified.

The science of classification is called taxonomy and deals with the separation of living organisms into interrelated groups. Bergey's Manual has been the official, internationally accepted reference for bacterial classification since 1923. The current edition, Bergey's Manual

of Systematic Bacteriology, arranges related bacteria into 33 groups called sections rather than into the classical taxonomic groupings of phylum, class, order, and family. The interrelationship of the organisms in each section is based on characteristics such as morphology, staining reactions, nutrition, cultural characteristics, physiology, cellular chemistry, and biochemical test results for specific metabolic end products.

At this point you have developed sufficient knowledge of staining methods, isolation techniques, microbial nutrition, biochemical activities, and characteristics of microorganisms to be able to work independently in attempting to identify the genus of an unknown culture. Characteristics of the major organisms that have been used in experiments thus far are given in the Table given below:

Requirements:

Number coded 24- to 48-hour agar slant culture of one unknown pure culture.

Two Trypticase soy agar slants, and one each of the following: Phenol red sucrose broth, Phenol red lactose broth, Phenol red dextrose broth, SIM agar deep tube, MR-VP broth, Tryptic nitrate broth, Simmons citrate agar slant, Urea broth, Litmus milk, Trypticase soy agar plate, Nutrient gelatin deep tube, Starch agar plate, and Tributyrin agar plate.

Reagents:

Crystal violet; Gram's iodine; 95% ethyl alcohol;
Safranin; methyl red; 3% hydrogen peroxide;
Barritt's reagent, Solutions A and B; Kovac's reagent;
Zinc powder; and p-aminodimethylaniline oxalate.

Equipment:

Bunsen burner,
Inoculating loop and needle
Staining tray
Immersion oil
Microscope
Glass marker

Procedure:

1. Perform a Gram stain of the unknown organism. Observe and record in the Laboratory notebook.
2. Chart the reaction and the morphology and arrangement of the cells.
3. Using aseptic inoculation technique, inoculate two Trypticase soy agar slants by means of a streak inoculation.
4. Following incubation, you will use one slant culture to determine the cultural characteristics of the unknown microorganism.
5. You will use the second as a stock. Subculture it, if necessary to repeat any of the tests.
6. Utmost care in aseptic technique so as not to contaminate cultures and thereby obtain spurious results, inoculate the media for the following biochemical tests:

Sr. No.	Medium	Test
1.	Phenol red lactose broth	Carbohydrate fermentation
2.	Phenol red dextrose broth	
3.	Phenol red sucrose broth	
4.	Litmus milk	Litmus milk reactions
5.	SIM medium	Indole production
6.		H_2S production
7.	Tryptic nitrate broth	Nitrate reduction
8.	MR-VP broth	Methyl red test Voges-Proskauer test
9.	Simmons citrate agar slant	Citrate utilization
10.	Urea broth	Urease activity
11.	Trypticase soy agar slant	Catalase activity
12.	Tributyrin agar plate	Lipid hydrolysis
13.	Nutrient gelatin deep tube	Gelatin liquefaction
14.	Starch agar plate	Starch hydrolysis
15.	Trypticase soy agar plate	Oxidase test

7. Incubate all cultures at 37°C for 24 to 72 hours.
8. Examine a Trypticase soy agar slant culture and determine the cultural characteristics of your unknown organism. Record your results in the Lab notebook.
9. Perform biochemical tests on the remaining cultures, making reference to the specific laboratory exercise for each test. Record your observations and results.

Observation:

Based on your results, identify the genus and species of the unknown organism.

Note: Results may vary depending on the strains of each species used and the length of time the organism has been maintained in stock culture. The observed results may not be identical to the expected results. Therefore choose the organism that best fits the results summarized in Table below.

Cultural and Biochemical characteristics of unknown organisms: Comparison table

ORGANISM	GRAM STAIN	AGAR SLANT CULTURAL CHARACTERISTICS	LITMUS MILK REACTION	FERMENTATION			H_2S PRODUCTION	NO_3 REDUCTION	INDOLE PRODUCTION	MR REACTION	VP REACTION	CITRATE USE	UREASE ACTIVITY	CATALASE ACTIVITY	OXIDASE ACTIVITY	GELATIN LIQUEFACTION	STARCH HYDROLYSIS	LIPID HYDROLYSIS
				LACTOSE	DEXTROSE	SUCROSE												
Escherichia coli	Rod −	White, moist, glistening growth	Acid, curd ±, gas ±, reduction ±	AG	AG	A±	−	+	+	+	−	−	−	+	−	−	−	−
Enterobacter aerogenes	Rod −	Abundant, thick, white, glistening growth	Acid	AG	AG	AG±	−	+	−	−	+	+	−	+	−	−	−	−
Klebsiella pneumoniae	Rod ├	Slimy, white, somewhat translucent, raised growth	Acid, gas, curd ±	AG	AG	AG	−	+	−	±	±	+	+	+	−	−	−	−
Shigella dysenteriae	Rod −	Thin, even, grayish growth	Alkaline	−	A	A±	−	+	±	+	−	−	−	+	−	−	−	−
Salmonella typhimurium	Rod −	Thin, even, grayish growth	Alkaline	−	AG±	A±	+	+	−	+	−	+	−	+	−	−	−	−
Proteus vulgaris	Rod −	Thin, blue-gray, spreading growth	Alkaline	−	AG	AG±	+	+	+	+	−	±	+	+	−	+	−	−
Pseudomonas aeruginosa	Rod −	Abundant, thin, white growth, with medium turning green	Rapid peptonization	−	−	−	−	+	−	−	−	+	−	+	+	+ Rapid	−	+
Alcaligenes faecalis	Rod* −	Thin, white, spreading, viscous growth	Alkaline	−	−	−	−	−	−	−	−	±	−	+	+	−	−	−
Staphylococcus aureus	Cocci +	Abundant, opaque, golden growth	Acid reduction ±	A	A	A	−	+	−	+	±	−	−	+	−	+	−	+
Lactococcus lactis	Cocci +	Thin, even growth	Acid, rapid reduction with curd	A	A	A	−	−	−	+	−	−	−	−	−	−	−	−
Micrococcus luteus	Cocci +	Soft, smooth, yellow growth	Alkaline	−	−	−	−	±	−	−	−	−	+	+	−	+ Slow	−	−
Corynebacterium xerosis	Rod +	Grayish, granular, limited growth	Alkaline	−	A±	A±	−	+	−	−	−	−	−	+	−	−	−	−
Bacillus cereus	Rod +	Abundant, opaque, white waxy growth	Peptonization	−	A	A	−	+	−	−	±	−	−	+	−	+ Rapid	+	±

Note: AG — Acid and gas; ± — Variable reaction; Rod* — Coccobacillus

Chapter - 16
Enumeration of Bacteria

Many approaches are commonly employed for enumerating bacteria, including measurements of the *direct microscopic count, culture turbidity, dry weight of cells,* etc. In a microbiology lab, we frequently determine the total viable count in a bacterial culture. The methods of enumeration in microbes can be divided into four categories. Direct methods involve counting the microbes, while indirect methods involve estimation. Viable methods only count cells that are metabolically active, while total counts include dead and inactive cells.

Direct/Viable:

A direct/viable method involves a standard plate count, in which repeated dilutions of a sample are counted to calculate the count in the original sample.

Indirect/Viable:

Indirect/viable methods such as MPN (most probable number) involve making a statistical inference about the microbe count based on patterns of growth.

Direct/Total:

The microbes are counted with the aid of fluorescent stains and dyes, which make the microbes visible with the aid of a fluorescent microscope.

Indirect/Total:

Spectroscopy is a form of indirect/total enumeration, which involves estimating the amount of microbes based on the amount of light passed through the culture by a spectrophotometer.

Many methods have been devised to accomplish this, including direct microscopic counts, use of an electronic cell counter such as the Coulter's Counter, chemical methods for estimating cell mass or cellular constituents, turbidimetric measurements for increases in cell mass, and the serial dilution–agar plate method.

A. Direct Microscopic Counts:

Direct microscopic counts require the use of a specialized slide called the Petroff-Hausser counting chamber, in which an aliquot of a bacterial suspension is counted and the total number of cells is determined mathematically. The Petroff- Hausser counting chamber is a thick glass microscope slide with a chamber 0.02 mm (1/50 mm) deep in the center. The chamber contains an etched grid and has improved Neubauer rulings (1/400 square mm). The slide and the counting chamber are shown below.

The rulings cover 9 mm2. The boundary lines (Neubauer rulings) are the center lines of the groups of three. The center square millimeter is ruled into groups of 16 small squares, and each group is separated by triple lines, the middle one of which is the boundary. The ruled surface is 0.02 mm below the cover glass, which makes the volume over a square millimeter 0.02 mm3 (cubic mm). All cells are counted in this square millimeter.

The number of bacterial cells counted is calculated as follows:

The number of cells per mm = number of cells counted x dilution x 50,000

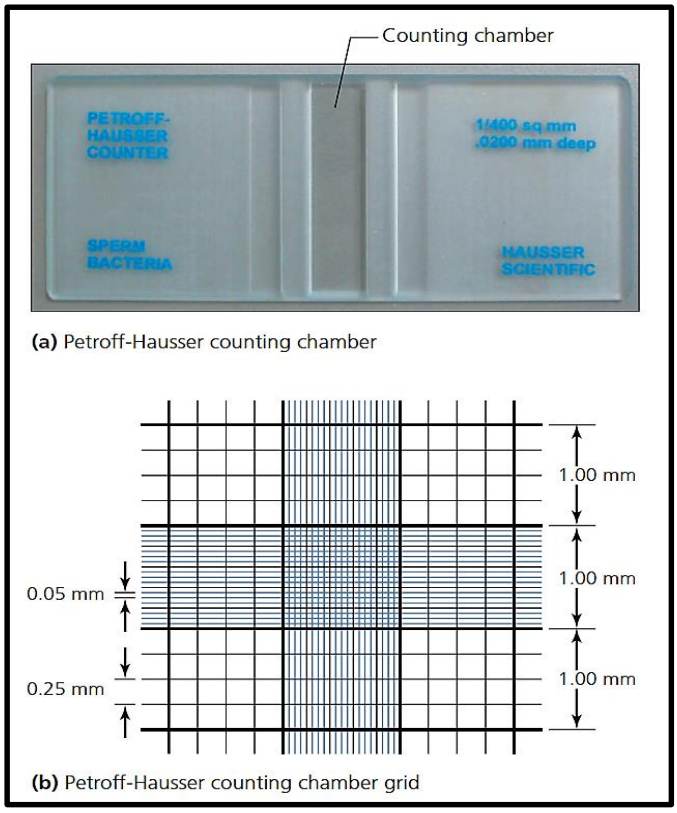
[The factor of 50,000 is used in order to determine the cell count for 1 ml: 1 ml = 1000 mm^3 =

(50 times the chamber depth of 0.02 mm) : 1000.]

Although rapid, a direct count has the disadvantages that both living and dead cells are counted and that it is not sensitive to populations of fewer than 1 million cells.

Breed smears are used mainly to quantitate bacterial cells in milk. Using stained smears confined to a 1-square-millimeter ruled area of the slide, the total population is determined mathematically.

This method also fails to discriminate between viable and dead cells.



Electronic Cell Counters:

The Coulter Counter is an example of an instrument capable of rapidly counting the number of cells suspended in a conducting fluid that passes through a minute orifice through which an electric current is flowing. Cells, which are nonconductors, increase the electrical resistance of the conducting fluid, and the resistance is electronically recorded, enumerating the number of organisms flowing through the orifice. In addition to its inability to distinguish between living and dead cells, the apparatus is also unable to differentiate inert particulate matter from cellular material.

B. Chemical Methods:

While not considered means of direct quantitative analysis, chemical methods may be used to indirectly measure increases both in protein concentration and in DNA production. In addition, cell mass can be estimated by dry weight determination of a specific aliquot of the culture. Measurement of certain metabolic parameters may also be used to quantitate bacterial populations. The amount of oxygen consumed (oxygen uptake) is directly proportional to the increasing number of vigorously growing aerobic cells such as in Methylene Blue Reductase test,

and the rate of carbon dioxide production is related to increased growth of anaerobic organisms.

C. Spectrophotometric Analysis:

Increased turbidity in a culture is another index of growth. With turbidimetric instruments, the amount of transmitted light decreases as the cell population increases, and the decrease in radiant energy is converted to electrical energy and indicated on a galvanometer. This method is rapid but limited because sensitivity is restricted to microbial suspensions of 10 million cells or greater.

D. Serial Dilution-Agar Plate Analysis:

While all these methods may be used to enumerate the number of cells in a bacterial culture, the major disadvantage common to all is that the total count includes dead as well as living cells. Sanitary and medical microbiology at times require determination of viable cells. To accomplish this, the serial dilution–agar plate technique is used. Briefly, this method involves serial dilution of a bacterial suspension in sterile water blanks, which serve as a diluent of known volume. Once diluted, the suspensions are placed on suitable nutrient media.

The **pour-plate technique** is usually employed. Molten agar, cooled to 45°C, is poured into a Petri dish containing a specified amount of the diluted sample. Following addition of the melted-then cooled agar, the cover is replaced, and the plate is gently rotated in a circular motion to achieve uniform distribution of microorganisms. This procedure is repeated for all dilutions to be plated. Dilutions should be plated in duplicate for greater accuracy, incubated overnight, and counted on a Quebec colony counter either by hand or by an electronically modified version of this instrument.

Figure 76: Quebec colony counter

Plates suitable for counting must contain not fewer than 30 nor more than 300 colonies. The total count of the suspension is obtained by multiplying the number of cells per plate by the dilution factor, which is the reciprocal of the dilution.

Experiment 43

Aim:

Enumeration of bacteria by pour-plate/spread plate technique using serially diluted culture samples.

Principle:

The most commonly used technique for enumerating bacterial colony forming units (cfu) involves serial dilution of samples followed by spreading 0.1 ml of each dilution onto agar media. Subsequent to incubation, colonies are counted and bacterial concentrations in the

original sample estimated. Two types of methods are usually followed:

1. **Pour Plate Technique:**

In this technique, 1 ml of the bacteria suspension is poured onto a sterilised petri dish and then liquefied nutrient agar medium is poured over it. The petri dish is swirled gently, so as to allow the suspension to mix with the medium uniformly. It is allowed to cool and solidify.

2. **Spread Plate Technique:**

In this technique 0.1 ml of the bacteria suspension is dropped onto a prepared agar plate. Then, the drop of suspension is spread uniformly on the agar plate by a sterilised L- shaped glass spreader.

Requirements:

24- to 48-hour nutrient broth culture of *Escherichia coli*

Six 20-ml nutrient agar deep tubes and seven sterile 9-ml distilled water blanks

Hot plate, water bath, thermometer, test tube rack,

Bunsen burner, sterile 1-ml serological pipettes, mechanical pipetting device, sterile Petri dishes, Quebec colony counter, disinfectant solution in a 500-ml beaker, glassware marking pencil, turntable, L- shaped glass spreader.

Beaker with 95% alcohol.

Procedure:

1. Liquefy six agar deep tubes in an autoclave or by boiling. Cool the molten agar tubes and maintain in a water bath at 45°C.
2. Label the *E. coli* culture tube with the number 1 and the seven 9-ml water blanks as numbers 2 through 8. Place the labeled tubes in a test tube rack. Label the Petri dishes 1A, 1B, 2A, 2B, 3A, and 3B.
3. Mix the E. coli culture (Tube 1) by rolling the tube between the palms of your hands to ensure even dispersal of cells in the culture.
4. With a sterile pipette, aseptically transfer 1 ml from the bacterial suspension, Tube 1, to water blank Tube 2. Discard the pipette in the beaker of disinfectant. The culture has been diluted 10 times to 10^{-1}.
5. Mix Tube 2 and, with a fresh pipette, transfer 1 ml to Tube 3. Discard the pipette. The culture has been diluted 100 times to 10^{-2}.
6. Mix Tube 3 and, with a fresh pipette, transfer 1 ml to Tube 4. Discard the pipette. The culture has been diluted 1000 times to 10^{-3}.
7. Mix Tube 4 and, with a fresh pipette, transfer 1 ml to Tube 5. Discard the pipette. The culture has been diluted 10,000 times to 10^{-4}.
8. Mix Tube 5 and, with a fresh pipette, transfer 0.1 ml of this suspension to Plate 1A. Return the pipette to Tube 5 and transfer 1 ml to Tube 6. Discard the pipette. The culture has been diluted 100,000 times to 10^{-5}.
9. Mix Tube 6 and, with a fresh pipette, transfer 1 ml of this suspension to Plate 1B. Return the pipette to Tube 6 and transfer 0.1 ml to Plate 2A. Return the pipette to Tube 6 and transfer 1 ml to Tube 7. Discard the pipette. The culture has been diluted 1,000,000 times to 10^{-6}.
10. Mix Tube 7 and, with a fresh pipette, transfer 1 ml of this suspension to Plate 2B. Return

the pipette to Tube 7 and transfer 0.1 ml to Plate 3A. Return the pipette to Tube 7 and transfer 1 ml to Tube 8. Discard the pipette. The culture has been diluted 10,000,000 times to 10^{-7}.

11. Mix Tube 8 and, with a fresh pipette, transfer 1 ml of this suspension to Plate 3B. Discard the pipette. The dilution procedure is now complete.
12. Check the temperature of the molten agar medium to be sure the temperature is 45 °C. Remove a tube from the water bath and wipe the outside surface dry with a paper towel. Using the pour-plate technique, pour the agar into Plate 1A and rotate the plate gently to ensure uniform distribution of the cells in the medium.
13. Repeat Step 12 for the addition of molten nutrient agar to Plates 1B, 2A, 2B, 3A, and 3B.
14. Once the agar has solidified, incubate the plates in an inverted position at 37 °C for 24 hours.

Note: If desired, the spread-plate technique may be substituted for the agar pour-plate method described in the earlier experiment. In this case, the dilutions may be placed on the surface of the hardened agar with a sterile pipette and distributed over the surface by means of a bent glass rod and turntable. Following incubation, bacterial cells are counted. The resultant cell counts should be the same with either system. The main difference is that there will be no subsurface colonies in the spread-plate method.

Using a Quebec colony counter and a mechanical hand counter, observe all colonies on plates. Statistically valid plate counts are only obtained from bacterial cell dilutions that yield between 30 and 300 colonies.

Plates with more than 300 colonies cannot be counted and are designated as too numerous to count- **TNTC**; plates with fewer than 30 colonies are designated as too few to count- **TFTC**. Count only plates containing between 30 and 300 colonies. Remember to count all subsurfaces as well as surface colonies.

The number of organisms per ml of original culture is calculated by multiplying the number of colonies counted by the dilution factor:

Number of cells per ml = number of colonies x dilution factor

Since the dilutions plated are replicates of each other, determine the average of the duplicate bacterial counts per ml of sample.

Record your observations and calculate bacterial counts per ml of sample in the Lab Notebook.

Calculations:

Colonies per plate = 52

Dilution factor = 1:1 x 10^7 (1:10,000,000)

Volume of dilution added to plate = 1 ml

52 x 10,000,000 = 520,000,000 or

(5.2 x 10^8) CFUs/ml (colony-forming units)

Advantages of the serial dilution–agar plate technique are as follows:

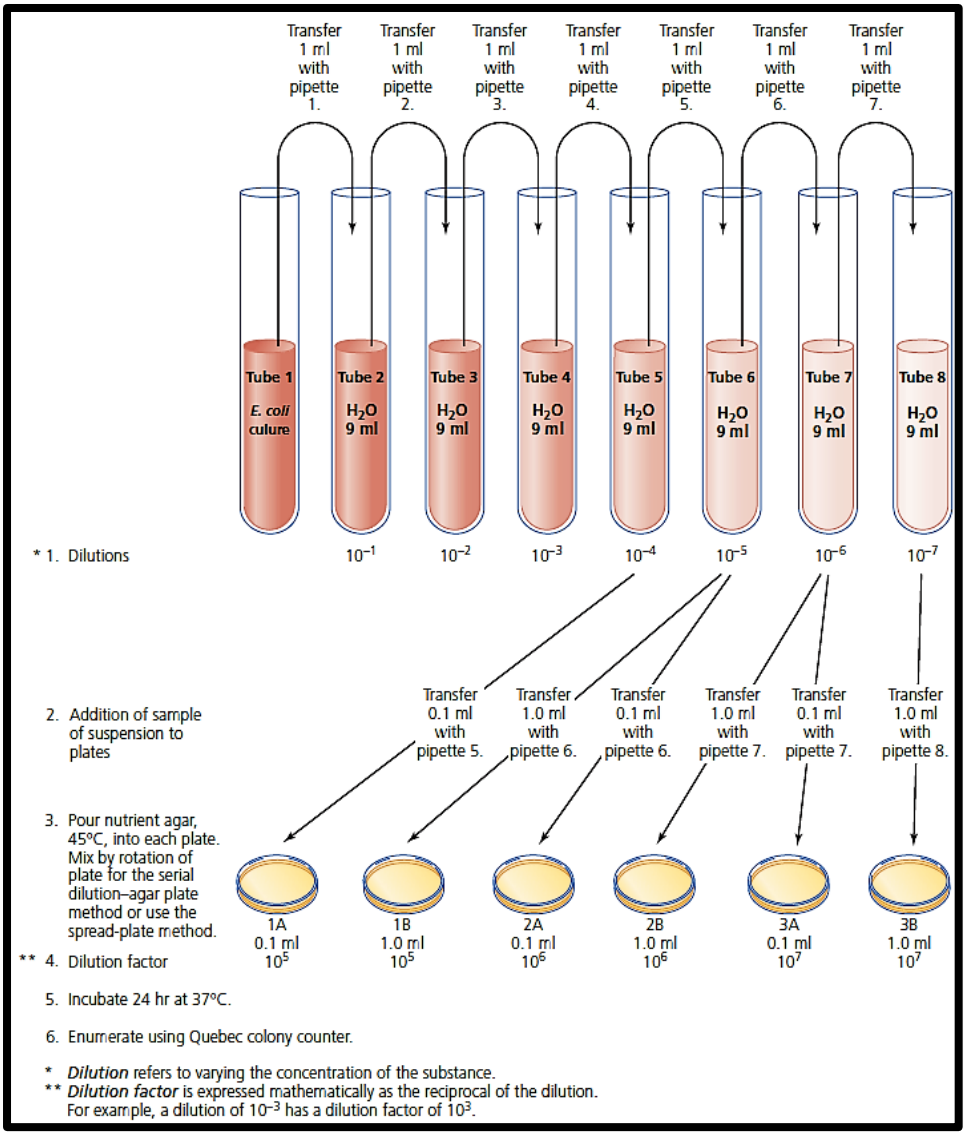
1. Only viable cells are counted.
2. It allows isolation of discrete colonies that can be subcultured into pure cultures, which may then be easily studied and identified.

Disadvantages of this method are as follows:

1. Overnight incubation is necessary before colonies develop on the agar surface.
2. More glassware is used in this procedure.
3. The need for greater manipulation may result in erroneous counts due to errors in dilution or plating.

Agar plating method for viable cell counts using dilutions 1×10^5, 1×10^6, 1×10^7, and 1×10^8

Procedure:



Chapter - 17
Control of Microorganisms by Physical and Chemical Methods

Control of microorganisms is essential in the home, industry, and medical fields to prevent and treat diseases and to avoid the spoilage of foods and other industrial products. Common methods of control involve chemical and physical agents that adversely affect microbial structures and functions, thereby producing a microbicidal or microbiostatic effect. A microbicidal effect is one that kills the microbes immediately; a microbiostatic effect inhibits the reproductive capacities of the cells, retards their growth and maintains the microbial population at a constant size.

Chemical Methods for Control of Microbial Growth:

1. **Antiseptics:** Chemical substances used on living tissue that kill or inhibit the growth of vegetative microbial forms.
2. **Disinfectants:** Chemical substances that kill or inhibit the growth of vegetative microbial forms on non living materials.
3. **Chemotherapeutic agents:** Chemical substances that destroy or inhibit the growth of microorganisms in living tissues.

Physical Methods for Control of Microbial Growth:

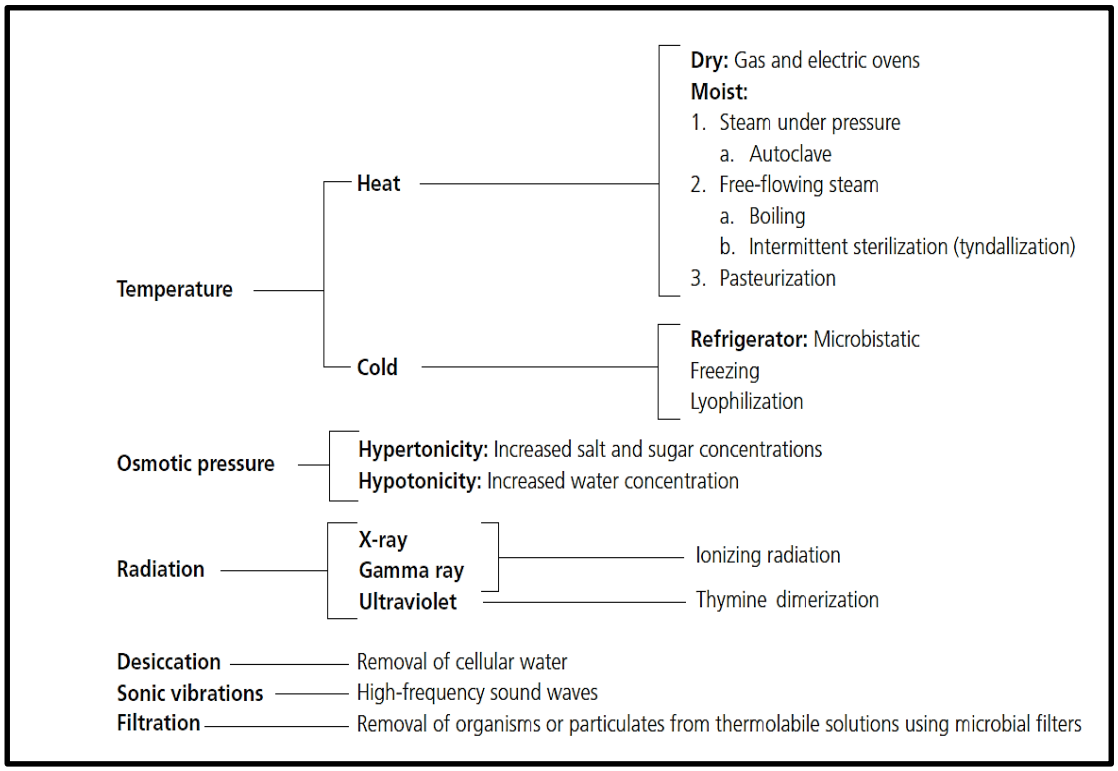
The modes of action of the different chemical and physical agents on microbial growth vary depending on the type of microorganisms. They produce damaging effects to essential cellular structures or molecules and cause cell death or inhibition of growth. These damages can result in malfunction of the cell wall, cell membrane, cytoplasm, enzymes, or nucleic acids.

The adverse effects manifest themselves in the following ways.

a. **Cell-wall injury:** This occurs in one of two ways. First, lysis of the cell wall will leave the wall-less cell, called a protoplast, susceptible to osmotic damage, and a hypotonic environment may cause lysis of the protoplast. Second, certain agents inhibit cell wall synthesis, which is essential during microbial cell reproduction. This incomplete cell wall synthesis results in lysis of an unprotected protoplast.
b. **Cell-membrane damage:** This may be the result of lysis of the membrane, which will cause immediate cell death. Also, the selective nature of the membrane may be affected without causing its complete disruption. As a result, there may be a loss of essential cellular molecules or interference with the uptake of nutrients. In both cases, metabolic processes will be adversely affected.
c. **Alteration of the colloidal state of cytoplasm:** Certain agents cause denaturation of cytoplasmic proteins. Denaturing processes are responsible for enzyme inactivation and cellular death by irreversibly rupturing the molecular bonds of these proteins and making them biologically inactive.
d. **Inactivation of cellular enzymes:** Enzymes may be inactivated competitively or noncompetitively. Noncompetitive inhibition is irreversible and occurs following the application of some physical agent, such as mercuric chloride ($HgCl_2$) that results in the uncoiling of the protein molecule, rendering it biologically inactive. Competitive

inhibition occurs when a natural substrate is forced to compete for the active site on an enzyme surface which can block the enzyme's ability to create end products. Competitive inhibitors are reversible.

e. **Interference with the structure and function of the DNA molecule:** The DNA molecule is the control center of the cell and may also represent a cellular target area for destruction or inhibition. Some agents have an affinity for DNA and cause breakage or distortion of the molecule, thereby interfering with its replication and role in protein synthesis. **Figure 78** below illustrates all the physical methods used for the control of microbial growth. However, awareness of the mode of action of the physical and chemical agents is absolutely essential for their proper selection and application in microbial control.



Physical methods used for the control of microorganisms

The experiments in this lab manual are designed to acquaint you more fully with several commonly employed agents and their uses.

Moist Heat as a physical control for the control of microorganisms:

Temperature has an effect on cellular enzyme systems and therefore a marked influence on the rate of chemical reactions and thus the life and death of microorganisms. Despite the diversity among microorganisms' temperature requirements for growth, extremes in temperature can be used in microbial growth control. Sufficiently low temperatures will inactivate enzymes and produce a static effect. High temperatures destroy cellular enzymes, which become irreversibly denatured.

Two methods of comparison are used: the thermal death point and the thermal death time.

The thermal death point (TDP) is the temperature at which an organism is killed in 10 minutes. The thermal death time (TDT) is the time required to kill a suspension of cells or spores at a given temperature. Since various factors such as pH, moisture, composition of medium, and age of cells will greatly influence results, these variables must be clearly stated. The application of heat is a common means of destroying microorganisms. Both dry and moist heat is effective. However, moist heat, which (because of the hydrolyzing effect of water and its greater penetrating ability) causes coagulation of proteins, kills cells more rapidly and at lower temperatures than does dry heat. Sterilization, the destruction of all forms of life, is accomplished in 15 minutes at 121°C with moist heat (steam) under pressure; dry heat requires a temperature of 160°C to 180°C for 1½ to 3 hours. Microbes show differences in their resistance pattern to moist heat. Bacterial spores require temperatures above 100°C for destruction, whereas most bacterial vegetative cells are killed at temperatures of 60°C to 70°C in 10 minutes. Fungi can be killed at 50°C to 60°C, and fungal spores require 70°C to 80°C for 10 minutes for destruction. Because of this variability, moist heat can either sterilize or disinfect. Common applications include steam under pressure (autoclaving), intermittent steam at 100°C (tyndallization), and the use of lower temperatures (pasteurization).

Steam under pressure requires the use of an autoclave, a double-walled metal vessel that allows steam to be pressurized in the outer jacket. At a designated pressure, the saturated steam is released into the inner chamber, from which all the air has been evacuated. The steam under pressure in the vacuumed inner chamber is now capable of achieving temperatures in excess of 100°C. The temperature is determined by the pounds of pressure applied per square inch.

Pasteurization exposes fairly thermolabile products such as milk, wine, and beer for a given period of time to a temperature that is high enough to destroy pathogens and some spoilage causing microorganisms that may be present, without necessarily destroying all vegetative cells. There are three types of pasteurization: The high-temperature, short-time (HTST) procedure requires a temperature of 72°C for 15 seconds. The low-temperature, long-time (LTLT) method requires 62.8°C for 30 minutes, and the ultra high temperature (UHT) approach occurs at 138°C for 2 seconds.

Experiment 44

Aim:

To determine the lethal effect of temperature on microbial growth by the application of moist heat

Principle:

Moist heat as a physical method for the control of microbial growth is an effective method used as a disinfection as well as sterilization. Steam under reduced pressure can be applied to the contaminated substance at a temperature of 100°C, which is achieved by boiling water. This exposure to boiling water for 30 minutes will result in disinfection only; all vegetative cells will be killed, but not necessarily the more heat resistant spores. Another procedure is tyndallization, also known as intermittent or fractional sterilization. This method uses exposure of the contaminated material to live steam at 100°C for 20 minutes for 3 consecutive days with intermittent incubation at 37°C. The steaming kills all vegetative cells.

Any spores that may be present germinate during the period of incubation and are destroyed during subsequent exposure to a temperature of 100°C. Repeating this procedure for 3 days ensures germination of all spores and their destruction in the vegetative form. Because tyndallization requires so much time, it is used only for sterilization of materials that are composed of thermolabile chemicals and that might be subject to decomposition at higher temperatures.

In this experiment, use of moist heat is achieved by boiling the microbial culture in a water bath at respective temperatures and then following incubation of these organisms to study the effect on microbial growth.

Requirements:

24- hour nutrient broth culture of *Escherichia coli*

Nutrient agar plates (05), and one 10-ml tube of nutrient broth.

Bunsen burner, 1000-ml beaker (water bath), tripod Stand and wire gauze screen with heat-resistant pad,

Thermometer, sterile test tubes,

Glass marker

Inoculating loop

Incubator

Procedure:

1. Label the bottom of each of the nutrient agar plates, indicating the heat temperatures to be used: 25°C (control), 40°C, 60°C, 80°C, and 100°C.
2. Using aseptic technique, inoculate the nutrient agar labeled 25°C by making a single-line loop inoculation of *E. coli*
3. Using a sterile pipette, transfer 10 ml of each culture to four sterile test tubes labeled with the temperature (40°C, 60°C, 80°C, and 100°C).
4. Set up the water bath on a tripod, inserting the thermometer in an uncapped tube of nutrient broth.
5. Slowly heat the water to 40°C; check the thermometer frequently to ensure that it does not exceed the desired temperature. Place the culture of the *E.coli* into the beaker and maintain the temperature at 40°C for 10 minutes. Remove the culture and aseptically inoculate on the nutrient agar plate labeled 40°C.
6. Raise the water bath temperature to 60°C and repeat Step 6 for the inoculation of the nutrient agar plate labeled 60°C.
7. Raise the water bath temperature to 80°C and repeat Step 6 for the inoculation of the nutrient agar plate labeled 80°C.
8. Raise the water bath temperature to 100°C and repeat Step 6 for the inoculation of the nutrient agar plate labeled 100°C.
9. Incubate the nutrient agar plate in an inverted position for 24 to 48 hours at 37°C

Observation:

1. Observe all plates for the growth of the *E.coli* at each of the temperatures.

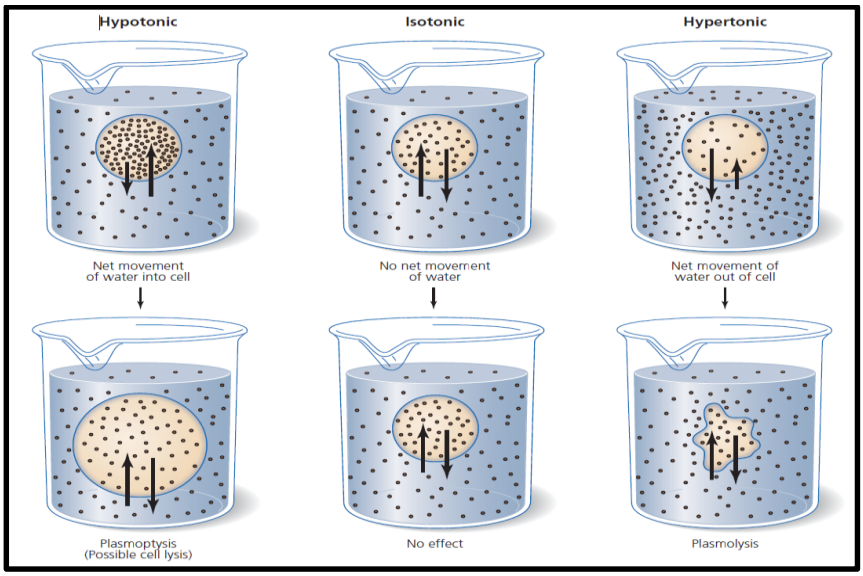
Osmotic pressure as a physical control for the control of microorganisms:

Osmosis is the net movement of water molecules (solvent) across a semipermeable

membrane from a solution of their higher concentration to a solution of their lower concentration. The relative water concentrations of two solutions are determined by their solute concentrations. The hypertonic solution possesses a higher osmotic pressure and a higher solute concentration, and therefore it has a lower water concentration; it tends to draw in water. The hypotonic solution possesses a lower osmotic pressure and solute concentration, and therefore a higher water concentration; it tends to lose water. If two solutions have equal concentrations of solutes and therefore equal water concentrations, there is no osmosis and the solutions are isotonic.

The bacterial cell and its environment represent two solutions separated by the semipermeable cell membrane. The cell's cytoplasm contains colloidal and solute particles dispersed in water, as does the cell's environment. The osmotic pressure of the environment in relation to that of the cytoplasm of the cell plays a vital role in the life and death of a cell. In a hypertonic, high-pressure environment, all cells lose water by osmosis and become shriveled. This effect is called **plasmolysis**. As water is necessary for the occurrence of many chemical reactions, water loss adversely affects cell metabolism and reproduction.

In a hypotonic, low-pressure environment, cells take in water and become swollen. This phenomenon is called **plasmoptysis**. In an environment with sufficiently low osmotic pressure, animal cells undergo lysis, which causes their deaths. Microorganisms possess rigid cell walls and are not usually susceptible to lysis in hypotonic environments and usually prefer a slightly hypotonic environment to maintain them in a turgid state. The effects of environmental osmotic pressure on cells are illustrated in Figure below.



Experiment 45

Aim:

To determine the possible effect of osmotic pressure environments on microorganisms

Principle:

Osmotic pressure can be used as an antimicrobial agent. Microorganisms are not usually adversely affected by low environmental osmotic pressure because of their small sizes and the presence of rigid cell walls. However, hypertonicity is a commonly used method of inhibiting

microbial growth. Because of their varied habitats, microorganisms are generally well adapted to exist in all types of osmotic pressure environments. Different groups of microorganisms require different degrees of salinity for growth, and they can adjust to salt concentrations of 0.5% to 3%. Concentrations of 10% to 15% are inhibitory to the growth of most microbes, except for halophiles, which require high salinity concentrations for growth. This sensitivity is the basis of food preservation by the process of salting.

Requirements:

24- nutrient broth cultures of *Staphylococcus aureus* and *Escherichia coli*

One nutrient agar plate of each of the following sodium chloride concentrations: 0.85%, 5%, 10%, 15%, and 25%.

Bunsen burner, inoculating loop

Glass marker

Incubator

Procedure:

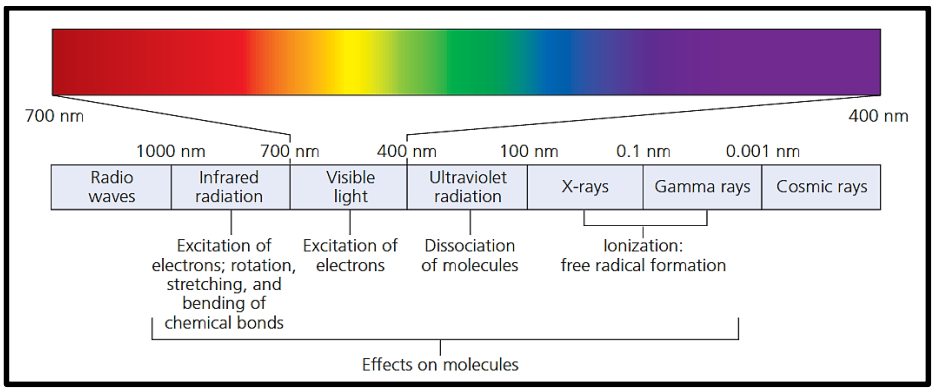
1. Divide the bottom of each of the five nutrient agar plates into two sections with a glass marker.
2. Label each of the two sections on each plate with the name of the organism to be inoculated and sodium chloride concentration used.
3. Using aseptic technique, inoculate each of the agar plates with the test organisms by making a single-line loop inoculation of each organism in its labeled section.
4. Incubate all plates in an inverted position for 4 to 5 days at 25 °C.

Observation:

Observe each of the nutrient agar plate cultures for the growth of each of the microbial species.

Ultra violet radiation as a physical control for the control of microorganisms:

Certain forms of electromagnetic radiation are capable of producing a lethal effect on cells and therefore can be used for microbial control. Electromagnetic radiations that possess sufficient energy to be microbicidal are the short wavelength radiations, that is, 300 nm and below. These include UV, gamma rays, and x-rays. The high-wavelength radiations, those above 300 nm, have insufficient energy to destroy cells. The electromagnetic spectrum and its effects on molecules are illustrated below.



Electromagnetic Spectrum and its effect on molecules

Gamma radiation, originating from unstable atomic nuclei, and **x-radiation**, originating from outside of the atomic nucleus, is representative of ionizing forms of radiation. Both transfer their energy through quanta (photons) to the matter through which they pass, causing excitation and the loss of electrons from molecules in their paths. This injurious effect is nonspecific in that any molecule in the path of the radiation will undergo ionization. Essential cell molecules can be directly affected through loss of their chemical structures and activity brought about by the ionization.

Water, the most abundant chemical constituent of cells, undergoes radiation breakdown, with the ultimate production of highly reactive H^+, OH^-, and, in the presence of oxygen, HO_2 free radicals. These may combine with each other, frequently forming hydrogen peroxide (H_2O_2), which is highly toxic to cells lacking catalase or other peroxidases, or the highly reactive free radicals may combine with any cellular constituents, again resulting in cell damage. Because of their high energy content and therefore ability to penetrate matter, x-ray and gamma radiations can be used as means of sterilization, particularly of thermolabile materials. They are not commonly used, however, because of the expense of the equipment and the special facilities necessary for their safe use.

Experiment 46

Aim:

To determine the microbicidal effect of ultraviolet (UV) radiation on microorganisms

Principle:

Ultraviolet light, which has lower energy content than ionizing radiations, is capable of producing a lethal effect in cells exposed to the low penetrating wavelengths in the range of 210 nm to 300 nm. Cellular components capable of absorbing ultraviolet light are the nucleic acids; DNA is the primary site of damage. As the pyrimidines especially absorb ultraviolet wavelengths, the major effect of this form of radiation is thymine dimerization, which is the covalent bonding of two adjacent thymine molecules on one nucleic acid strand in the DNA molecule. This dimer formation distorts the configuration of the DNA molecule, and the distortion interferes with DNA replication and transcription during protein synthesis. Some cell types, including some microorganisms, possess enzyme systems for the repair of radiation-induced DNA damage. Two different systems are found in the microorganisms:

1. The excision repair system, which functions in the absence of light; and

2 Photoreactivation, the light repair system, which is made operational by exposure of the irradiated cells to visible light in the wavelength range of 420 nm to 540 nm. The visible light serves to activate an enzyme photolyase that splits the dimers and reverses the damage.

Ultraviolet radiation, because of its low penetration ability, cannot be used as a means of sterilization, and its practical application is only for surface or air disinfection.

Requirements:

24- hour nutrient broth culture of *E. coli* and *S. marcescens*

Nutrient agar plates (10)

Bunsen burner

Inoculating loop,

Ultraviolet radiation source (254 nm)
Glass marker

Procedure:

1. Divide all nutrient agar plates into two sections by labelling the bottom of each plate with a glass marker.
2. Label each of the sections on each plate with the name of the organism to be inoculated.
3. Using aseptic technique, inoculate all the plates by means of a streak inoculation specifically as shown in the following illustration:
4. Label the cover of each inoculated plate with the exposure time to ultraviolet radiation as 0 second (control), 15 seconds, 30 seconds, 45 seconds, 60 seconds,75 seconds, 2 minute, and 3 minutes. Label two plates as 5 minutes; one of these plates will serve as the irradiated, covered control.
5. Irradiate all inoculated plates for the designated period of time by placing them 12 inches below the ultraviolet light source. Make sure first to remove all Petri dish covers except that of the 5-minute irradiated control plate.
6. Incubate all plates in an inverted position for 4 to 5 days at 25 °C.

Observation:

Observe each of the nutrient agar plates for growth of the test organism.

Chemotherapeutic agents as a chemical control for the control of microorganisms:

Chemotherapeutic agents are chemical substances used in the treatment of infectious diseases. Their mode of action is to interfere with microbial metabolism, thereby producing a bacteriostatic or bactericidal effect on the microorganisms, without producing a like effect in host cells. Chemotherapeutic agents act on a number of cellular targets. Their mechanisms of action include inhibition of cell-wall synthesis, inhibition of protein synthesis, inhibition of nucleic acid synthesis, disruption of the cell membrane, and inhibition of folic acid synthesis. These drugs can be classified into two categories:

1. **Antibiotics** are synthesized and secreted by some true bacteria, actinomycetes, and fungi that destroy or inhibit the growth of other microorganisms. Today, some antibiotics are laboratory synthesized or modified; however, their origins are living cells.
2. **Synthetic drugs** are synthesized in the laboratory.

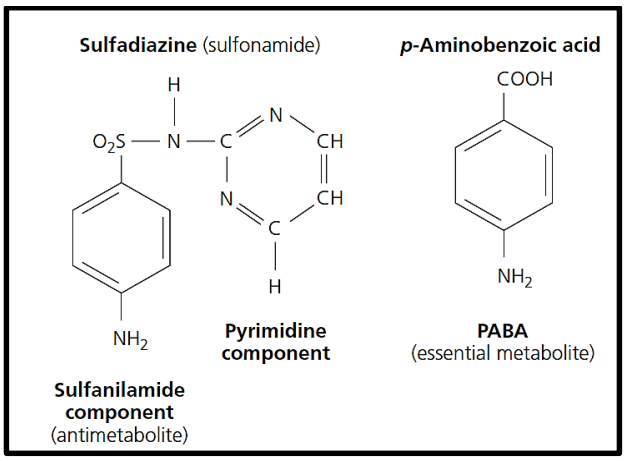
To determine a therapeutic drug of choice to be used, one must know its mode of action, possible adverse side effects in the host, and the scope of its antimicrobial activity. The specific mechanism of action varies among different drugs, and the short-term or long-term use of many drugs can produce systemic side effects in the host.

Synthetic Agents:

Sulfadiazine (a sulfonamide) produces a static effect on a wide range of microorganisms by a mechanism of action called competitive inhibition. The active component of the drug, sulfanilamide, acts as an antimetabolite that competes with the essential metabolite, p-aminobenzoic acid (PABA), during the synthesis of folic acid in the microbial cell. Folic acid is an essential cellular coenzyme involved in the synthesis of amino acids and purines. Many microorganisms possess enzymatic pathways for folic acid synthesis and can be adversely affected by sulfonamides. Human cells lack these enzymes, and the essential folic acid enters the

cells in a preformed state. Therefore, these drugs have no competitive effect on human cells.

The similarity between the chemical structure of the antimetabolite sulfanilamide and the structure of the essential metabolite PABA is shown below.



Antibiotics:

An antibiotic is a type of antimicrobial substance active against bacteria. It is the most important type of antibacterial agent for fighting bacterial infections, and antibiotic medications are widely used in the treatment and prevention of such infections.They may either kill or inhibit the growth of bacteria. There are different types of antibiotics. Each type is only effective against certain bacteria.

An antibiotic sensitivity test can help find out which antibiotic will be most effective in treating your infection. The test can also be helpful in finding a treatment for antibiotic-resistant infections. Antibiotic resistance happens when standard antibiotics become less effective or ineffective against certain bacteria. Antibiotic resistance can turn once easily treatable diseases into serious, even life-threatening illnesses.

The Kirby-Bauer procedure is used for the evaluation of the antimicrobial activity of chemotherapeutic agents.

Experiment 47

Aim:

To determine antibiotic sensitivity of an organism against antibiotics by Kirby-Bauer method.

Principle:

The available chemotherapeutic agents vary in their scope of antimicrobial activity. Some have a limited spectrum of activity, being effective against only one group of microorganisms. Others exhibit broad-spectrum activity against a range of microorganisms. The drug susceptibilities of many pathogenic microorganisms are known, but it is sometimes necessary to test several agents to determine the drug of choice. A standardized diffusion procedure with filter paper discs on agar, known as the Kirby-Bauer method, is frequently used to determine the drug susceptibility of microorganisms isolated from infectious processes. This method allows the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that results from diffusion of the agent into the medium surrounding the disc.

In this procedure, filter-paper discs of uniform size are impregnated with specified concentrations of different antibiotics and then placed on the surface of an agar plate that has been seeded with the organism to be tested. The medium of choice is Mueller-Hinton agar, with a pH of 7.2 to 7.4, which is poured into plates to a uniform depth of 5 mm and refrigerated after solidification. Prior to use, the plates are transferred to an incubator at 37 °C for 10 to 20 minutes to dry off the moisture that develops on the agar surface. The plates are then heavily inoculated with a standardized inoculum by means of a cotton swab to ensure the confluent growth of the organism. The discs are aseptically applied to the surface of the agar plate at well-spaced intervals. Once applied, each disc is gently touched with a sterile applicator stick to ensure its firm contact with the agar surface.

Following incubation, the plates are examined for the presence of growth inhibition, which is indicated by a clear zone surrounding each disc. The susceptibility of an organism to a drug is assessed by the size of this zone, which is affected by other variables such as:

1. The ability and rate of diffusion of the antibiotic into the medium and its interaction with the test organism.
2. The number of organisms inoculated.
3. The growth rate of the organism.

Requirements:

0.85% saline suspensions of *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Proteus vulgaris, Bacillus cereus,* and *Enterococcus faecalis* adjusted to an absorbance of 0.1 at 600 nm.

Mueller- Hinton agar plates (07).

Antimicrobial-Sensitivity Discs Penicillin G, 10 μg; Streptomycin, 10 μg; Tetracycline, 30 μg; Chloramphenicol, 30 μg; Gentamicin, 10 μg; Vancomycin, 30 μg; Sulfanilamide, 300 μg.

Sensi-Disc™ dispensers or forceps, Bunsen burner, sterile cotton swabs

Glass marker

Millimeter ruler

Procedure:

1. Label the covers of each of the agar plates with the name of the test organism to be inoculated.
2. Using aseptic technique, inoculate all agar plates with their respective test organisms as follows:
3. Dip a sterile cotton swab into a well-mixed saline test culture and remove excess inoculum by pressing the saturated swab against the inner wall of the culture tube.
4. Using the swab, streak the entire agar surface horizontally, vertically, and around the outer edge of the plate to ensure a heavy growth over the entire surface.
5. Allow all culture plates to dry for about 5 minutes.
6. Using the Sensi-Disc dispenser, apply the antibiotic discs by placing the dispenser over the agar surface and pressing the plunger, depositing the discs simultaneously onto the agar surface Or, if dispensers are not available, distribute the individual discs at equal distances with forceps dipped in alcohol and flamed.

7. Gently press each disc down with the wooden end of a cotton swab or with sterile forceps to ensure that the discs adhere to the surface of the agar. **Note:** Do not press the discs into the agar.
8. Incubate all plate cultures in an inverted position at 37 °C for 24 to 48 hours.

Observation:

Examine all plate cultures for the presence or absence of a zone of inhibition surrounding each disc. Using a ruler graduated in millimeters, carefully measure each zone of inhibition to the nearest millimeter.

A measurement of the diameter of the zone of inhibition in millimeters is made, and its size is compared to that contained in a standardized chart shown in Table below. Compare your results with **Table** and determine the susceptibility of each test organism to the chemotherapeutic agent. Record your results in the Lab notebook.

Based on this comparison, the test organism is determined to be resistant, intermediate, or susceptible to the antibiotic.

		ZONE DIAMETER, NEAREST WHOLE mm		
ANTIMICROBIAL AGENT	**DISC CONTENT**	**RESISTANT**	**INTERMEDIATE**	**SUSCEPTIBLE**
Ampicillin				
when testing gram-negative bacteria	10 µg	≤ 13	14–16	≥ 17
when testing gram-positive bacteria	10 µg	≤ 28	—	≥ 29
Carbenicillin				
when testing *Pseudomonas*	100 µg	≤ 13	14–16	≥ 17
when testing other gram-negative organisms	100 µg	≤ 19	20–22	≥ 23
Cefoxitin	30 µg	≤ 14	15–17	≥ 18
Cephalothin	30 µg	≤ 14	16–17	≥ 18
Chloramphenicol	30 µg	≤ 12	13–17	≥ 18
Clindamycin	2 µg	≤ 14	15–20	≥ 21
Erythromycin	15 µg	≤ 13	14–22	≥ 23
Gentamicin	10 µg	≤ 12	13–14	≥ 15
Kanamycin	30 µg	≤ 13	14–17	≥ 18
Methicillin when testing staphylococci	5 µg	≤ 9	10–13	≥ 14
Novobiocin	30 µg	≤ 17	18–21	≥ 22
Penicillin G				
when testing staphylococci	10 units	≤ 28	—	≥ 29
when testing other bacteria	10 units	≤ 14	—	≥ 15
Rifampin	5 µg	≤ 16	17–19	≥ 20
Streptomycin	10 µg	≤ 11	12–14	≥ 15
Tetracycline	30 µg	≤ 14	15–18	≥ 19
Tobramycin	10 µg	≤ 12	13–14	≥ 15
Trimethoprim/sulfamethoxazole	1.25/23.75 µg	≤ 10	11–15	≥ 16
Vancomycin				
when testing enterococci	30 µg	≤ 14	15–16	≥ 17
when testing *Staphylococcus* spp.	30 µg	—	—	≥ 15
Sulfonamides	250 or 300 µg	≤ 12	—	≥ 17
Trimethoprim	5 µg	≤ 10	—	≥ 16

Source: Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Disk Susceptibility Tests*, Tenth Edition, 2008.

Experiment 48

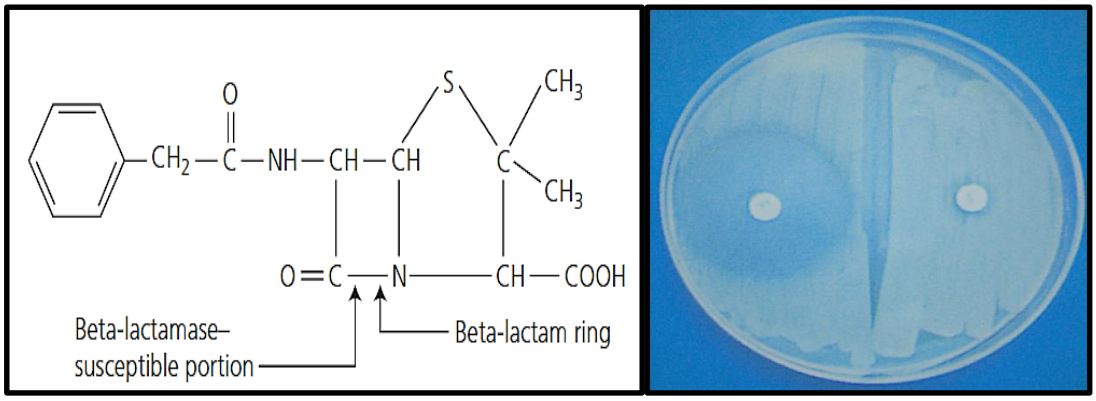
Aim:

To determine the minimal inhibitory concentration (MIC) of penicillin

Principle:

In addition to the Kirby-Bauer paper disc–agar diffusion method, the broth tube dilution method may be used to determine the susceptibility of an organism to an antibiotic. In this method, dilutions of the antibiotic are prepared in the broth medium and minimal inhibitory concentration (MIC) is determined for the antibiotic under investigation. The MIC is the lowest concentration of an antimicrobial agent that inhibits the growth of the test microorganism. Quantitative data of this nature may be used by a clinician to establish effective antimicrobial regimens for the treatment of a bacterial infection in a host. This data is of particular significance when the toxicity of the antibiotic is known to produce major adverse effects in host tissues.

Penicillin is a potent antibiotic produced by the mold Penicillium chrysogenum . Sir Alexander Fleming's discovery of penicillin in 1928 provided the world with the first clinically useful antibiotic in the fight to control human infection. The activity of this antibiotic is associated with the β-lactam ring within its molecular structure. Shortly after the clinical introduction of benzylpenicillin (penicillin G), pathogenic organisms such as Staphylococcus aureus were found to be resistant to this "wonder drug." Research revealed that some organisms were genetically capable of producing β-lactamase (penicillinase), an enzyme that breaks a bond in the β-lactam ring portion of the molecule. When the integrity of this ring is compromised, the inhibitory activity of the antibiotic is lost. Penicillinase activity is illustrated below.



Chemical Structure of Benzyl Penicillin and Penicillinase activity:
Penicillin sensitive (left side) & penicillin resistance (right side)

In this experiment, the MIC of penicillin will be determined against penicillin-sensitive and penicillinase-producing strains of *Staphylococcus aureus*. The procedure to be followed involves specific concentrations of the penicillin prepared by means of a twofold serial dilution technique in an enriched broth medium. The tubes containing the antibiotic dilutions are then inoculated with a standardized concentration of the test organism Table below illustrates the protocol for the antibiotic serial dilution–broth medium setup.

	TUBE NUMBER									
Additions (ml) to:	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Medium	0	2	2	2	2	2	2	2	2	2
Penicillin	2	2	Serial dilution (See protocol)							0
Test culture	2	2	2	2	2	2	2	2	2	2
Total volume	4	4	4	4	4	4	4	4	4*	4
Penicillin (µg/ml)	50	50	25	12.5	6.25	3.12	1.56	0.78	0.39	0
Control	(–)									(+)
*After 2 ml discarded										

Following incubation, spectrophotometric absorbance readings will be used to determine the presence or absence of growth in the cultures. The culture that shows no growth in the presence of the lowest concentration of penicillin represents the minimal inhibitory concentration of this antibiotic against *S. aureus.*

Requirements:

1:1000 brain heart infusion (BHI) broth dilutions of 24-hour BHI broth cultures of Staphylococcus aureus ATCC® 27661™ (penicillin-sensitive strain) and Staphylococcus aureus ATCC 27659 (penicillinase- producing strain).

40 ml of brain heart infusion broth in a 100-ml Erlenmeyer flask and 10 ml of sterile aqueous crystalline penicillin G solution (100 µg/ml).

Sterile 13x100mm test tubes, test tube racks, sterile 2-ml and 10-ml pipettes, mechanical pipetting device, Bunsen burner, spectrophotometer, glass marker, and Dettol solution in a 500-ml beaker.

Procedure:

1. Into each of two test tube racks, place a set of 10 sterile 13 x 100mm test tubes labeled 1 through 10. Label one rack Set I-penicillin sensitive and the other rack Set II-penicillin resistant.

   Refer to Table for Steps 2 through 7.
2. Using a sterile 10-ml pipette and mechanical pipetting device, add 2 ml of BHI broth to the tubes labeled 2 through 10 in Sets I and II. **Note:** Discard the pipette into the beaker of disinfectant.
3. With a 2-ml sterile pipette, add 2 ml of the penicillin solution to Tubes 1 and 2 in Sets I and II. Discard the pipette. **Note:** Mix the contents of the tubes well.
4. **Set I Serial Dilution**: Using a sterile 2-ml pipette, transfer 2 ml from Tube 2 to Tube 3. Mix well and transfer 2 ml from Tube 3 to Tube 4.
5. Continue this procedure through Tube 9 into beaker. Discard 2 ml from Tube 9.
   Tube 10 receives no antibiotic and serves as a positive control. Discard the pipette. **Note:** Remember to mix the contents of each tube well between transfers.
6. **Set II Serial Dilution:** Using a sterile 2-ml pipette, repeat Step 4.
7. Using a sterile 2-ml pipette, add 2 ml of the 1:1000 dilution of the *S. aureus* ATCC 27661 (penicillin-sensitive strain) to all tubes in Set I. Discard the pipette.
8. Repeat Step 6 to inoculate all the tubes in Set II with the 1:1000 dilution of *S. aureus* ATCC 27659 (penicillinase-producing strain). Discard the pipette.

9. Incubate both sets of tubes for 12 to 18 hours at 37°C.
10. Use the spectrophotometer to take the absorbance readings for Tubes 2 through 10 in Sets I and II. Use the Number 1 tubes, the negative controls, as your blanks to adjust the spectrophotometer.
11. Record your absorbance readings in the chart in the Lab notebook.

Antiseptics and disinfectants:

Antiseptics and disinfectants are chemical substances used to prevent contamination and infection. Many are available commercially for disinfection and asepsis.

Table 3: The major groups of antimicrobial agents, their modes and ranges of action, and their practical uses

AGENT	MECHANISM OF ACTION	USE
Phenolic Compounds		
Phenol	1. Germicidal effect caused by alteration of protein structure resulting in protein denaturation. 2. Surface-active agent (surfactant) precipitates cellular proteins and disrupts cell membranes. (Phenol has been replaced by better disinfectants that are less irritating, less toxic to tissues, and better inhibitors of microorganisms.)	1. 5% solution: Disinfection. 2. 0.5% to 1% solutions: Antiseptic effect and relief of itching as it exerts a local anesthetic effect on sensory nerve endings.
Cresols	1. Similar to phenol. 2. Poisonous and must be used externally. 3. 50% solution of cresols in vegetable oil, known as Lysol®.	2% to 5% Lysol solutions used as disinfectants.
Hexachlorophene	Germicidal activity similar to phenol. (This agent is to be used with care, especially on infants, because after absorption it may cause neurotoxic effects.)	1. Reduction of pathogenic organisms on skin; added to detergents, soaps, lotions, and creams. 2. Effective against gram-positive organisms. 3. An antiseptic used topically.
Resorcinol	1. Germicidal activity similar to that of phenol. 2. Acts by precipitating cell protein.	1. Antiseptic. 2. Keratolytic agent for softening or dissolving keratin in epidermis.
Hexylresorcinol	Germicidal activity similar to that of phenol.	1. Treatment of worm infections. 2. Urinary antiseptic.

AGENT	MECHANISM OF ACTION	USE
Thymol	1. Related to the cresols. 2. More effective than phenol.	1. Antifungal activity. 2. Treatment of hookworm infections. 3. Mouthwashes and gargle solutions.
Alcohols Ethyl: CH_3CH_2OH Isopropyl: $(CH_3)_2CHOH$	1. Lipid solvent. 2. Denaturation and coagulation of proteins. 3. Wetting agent used in tinctures to increase the wetting ability of other chemicals. 4. Germicidal activity increases with increasing molecular weight.	Skin antiseptics: Ethyl—50% to 70%. Isopropyl—60% to 70%.
Halogens Chlorine compounds: Sodium hypochlorite (Dakin's fluid): NaOCl Chloramine: $CH_3C_6H_4SO_2NNaCl$	1. Germicidal effect resulting from rapid combination with proteins. 2. Chlorine reacts with water to form hypochlorous acid, which is bactericidal. 3. Oxidizing agent. 4. Noncompetitively inhibits enzymes, especially those dealing with glucose metabolism, by reacting with SH and NH_2 groups on the enzyme molecule.	1. Water purification. 2. Sanitation of utensils in dairy and restaurant industries. 3. Chloramine, 0.1% to 2% solutions, for wound irrigation and dressings. 4. Microbicidal.
Iodine compounds: Tincture of iodine Povidone-iodine solution (Betadine®)	1. Mechanism of action is not entirely known, but it is believed that it precipitates proteins. 2. Surface-active agent.	1. Tinctures of iodine are used for skin antisepsis. 2. Treatment of goiter. 3. Effective against spores, fungi, and viruses.
Heavy Metals Mercury compounds: Inorganic: Mercury bichloride Mercurial ointments	1. Mercuric ion brings about precipitation of cellular proteins. 2. Noncompetitive inhibition of specific enzymes caused by reaction with sulfhydryl group (SH) on enzymes of bacterial cells.	1. Inorganic mercurials are irritating to tissues, toxic systemically, adversely affected by organic matter, and incapable of acting on spores. 2. Mercury compounds are mainly used as disinfectants of laboratory materials.
Organic mercurials: Mercurochrome (merbromin) Merthiolate (thimerosal) Metaphen (nitromersol) Merbak (acetomeroctol)	1. Similar to those of inorganic mercurials, but in proper concentrations are useful antiseptics. 2. Much less irritating than inorganic mercurials.	1. Less toxic, less irritating; used mainly for skin asepsis. 2. Do not kill spores.
Silver compounds: Silver nitrate	1. Precipitate cellular proteins. 2. Interfere with metabolic activities of microbial cells. 3. Inorganic salts are germicidal.	Asepsis of mucous membrane of throat and eyes.

AGENT	MECHANISM OF ACTION	USE
Surface-Active Agents		
Wetting agents: Emulsifiers, soaps, and detergents	1. Lower surface tension and aid in mechanical removal of bacteria and soil. 2. If active portion of the agent carries a negative electric charge, it is called an anionic surface-active agent. If active portion of the agent carries a positive electric charge, it is called a cationic surface-active agent. 3. Exert bactericidal activity by interfering with or by depressing metabolic activities of microorganisms. 4. Disrupt cell membranes. 5. Alter cell permeability.	Weak action against fungi, acid-fast microorganisms, spores, and viruses.
Cationic agents: Quaternary ammonium compounds Benzalkonium chloride	1. Lower surface tension because of keratolytic, detergent, and emulsifying properties. 2. Their germicidal activities are reduced by soaps.	1. Bactericidal, fungicidal; inactive against spores and viruses. 2. Asepsis of intact skin. 3. Disinfectant for operating-room equipment. 4. Dairy and restaurant sanitization.
Anionic agents: Tincture of green soap Sodium tetradecyl sulfate	1. Neutral or alkaline salts of high-molecular-weight acids. Common soaps included in this group. 2. Exert their maximum activity in an acid medium and are most effective against gram-positive cells. 3. Same as all surface-active agents.	1. Cleansing agent. 2. Sclerosing agent in treatment of varicose veins and internal hemorrhoids.
Acids (H^+) Alkali (OH^-)	1. Destruction of cell wall and cell membrane. 2. Coagulation of proteins.	Disinfection; however, of little practical value.
Formaldehyde (liquid or gas)	Alkylating agent causes reduction of enzymes.	1. Room disinfection. 2. Alcoholic solution for instrument disinfection. 3. Specimen preservation.
Ethylene Oxide	Alkylating agent causes reduction of enzymes.	Sterilization of heat-labile material.
β-Propiolactone (liquid or gas)	Alkylating agent causes reduction of enzymes.	1. Sterilization of tissue for grafting. 2. Destruction of hepatitis virus. 3. Room disinfection.
Basic Dyes Crystal violet	Affinity for nucleic acids; interfere with reproduction in gram-positive organisms.	1. Skin antiseptic. 2. Laboratory isolation of gram-negative bacteria.

The efficiency of all disinfectants and antiseptics is influenced by a variety of factors, including the following:

1. Concentration: The concentration of a chemical substance markedly influences its effect on microorganisms, with higher concentrations producing a more rapid death. Concentration cannot be arbitrarily determined; the toxicity of the chemical to the tissues being treated and the

damaging effect on non living materials must also be considered.

2. Length of exposure: All microbes are not destroyed within the same exposure time. Sensitive forms are destroyed more rapidly than resistant ones. The longer the exposure to the agent, the greater its antimicrobial activity. The toxicity of the chemical and environmental conditions must be considered in assessing the length of time necessary for disinfection or asepsis.

3. Type of microbial population to be destroyed: Microorganisms vary in their susceptibility to destruction by chemicals. Bacterial spores are the most resistant forms. Capsulated bacteria are more resistant than non capsulated forms; acid-fast bacteria are more resistant than non–acid-fast; and older, metabolically less-active cells are more resistant than younger cells. Awareness of the types of microorganisms that may be present will influence the choice of agent.

4. Environmental conditions: Conditions under which a disinfectant or antiseptic affects the chemical agent are as follows:

a. Temperature: Cells are killed as the result of a chemical reaction between the agent and cellular component. As increasing temperatures increase the rate of chemical reactions, application of heat during disinfection markedly increases the rate at which the microbial population is destroyed.

b. pH: The pH conditions during disinfection may affect not only the microorganisms but also the compound. Extremes in pH are harmful to many microorganisms and may enhance the antimicrobial action of a chemical.

Deviation from a neutral pH may cause ionization of the disinfectant; depending on the chemical agent, this may serve to increase or decrease the chemical's microbicidal action.

c. Type of material on which the microorganisms exist: The destructive power of the compound on cells is due to its combination with organic cellular molecules. If the material on which the microorganisms are found is primarily organic, such as blood, pus, or tissue fluids, the agent will combine with these extracellular organic molecules, and its antimicrobial activity will be reduced.

Numerous laboratory procedures are available for evaluating the antimicrobial efficiency of disinfectants or antiseptics. They provide a general rather than an absolute measure of the effectiveness of any agent because test conditions frequently differ considerably from those seen during practical use. The agar plate-sensitivity method, a commonly employed procedure, is presented.

Experiment 49

Aim:

To evaluate the effectiveness of antiseptic agents against selected test organisms

Principle:

The effectiveness of antiseptic agents against microbes is determined by heavy inoculation of an agar plate with the test organism. Sterile, filter-paper discs are impregnated with a different antiseptic (10µl) and equally spaced on the inoculated agar plate. Following incubation, the agar plate is examined for zones of inhibition (areas of no microbial growth) surrounding the discs.

A zone of inhibition is indicative of microbicidal activity against the organism. Absence

of a zone of inhibition indicates that the chemical was ineffective against the test organism. However, the size of the zone of inhibition is not indicative of the degree of effectiveness of the chemical agent.

Requirements:

24- to 48-hour Trypticase soy broth cultures of *Escherichia coli, Bacillus cereus, Staphylococcus aureus, and Mycobacterium smegmatis*, and a 7-day-old Trypticase soy broth culture of *Bacillus cereus.*

Trypticase soy agar plates (05).

Antiseptics/Disinfectants

10 ml of each of the following dispensed in 25-ml beakers: tincture of iodine, 3% hydrogen peroxide, 70% isopropyl alcohol, and 5% chlorine bleach

Sterile Sensi-Discs; forceps; sterile cotton swabs; Bunsen burner; and glass marker.

Procedure:

1. Aseptically inoculate the appropriately labeled agar plates with their respective test organisms by streaking each plate in horizontal and vertical directions and around the edge with a sterile swab.
2. Appropriately label the Sensi-Discs according to the chemical agents to be used.
3. Using forceps dipped in alcohol and flamed, expose five discs by placing them into the solution of one of the chemical agents. Drain the saturated discs on absorbent paper immediately prior to placing one on each of the inoculated agar plates. Place each disc approximately 2 cm in from the edge of the plate. Gently press the discs down with the forceps so that they adhere to the surface of the agar.
4. Impregnate the well labelled remaining discs as described in Step 3. Place one of each of the three remaining colored discs on the surface of each of the five inoculated agar plates equidistant from each other around the periphery of the plate.
5. Incubate all plate cultures in an inverted position for 24 to 48 hours at 37 °C.

Observation:

Observe all the plates for the presence of a zone of inhibition surrounding each of the impregnated discs. Record your observations in your Lab notebook.

Oligodynamic Action

The ability of small amounts of heavy metals to exert a lethal effect on bacteria is designated as oligodynamic action (Greek: oligos, small; dynamis, power). The effectiveness of these small amounts of metal is probably due to the high affinity of cellular proteins for the metallic ions. Although the concentration of ions in solution may be miniscule (a few parts per million), cells die due to the cumulative effects of ions within the cell. Similarly, silver inactivates enzymes by binding with sulfhydryl groups to form silver sulfides or sulfhydryl-binding propensity of silver ion disrupts cell membranes, disables proteins and inhibits enzyme activities.

Experiment 50

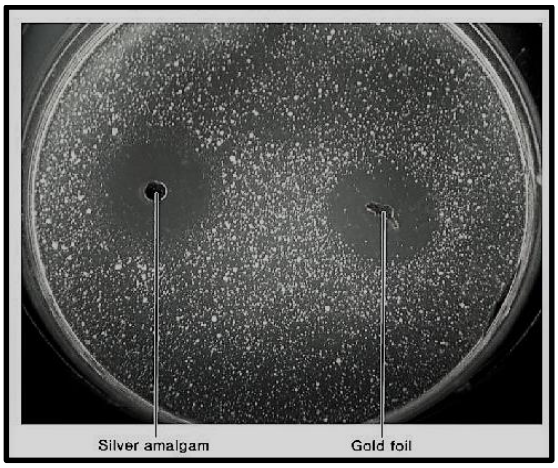
Aim:

To study the oligodynamic effect of heavy metals on the growth of microorganisms

Principle:

Many metallic elements have been observed to inhibit the growth of bacteria and to inactivate enzymes. Oligodynamic metals, such as silver and copper, have been utilized since long times as disinfectants for non-spore-forming bacteria and viruses. The antimicrobial activities are shown by metals such as mercury, silver, copper, lead, zinc, gold, aluminum and other metals, and the concentration of the metal needed for this antimicrobial effect is miniscule.

The success of silver amalgam fillings to prevent secondary dental decay in teeth over long periods of time is due to the small amounts of silver and mercury ions that diffuse into adjacent tooth dentin. However, its success in this respect is much debated and of concern that its toxicity may cause long-term injury to patients. In addition to its value (or harm) as a dental restoration material, oligodynamic action of certain other heavy metals has been applied to water purification, ointment manufacture,the treatment of bandages and in the preservation of juices and foods.



Effect of Silver amalgam and gold foil on microorganisms

In this experiment, the oligodynamic action of three metals (copper, silver, and aluminum) are carried out.

Requirements:

Nutrient agar molten
Sterile Petri plate
Bunsen burner and forceps
Acid alcohol
Broth culture of *E. coli* and *S. aureus*
Metallic disks (copper, silver, aluminum)
Water bath

Principle:

1. Liquefy a tube of nutrient agar, cool to 50 °C, and inoculate with either E. coli or S. aureus (odd: *E. coli*; even: *S. aureus*).

2. Pour half of the medium from each tube into a sterile Petri plate and leave the other half in a water bath (50 °C). Allow agar to solidify in the plate.
3. Clean three metallic disks, one at a time, and place them on the agar, evenly spaced, as soon as they are cleaned.
4. Pour the remaining seeded agar from the tube over the metal disks. Incubate at 37 °C for 48 hours.
5. After incubation, compare the zones of inhibition and record your results in a Lab notebook.

Observation:

Observe the plates for the antimicrobial effect of Copper, Silver and Aluminium on microbial growth.

Chapter - 18
Cultivation of Economically Important Microbes: Probiotics

The World Health Organization (WHO) defines "probiotics" as "live microorganisms that, when administered in adequate amounts, confer a perceived health benefit on the host". These intentionally ingested microorganisms consist mainly of bacteria but also include yeasts. Products containing dead microorganisms and those made by microorganisms are, by definition, not probiotics.

Probiotics (Greek pro, for, and bios, life) confer various benefits especially to individuals who experience major changes in their normal microflora due to disease, surgery, or other medical treatments, or whose normal microflora changes for other reasons, such as poor diet. Oral administration of probiotic organisms reestablish the natural balance of gastrointestinal flora and return the host to normal health and nutrition.

Probiotic microorganisms are host-specific; thus a strain selected as a probiotic in one animal may not be suitable in another species. Probiotics are subcategorized into probiotic drugs, probiotic foods (e.g., foods, food ingredients, and dietary supplements), direct-fed microbials (probiotics for animal use), and designer probiotics (genetically modified probiotics).

Probiotics should not be confused with **prebiotics** which are food ingredients, typically complex carbohydrates (mostly consisting of nonstarch polysaccharides and oligosaccharides) that escape digestion in the upper gastrointestinal tract and are available for microorganisms living in the colon.

Prebiotics:

Most prebiotics are used as food ingredients in chocolates, biscuits, cereals, spreads, and dairy products. Commonly known prebiotics are:

1. Oligofructose
2. Inulin
3. Galacto-oligosaccharides
4. Lactulose
5. Breast milk oligosaccharides

The prebiotic oligofructose is found naturally in many foods, such as wheat, onions, bananas, honey, garlic, and leeks. Oligofructose can also be isolated from chicory root or synthesized enzymatically from sucrose. Inulin is a prebiotic fiber that occurs naturally in asparagus, garlic, onions, wheat, garlic, leeks, chicory, oats, soybeans, and Jerusalem artichokes. Galacto-oligosaccharides which are mainly used in infant milk formula are made up of plant sugars linked in chains. They are found naturally in dairy products, beans, and certain root vegetables. Lactulose is a man-made sugar that contains two naturally occurring sugars, galactose, and fructose. Lactulose is produced industrially by isomerization of lactose via a 1,2-enediol intermediate.

Health Benefits of Prebiotics:

The use of both prebiotics and/or probiotics is intended to influence the gut environment for the benefit of human health and their beneficial effects extend beyond the gut. Fermentation of oligofructose in the colon results in a large number of physiologic effects, including:

- Increasing the number of bifidobacteria in the colon

- Increasing calcium absorption
- Increasing fecal weight
- Shortening gastrointestinal transit time
- Possibly lowering blood lipid levels

Prebiotics selectively stimulate the growth of selective bacterial genera such as bifidobacteria and lactobacilli in the colon. The increase in colonic bifidobacteria benefits human health by producing digestive enzymes, vitamins, reducing blood ammonia levels, and inhibiting potential pathogens. Lactulose is a synthetic disaccharide used as a drug for the treatment of constipation and hepatic encephalopathy.

Commercial products containing both prebiotic sugars and probiotic organisms are often called "synbiotics." Synbiotics are appropriate combinations of prebiotics and probiotics and exert both a prebiotic and probiotic effect.

Probiotics vs. Antibiotics

The term "probiotic" literally means "for life" and "antibiotic" literally means "opposing life" are two opposing categories of supplements or drugs. Antibiotics (Greek anti, against, and bios, life) are used to prevent and treat infections caused by pathogenic bacteria whereas probiotics (Greek pro, for, and bios, life) are used to replenish good bacteria.

For example, during the course of antibiotics treatment, good bacteria of the gut are also wiped out, taking probiotics helps to restore the gut microbiome, thus restoring the healthy state of the gut.

A. Isolation of Probiotic Microorganisms:

A probiotic should contain a number of viable cells greater than 10^6 to 10^8 per dose to be efficacious. Seven microorganisms most often used in probiotic products are *Lactobacillus, Bifidobacterium, Saccharomyces, Streptococcus, Enterococcus, Escherichia,* and *Bacillus.*

These beneficial microorganisms are naturally present in fermented foods (such as yogurt, kefir, kimchi, etc), may be added to other food products, and also available as dietary supplements or as drugs. Probiotic microorganisms from commercial providers come in a variety of forms, including powders, pills, liquid suspensions, and food products.

Microorganisms selected for probiotic use should exhibit the following characteristics:

1. Adhere to the intestinal mucosa of the host
2. Be easily cultured
3. Be nontoxic and nonpathogenic to the host
4. Exert a beneficial effect on the host
5. Produce useful enzymes or physiological end products that the host can use
6. Remain viable for a long time
7. Withstand HCl in the host's stomach and bile salts in the small intestine

Health Benefits of Probiotics

Numerous clinical trials have proven the health benefits of probiotics but the exact mechanisms of the health benefits are not fully understood. Gut health is the most important target for probiotics. Prevention and treatment of different forms of diarrhea is one of the most successful and best-documented health benefits of probiotics.

Alterations in the composition of the intestinal microbiome have been associated with infections in the gastrointestinal tract, inflammatory bowel disease (IBD), and irritable bowel

syndrome (IBS). Growing evidence indicates that probiotics may be effective in the treatment of specific clinical IBD and IBS conditions but large trials are necessary before doctors can prescribe particular probiotics confidently on a routine basis as therapeutic agents.
Proven potential benefits of probiotics include:

1. Anticarcinogenic activity
2. Control of intestinal pathogens
3. Improvement of lactose use in individuals who have lactose intolerance
4. Reduction in the serum cholesterol concentration
5. Reduction of the risk of antibiotic-induced diarrhea

Experiment 51

Aim:

Isolation of Lactic acid bacteria for probiotics production

Principle:

Lactic acid and acetic acid bacteria are the common groups of bacteria involved in fermented foods and related products of fermentation. Many of the fermented products include cheese, pickles, and fermented sausages. Bacterial fermentation improves the aroma, flavor and nutritional qualities of the product. Lactic acid bacteria ferment carbohydrates to lactic acid. The acidic environment thus created greatly alters the undesirable microorganisms. These are responsible for the fermented vegetables (pickles), meat, milk etc. They are widely distributed among plants and plant products.

Lactic acid bacteria (LAB) are gram-positive, usually non-motile and non-sporulating bacteria that produce lactic acid as the major or sole product of fermentative metabolism. The cells often appear as chains under the microscope. Most lactic acid bacteria obtain energy only from the metabolism of sugars and related fermentable compounds and hence are usually restricted to habitats in which sugars are present. In general, LAB has complex nutritional requirements and needs amino acids, vitamins, purines, and pyrimidines. The genera that comprise LAB are at its core *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Streptococcus*.

***Requirements*:**

Any fermented product *viz.*, (curd, cheese, idli batter, buttermilk, pickles etc.)
MRS medium (de-Man, Rogosa and Sharpe's Agar)
Sterile water blanks (99ml & 9ml)
Sterile petri plates and pipettes.
Bunsen burner, inoculation loop
Incubator

MRS medium composition:

Glucose:	20.0g	Magnesium sulphate:	0.20g
Peptone:	10.0g	Manganese sulphate:	0.05g
Beef extract:	10.0g	Tween 80:	1ml
Yeast extract:	5.0g	Agar:	20 g
K_2HPO_4:	2.0g	Distilled water:	1000 ml.
Sodium acetate:	5.0g	pH:	6.0
Triammonium citrate:	2.0g		

Procedure:

1. Weigh one gram or one ml of the sample and dilute it in a 250 ml flask containing 99 ml of sterile water; this gives 10^{-2} dilution.
2. Make successive dilutions in the same way using 9 ml sterile distilled water blanks up to 10^{-4} dilution.
3. Transfer aseptically 1 ml of the sample dilution from 10^{-3} and 10^{-4} to sterile petri plates. Maintain three replications for each dilution in order to minimize handling error.
4. Now pour the melted and cooled media (MRS) into the Petri plates; immediately rotate the plates gently both in clockwise and anti-clockwise direction in order to spread the sample dilution evenly with the medium.
5. Incubate the plates upside-down position at 30± 4 ºC for 2 to 4 days.

Observation:

Observe the plates for colony formation. Count the number of colonies per plate and find out the mean cfu for each dilution. Observe the colony morphology. *Lactobacillus* sp. forms small, creamy, white colonies on MRS medium which are generally distinct from other bacterial colonies.

B. Screening of LAB for probiotic characters - Bile salt and pH.

Probiotics are live microorganisms beneficial to the host. Microorganisms like Yeast, *Lactobacillus, Bifidobacteria,* etc are the organisms of choice for probiotics production. Prebiotics are non digestible component which beneficially affects the host by selectively stimulating the growth or activity of one or a limited number of colonic bacteria, thereby improving the health of the host. It includes dietary fibre and carbohydrates. Strainsof *Lactobacillus* and *Bifidobacterium* have been extensively used as probiotic microorganisms for humans.

The mode of action of probiotics are:

a. Competitive exclusion
b. Production of bacteriocin
c. Production of organic acid
d. Altered absorption of intestinal mucosa

Experiment 52

Aim:

Screening of probiotics characteristics in Lactic acid bacteria

Principle:

Lactobacillus is one of the most studied organisms and extensively used in the food industry as a probiotic microorganism. An efficient probiotic microorganism should be able to:

1. survive the passage through digestive system
2. attach to intestinal epithelia and colonize
3. maintain good viability
4. utilize the nutrients and substances in normal diet
5. non pathogenic and non-toxic

6. capable of exerting a beneficial effect on host

In order to reach the colon in a viable state, they must cope with specific stress challenges throughout the gastrointestinal tract, among which the presence of bile in the upper parts of the small intestine is one of the main ones. The main components of bile are bile acids, which are produced and conjugated with the amino acids glycine or taurine in the liver, to generate conjugated bile salts. Bile is stored in the gallbladder and flows from there to the duodenum during digestion, facilitating the solubilization and absorption of dietary fats. Thus, under normal physiological conditions, our intestine holds a bile salt concentration gradient ranging from more than 40 mM to less than 1 mM – equivalent to a range between 2% and 0.05% – which is responsible, among other factors, for shaping the microbial community profile found in our gut.

Apart from its normal physiological function, bile is highly toxic for those microorganisms unadapted to the intestinal conditions. Therefore, enteric bacteria, including *Lactobacilli* and *Bifidobacteria*, must have evolved specific defense mechanisms to resist the deleterious action caused by these compounds. The strong lipophilic nature of the steroid ring makes the cell membrane the main target of these molecules, in which they disturb the lipid packaging and disrupt the proton motive force, causing cell death. Furthermore, since the unconjugated forms are weak acids, they can passively diffuse into the cell and, once inside, they are dissociated producing acidification of the cytoplasm. Other side effects induced by bile have been documented, including induction of oxidative stress and DNA repair mechanisms, alterations of sugar metabolism, and protein misfolding. The pH of gastric acid is 1.5 to 3.5 in the human stomach lumen. Hence it is necessary to assess the pH and bile salt tolerance of probiotic microorganisms prior to formulation development.

Requirements:

MRS broth
Bile Salt
LAB culture
Oakridge tubes
Phosphate buffered saline

Procedure:

A. Bile salt tolerance test (Al-Saleh *et al.*, 2006)

1. Estimate the ability of the selected lactic acid bacteria (LAB) strains to survive in bile salt by determining their growth rate in MRS broth containing different levels of bile salt.
2. Inoculate freshly prepared LAB strains at the rate of 1 per cent inoculum into sterile MRS broth amended with bile salt (0.3%, 0.5%, and 0.7%)
3. Incubate at 37 ^{o}C for 24 hours under aerobic conditions in the incubator at 120 rpm.
4. Measure the growth as optical density periodically at 0, 2, 4, 6, 8 and 24 hours spectrophotometrically at 620 nm.

B. pH tolerance (Buntin *et al.*, 2008)

1. Harvest LAB cells were grown in MRS broth at 37 ^{o}C under aerobic conditions.
2. Wash the cell pellets twice, and finally, resuspended in 10 ml of phosphate-buffered saline (PBS) in order to achieve 10^{9} CFU ml^{-1}
3. Inoculate phosphate-buffered saline of pH values of 2, 3 and 7.0 and 9.0 (adjusted using

5M HCl and NaOH)

4. Incubate at 37 ^{o}C, and determine the viability of the inoculated cells after exposure to the acidic environment.
5. The plate on the MRS agar plates for 0, 1, 2, 3 and 24 h and incubate at 37 ^{o}C for 48 hours.
6. Estimate the survival based on the number of colonies grown on the MRS agar plates on comparison with the initial cell concentration.

Observation:

Observe the MRS plate amended with different concentrations of bile salt ((0.3%, 0.5%, and 0.7%) and measure the absorbance in the MRS broth tubes for the maximum pH tolerance by the given LAB culture.

Experiment 53

Aim:

Screening of antimicrobial activity as probiotics characteristics in Lactic acid bacteria.

Principle:

Functional foods exert health benefits to the consumers and dominate the food market. These functional foods targeted towards improving the balance and activity of the intestinal microflora. Consumption of food containing live bacteria is the oldest and still most widely used way to increase the number of advantageous bacteria called "probiotics" in the intestinal tract. Noteworthy, there are a large number of probiotic foods that date back to ancient times which are mostly originated from fermented foods as well as cultured milk products. The quest to find food ingredients with valuable bioactive properties has encouraged interest in lactic acid bacteria (LAB) with probiotic attributes such as antimicrobial activity against pathogenic microorganisms antiviral activity], anti-yeast property, antimutagenic, antiplatelet aggregation, and antioxidant attributes etc. In general, it is believed that probiotics help keep up the balance between harmful and beneficial bacteria in the gut thus maintaining a healthy digestive system. The health benefits of probiotics have always been investigated with regard to their capability to sustain their availability, viability, digestibility, and rendering of their health benefits to the host without altering the safety and the organoleptic properties of the food in which they have been incorporated. Many viable probiotic strains with beneficial functional properties are available in the market as components of foods and beverages, in fermented dairy products like yogurt or as probiotic fortified foods as well as food preservatives.

The health claims of probiotics range from regulation of bowel activity and well-being to more specific actions such as the antagonistic effect on the gastroenteric pathogens like *Clostridium difficile, Campylobacter jejuni, Helicobacter pylori and Rotavirus* targeted. The mechanism of action of probiotics with anti-microbial properties is maybe due to:

1. Production of bacteriocins such as nicin for lowering the pH by producing acidic compounds like lactic acid.
2. Compete with other infectious bacteria for nutrients and cell-surface and help toward them off by inhibiting their colonization.
3. Active enzymes which inhibit other pathogenic bacteria.

Requirements:

LAB strains

MRS media

LB media

Pathogenic strains: *E. coli, S. aureus,* and *B. cereus*

Nutrient Agar plates

Procedure:

Preparation of sample filtrate:

1. Inoculate LAB isolates from slants to fresh 250 ml MRS broth and incubate at 37^{o}C for 48 hours.
2. Centrifuge the culture broth at 10,000 × g for 10 minutes.
3. Collect the supernatant after centrifugation and pass through 0.2 µm sterile syringe filter to obtain cell-free supernatant broths.

Growth of pathogens:

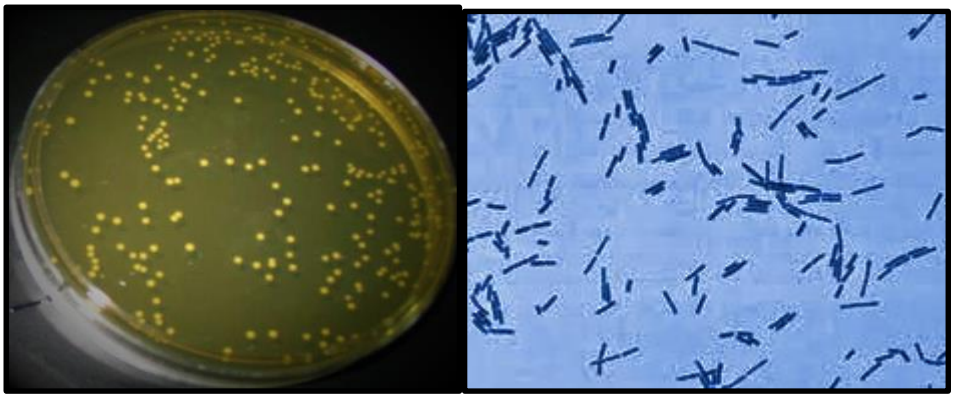
1. Inoculate pure cultures of foodborne pathogens, namely *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus* from slants to brain heart infusion broth and incubate at 37 ^{o}C for 24 hours.
2. Suspend the cell pellet in 9 ml saline solution.

Antimicrobial activity test by agar well diffusion method:

1. Prepare a lawn of the test microorganisms by spreading the cell suspension over the surface of nutrient agar plates with a sterile cotton swab.
2. Dry the plates and cut uniform wells using a sterile cork borer of diameter (5 mm)
3. Fill each well with the cell-free filtrate obtained from the LAB (60 µl) and incubate at 37^{o}C for 48 hours
4. Observe for the zone of inhibition in mm by ruler.

Observation:

Observe for the zone of inhibition and measure the diameter



Lactic acid Bacteria as probiotics: colony morphology and Shape (bacilli)

Chapter - 19
Cultivation of economically important microbes: Leavening agents

The use of yeast to make bread and alcohol has been recorded for thousands of years. Yeast, especially *Saccharomyces cerevisiae, have* been selected for decades for their dough-leaving characteristics. The yeast produces carbon dioxide that results in dough leavening and contributes to the flavor and crumb structure of bread. *S. cerevisiae* is capable of fermenting all sugars present in the dough, for example, glucose, fructose, sucrose and maltose with 8 times faster than other organisms.

Burrows (1970) listed four functions of yeast in bread making:

1. to increase dough volume by evolution of CO_2 during fermentation of the available carbohydrates in the flour
2. to develop structure and texture in the dough by the stretching due to expansion of gas bubbles
3. to improve flavor and
4. to add some nutritive values of bread.

Most ethanol for human consumption as beer or wine is produced by two common strains yeast, *S. carlsbergensis* and *S. cerevisiae*, which are characterized as bottom and top yeast fermentation respectively.

Experiment 54

Aim:

Isolation of yeast from grapes as leavening agent

Principle:

Yeast is a unicellular fungus, mostly classified under Ascomycetes. Yeast cells are usually spherical, oval or cylindrical and cell division generally takes place by budding. Yeasts usually flourish in habitats where sugars are present in abundant quantities such as in fruits, flowers and bark of trees.

The well known industrial uses of yeast are the production of alcoholic beverages and baking. Most important commercial yeasts are the bakers' and brewers' yeast which belong to the genus *Saccharomyces*; because they are easily manipulable eukaryotic cells. Strains of *Saccharomyces cerevisiae* are of greatest technical importance among known types of yeasts.

Requirements:

Grapes
YPS medium (Yeast extract -Peptone -Sodium succinate)
Sterile distilled water blanks (99ml & 9ml)
Sterile petri plates and pipettes.

Composition of YPS medium:

Yeast extract:	3g
Peptone:	3g
Sodium succinate:	2 g

Calcium chloride:	0.02g
Magnesium sulphate:	0.02g
pH:	6.8
Agar:	20g
Distilled water:	1000 ml

Procedure:

1. Weigh one gram sample of grapes, crush and dilute it in a 250 ml flask containing 99 ml of sterile water; this makes 10^{-2} dilution.
2. Shake well for 10- 15 min at room temperature to bring the surface microbial cells dissolved in water.
3. Transfer 1 ml of the aliquot from 10^{-2} dilution to 9 ml sterile water blank using a sterile pipette which gives 10^{-3} dilution.
4. Make successive dilutions, in the same way, using 9 ml sterile water blanks so as to get dilutions up to 10^{-5}.
5. Transfer aseptically 1 ml of the sample dilution from 10^{-3} and 10^{-4} to sterile Petri plates. Maintain three replicates for each dilution in order to minimize handling error.
6. Now pour the melted and cooled media (YPS) into the Petri plates; immediately rotate the plates gently both in clockwise and anti-clockwise direction in order to spread the sample dilution evenly with the medium.
7. Incubate the plates in an inverted position at room temperature for 2 to 7 days.

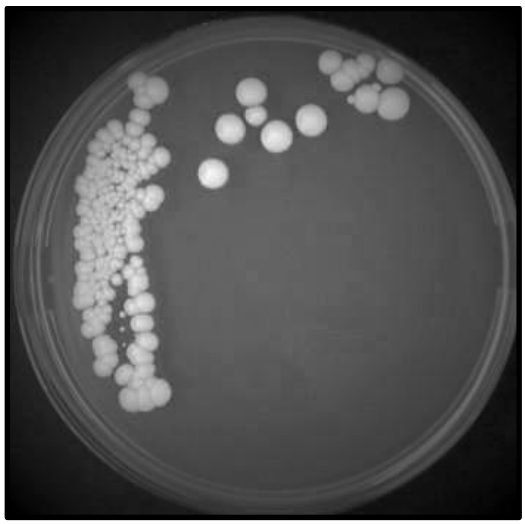
Yeast colonies appear as large, smooth and glistening colonies compared to bacterial colonies.

Observation:

Observe the plates for colony formation. Count the number of colonies per plate and find out the mean cfu for each dilution.

No. of yeast cells (CFU) per gram of the sample = Mean no. of CFUs' x dilution factor

Observe the colony morphology on the plates.



Yeast morphology

Experiment 55

Aim:

Study of yeast *Saccharomyces cerevisiae* as leavening agent in Bread making.

Principle:

Bread making by fermentation is an ancient process. Yeast, the most common one being*S. cerevisiae*, is used in baking as a leavening agent, where it converts the fermentable sugars present in the dough into carbon dioxide. This causes the dough to expand or rise as gas forms pockets or bubbles. When the dough is baked, the yeast dies and the air pockets "set", giving the baked product a soft and spongy texture. Most yeasts used in baking are of the same species common in alcoholic fermentation. In addition, *Saccharomyces exiguus* (also known as *S. minor*), a wild yeast found on plants, fruits, and grains, is occasionally used for baking. In bread making, the yeast initially respires aerobically, producing carbon dioxide and water. When the oxygen is depleted, anaerobic respiration begins, producing ethanol as a waste product; however, this evaporates during baking. Thus the leavening property of yeast depends on its metabolic activity to:

1. Produce CO_2
2. Sugar metabolism especially maltose

CO_2 production:

Yeast utilizes the hexose sugars, especially maltose, to produce CO_2, ethanol, and a variety of secondary metabolites such as esters, aldehydes, and amino acids that contribute to the development of flavor and aroma of the fermented food. The carbon dioxide thus produced is responsible for not only increasing the volume of dough (leavening process) through gas incorporation but also a valuable addition to the flavor and texture. Besides, the fermentation products like vitamins and amino acids are responsible for the health and nutritional benefits that are obtained from bread.

When yeast is used for making bread, it is mixed with flour, salt, and warm water or milk. The dough is kneaded until it is smooth and then left to rise, sometimes until it has doubled in size. Some bread doughs knock back after one rising and left to rise again by kneading. Longer rising time gives better flavor, but if it is left for too long initially, the yeast can fail to raise the bread in the final stages. The dough is then shaped into loaves, left to rise until it is to the correct size, and then baked.

Amylolytic activity:

Wheat flour dough contains different sources of fermentable sugars. There are some free saccharides naturally present in wheat flour. Their amounts are, however, relatively low ranging from approximately 0.05% (for glucose, fructose, and maltose) to 0.2% to 0.3% (for sucrose and raffinose). In wheat bran, however, relatively high sucrose concentrations (1.75% to 3.0%) are present. Therefore, the wholemeal contains higher concentrations of free saccharides than flour. Indeed, sucrose concentrations of 0.9% and 1.1% were reported for wholemeal dough.

Because of the relatively low amount of free saccharides in wheat flour, the majority of fermentable sugars in the dough are generated by degradation of damaged starch by amylases. Damaged starch refers to starch granules that are damaged during milling, and its amount ranges

from 5% to 8% (flour basis) in hard wheat flours obtained by roller milling. Damaged starch is degraded to the fermentable sugar maltose by amylases that act on the α-(1,4)- and/or α-(1,6)-linkages of the starch polymers. Two types of amylases are present in wheat flour: α-amylases and β-amylases. α-Amylases are endo-amylases that hydrolyze the α-(1,4)-linkages inside the starch chain more or less randomly, thereby generating oligosaccharides and α-limit dextrins. β-Amylases are exo-acting enzymes that cleave maltose from the non-reducing end of the starch chain. The ability of a flour-water suspension to produce maltose is known as the amylolytic activity of the flour. In unyeasted dough samples, the enzymatic degradation of damagedstarch leads to a very fast increase of maltose levels during mixing and the first minutes of incubation (0.1% in flour to 1% after mixing and 2% after 180 min of incubation).

Maltose levels in dough depend on the total damaged starch content and the α-amylase activity of the flour. Doughs prepared from flours with higher α-amylase activities or higher damaged starch contents will, therefore, contain higher maltose levels. Since α-amylase activity is often limited in wheat flour, fungal α-amylase or α-amylase from the malt is often added to wheat flour to increase the level of fermentable sugars in the dough. Hence it is essential for efficient amylase producing yeast strains for dough leavening.

Requirements:

Wheat flour:	1.0kg
Dehydrated yeast:	30g
Salt:	15g
Sugar:	200g
Water:	500-600 ml
Sterile petri plates	
Dropper	
Inoculating loop	

Composition of YPD broth:

Peptic digest of animal tissue:	20.0 g
Yeast extract:	10.0 g
Dextrose:	20.0 g
Distilled water:	1000 ml.
pH:	6.5

Starch agar medium (pH 7.0):

Starch (soluble):	20.0g
Peptone:	5.0g
Beef extract:	3.0g
Agar:	15.0g
Distilled water:	1000.0 ml

Gram's iodine solution

Procedure:

Yeast cultivation and Dough Leavening

1. Culture given yeast strains in conical flasks (250 ml media) in YPD medium
2. Incubate in the shaker at 30 °C for 72 hours

3. Collect yeast pellets after centrifugation at 10,000 rpm 5 min.
4. Weigh 50-gram wheat flour and mix with 1% salt
5. Dissolve 6% sugar in lukewarm water and add 0.6 g yeast pellets in the sugar solution to allow its activation.
6. Pour the activated yeast solution in the flour and mix well.
7. Use the dough without any yeast as a negative control.
8. Incubate the dough at 30 °C for 2 hours for proofing.
9. In order to assess the rise of the dough level, take 10 g of the dough mixture in measuring cylinder.
10. Incubate and note the level at every half hour (Karki et al., 2017).
11. Bake in a hot air oven at 180 °C for 20 minutes.

Amylolytic activity

1. Melt the starch agar medium, cool to 45 ^{o}C and pour into sterile Petri plates. Allow it to solidify.
2. Label each of the starch agar plates with the name of the organism to be inoculated.
3. Make a single streak of each organism into the centre of the respective plate aseptically.
4. Incubate the bacterial plates for 48 hours and fungal plates for 72-96 hours in an inverted position.
5. Flood the surface of the plates with iodine solution with a dropper for 30 seconds · Pour off the excess iodine solution.

Observation:

Compare the dough raising in yeasted and un-yeasted wheat flour. Examine the plates for the starch hydrolysis around the line of growth of each organism, *ie.*, the colour change of the medium.

Wheat flour kneaded with yeast:A. unyeasted dough; dough raised in volume B &C

Chapter - 20
Cultivation of economically important microbes: Wine

Wine is a product of alcoholic fermentation of fruit juices that are rich in fermentable sugars. It is a primary metabolite. Primary metabolites are the intermediary products produced by microorganisms during the primary growth phase and these are necessarily required for the growth of microbes. A typical microbial process in which the product is formed during the primary growth phase is alcohol (ethanol) fermentation. Ethanol is a product of anoxic metabolism of yeast and certain bacteria and is formed as part of energy metabolism. The process of preparation of wine is known as **vinification** and the branch of science that deals with study of wine is known as **enology** (American) or **oenology** (British).

Most fruit juices undergo natural fermentation caused by wild yeasts (*Saccharomyces ellipsoideus*) present on the fruit. From this, yeasts have been selected for more controlled production, and today alcoholic beverage production is one of the largest industries worldwide. The most important alcoholic beverages are wine, produced by fermentation of fruit juice; beer, produced by fermentation of malted grains, and distilled beverages produced by concentrating alcohol from fermentation by distillation.

Experiment 56

Aim:

Production of wine from grapes

Principle:

Most wines are made from grapes only. Wines are divided into 1. Dry wine - in this all sugars are perfectly fermented, 2. Sweet wines - in this some of the sugars are left unfermented or additional sugar is added after fermentation, 3. Fortified wines - in this brandy/alcoholic spirit is added after fermentation and 4. Sparkling wine - in this considerable CO_2 is present arising from final fermentation.

Most of the wines have alcoholic content of 11-12 per cent. Whereas, the dessert wines contain maximum alcoholic content of 19-21 per cent.

Requirements:

Graduated cylinder
Test tubes
Glass container
Rubber stopper
Tygon tubing
Hydro meter
Balance
Ripened grape fruits
Sucrose
Active dry wine yeast, strains of *Saccharomyces ellipsoideus*

Procedure:

Step I: Harvesting of fruits:

1. Appropriate variety of fruits and berries are harvested.
2. They must contain high amounts of fermentable sugars.
3. Grapes usually contain 5-25% total soluble sugar (Total soluble sugar- TSS).

Step II: Crushing and extraction:

1. Thus, obtained fruits are crushed and extracted mechanically.
2. This process releases juice and a little bit of pigment.
3. The whole mass is known as Must.
4. For white wine preparation, the skin is removed. The harvested fruits are de-steamed for white wine preparation which is not required for red wine preparation.
5. In the case of red wine, the steam gives a vegetable aroma due to the presence of 2 methoxy-3-isopropyl pyrazine.
6. Color is also extracted from steam.
7. In the case of red wine, the Must should be fermented.

Step III: Optimization:

1. The must is optimized for two parameters, TSS and pH.
2. The TSS is generally optimized between 17-22% and pH in between 3-4, depending on yeast strains to be used.
3. KNS (potassium metabisulphite) may or may not be added at this stage which is an antimicrobial compound against *Acetobacter* spp. and competitive yeast.
4. It also acts as an antioxidant and antifungal agent.

Step IV: Primary fermentation:

1. The optimized Must is inoculated with 2-10% of inoculum and fermentation is carried out under optimum temperature.
2. Red wine preparation= 22-27 ^{o}C for 3-5 days
3. White wine preparation= 10-21 ^{o}C for 7-14 days
4. During the fermentation, the content is mixed twice a day by punching the floating skin for proper aeration.
5. It also helps in color extraction.
6. This fermentation allows rapid multiplication of yeast cells as well as sugar fermentation to ethanol, when the TSS is decreased nearly about 9-10% then primary fermentation is terminated.

Step V: Pressing:

1. The skin of the must is taken out and pressed in order to release juice and alcohol.
2. The liquid is again transferred into the tank.
3. In case of white wine, pressing is carried out before fermentation.
4. During pressing the color of fruits and berries is extracted.

Step VI: Heat and cold sterilization:

1. The main aim of this technique is to remove the tartrate crystals (wine diamonds or wine crystals).

2. In the cold sterilization method, the fermented must is cooled to nearly freezing and kept for one to two weeks.
3. During this period, the crystals get separated or stirred in the wall of the fermenter and clear liquid is collected on the secondary fermented tank.
4. In heat stabilization technique, it is gently heated in between 50-60 ^{o}C for an hour and kept overnight.
5. The proteins get decanted. The clear contents are pumped out and the remaining turbid substance adsorbed on to bentonite.

Step VII: Secondary fermentation:

1. It is carried out in a stainless steel or oak barrel or concrete tank lined with plastic.
2. The stabilized, sterilized wine is now kept at 15-20 ^{o}C for 3-6 months under strict anaerobic conditions. Usually in case of sweet wine, the fermentation is terminated when sugar content is reduced to 4-6%.
3. During secondary fermentation, aroma is developed.
4. The aroma in wine is categorized into 3 types:
    a. Primary aroma - contributed by fruits or berries
    b. Secondary aroma - developed during secondary fermentation
    c. Tertiary aroma - developed during bottled ageing
5. The aroma compound may be volatile or non-volatile.
6. It is developed due to chemical reactions among acids (malic acid, citric acid etc), sugars, alcohols and phenolic compounds.

The main compound responsible for aroma is methoxypyrazine, monoterpenes, nor-isoprenoids, thiols, esters etc. among which ester is the principal one. Esters are produced by reaction between alcohols and acids which is very slow. It takes nearly one year for secondary fermentation. Before secondary fermentation malo-lactic fermentation occurs.

Malic acid (sharp sour) - Lactic acid bacteria (LAB) → Lactic acid

Step VIII: Laboratory testing:

After secondary fermentation, certain laboratory tests are conducted which includes bricks reading, bricks pH, titratable acidity, residual sugars, free or available sulfur, total sulfur, volatile acidity and alcohol percentage.

Step IX: Blending and fining:

1. It is most crucial to produce good quality wine having a special taste and aroma.
2. In the blending process, spices, extracts of aromatic plants, essential oils, fruit juices and other things are added in appropriate proportions.
3. Blending is kept trade secret in winery (wine industry).
4. In the fining process, tannins and microscopic particles are removed in order to make clear wine. For this purpose, wine is treated with gelatin, potassium caseinate, egg albumin, lysozymes, skimmed milk powder etc. or it is filtered through membrane filter or diatomaceous earth cellulose filter.
5. Finally, wine is clarified in order to remove pectin which is achieved with the use of pectinase enzyme.

Step X: Preservation:

1. Pasteurization technique and use of KMS (Potassium metabisulphite) are mainly used for preservation.
2. It kills sugar utilizing microorganisms.

Step XI: Bottling:

1. Finally, wine is aseptically filled in a bottle and the bottle is corked, which is usually made with oak.
2. Finally, the outside cork is sealed.
3. The bottled wine can be directly consumed or preserved.

DISTILLATION OF WINE:

These are the alcoholic beverages/drinks obtained by the distillation of wine or fermented cereals. It may be aged or unaged (i.e. the distilled liquor). Distilled liquor is commonly called spirits. They consist of more than 40% ethanol.

There are various types of distilled liquor.

The primary types are:

Whisky (Barley and others) → aged

Brandy (Wine distillation) → aged

Rum (fermented molasses) → aged

Vodka (fermented cereals) → not aged

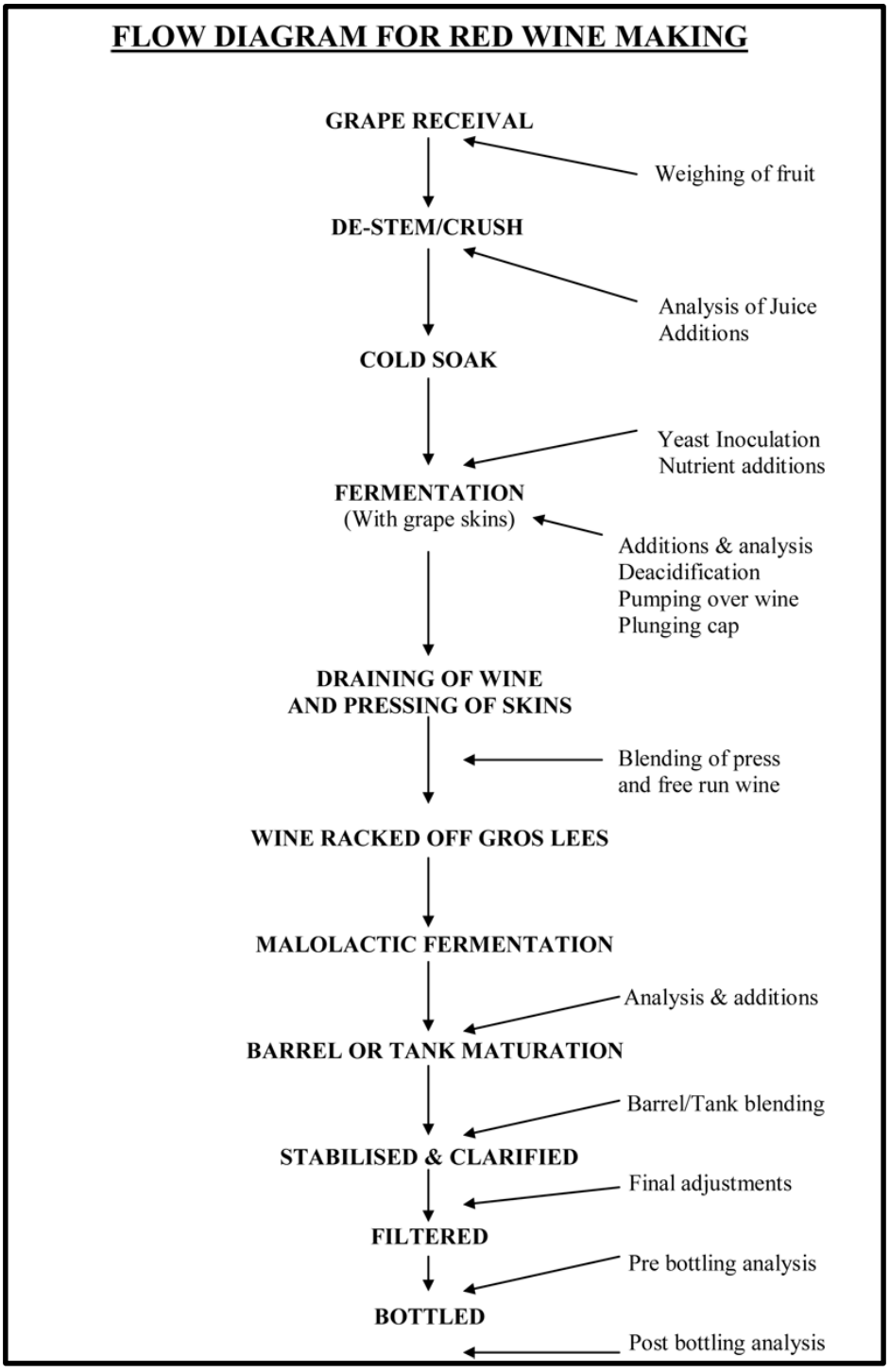
Gin (distillation of fermented cereals) → unaged but flavored

Composition of Must (approximately as follows (Haas, 1976)

1. Sugars (glucose and fructose) 15-25 %
2. Acids (Mainly tartaric and malic) 0.9-1.5 %
3. Tannins (for white wine) 0.02 – 0.04 %
4. Tannins (for red wine) 0.1 – 0.25 %
5. Pectins 0.1 – 0.15 %
6. Ash 0.30 – 0.50%
7. Aroma substances Trace
8. Nitrogenous substances Trace
9. Higher alcohols Trace
10. Aldehydes Trace

Typical composition of a wine

1. Extract (total solids) 2-3 %
2. Carbohydrates 0.03 – 0.5 %
3. Acids 0.5 –1 %
4. Ash 0.15-0.3 %
5. Amino acids Trace
6. Tannins Trace
7. Aroma substances Trace
8. Alcohol 6-9 wt. 8-13 vol



Observations:

Observe the wine for color, taste, flavor, pungency, clarity, etc.

Organoleptic Tests

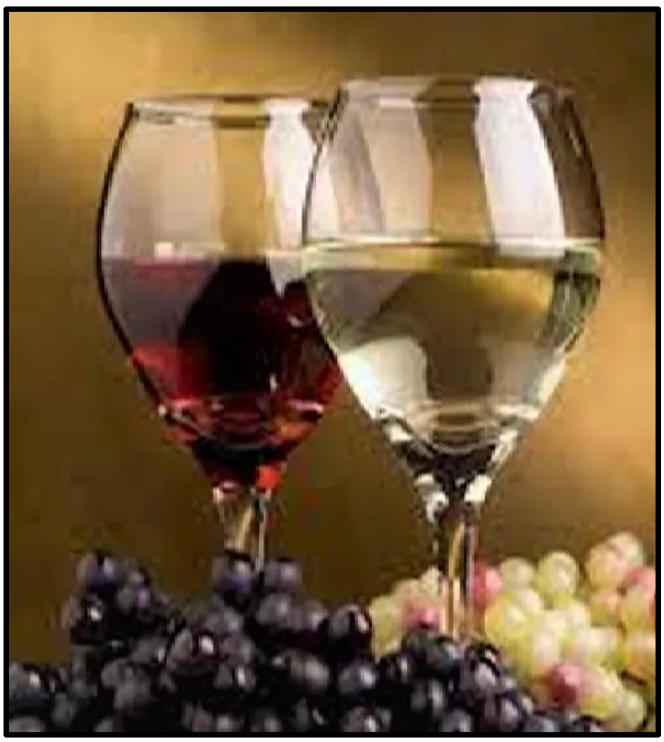
Appearance: White wines that have become brown are usually oxidized and over-aged in odor. A silky 'wavy' sheen in a hazy cloudy white or red dry wine accompanied by a characteristic odor is unmistakable evidence of bacterial spoilage. If red wine is high in acid, it will have a bright color. The older the wine, the more the red color shifts towards the brown or purplish-red. The old port is apt to be tawny in color; young port red or purplish-red. Angelica and Muscatel

should be light gold or golden amber in color. Madeira is light amber to brown, Chablis and Riesling should be pale yellow, Sauterne more so. Sherry may range from very pale to a dark amber, depending on the type.

Odour: Tasters should distinguish between bouquet and aroma. They designate bouquet as the odors developed by the wine in normal aging through esterification, oxidation, etc. and aroma as the odor derived from the fresh grapes. The age of the wine greatly affects the bouquet and with experience, the taster can tell much about the age of the wine by its bouquet. Slight vinegar souring or lactic souring can be detected.

Taste: Following factory examination, one proceeds to the evaluation of the taste. The four aspects of taste are sour, sweet, salty and bitter. The sour or acid taste is of value for all wine types, particularly table wines. A low acidity gives a wine of q flat or insipid taste. Sweetness is the most important for sweet table and dessert wines but is also especially critical for sparkling wines. Red wines all have some bitter taste.

Flavor: Flavour is made up, in part of bouquet and aroma and in part by taste evident chiefly to the tongue.



Red and White Wine

Chapter - 21
Cultivation of economically important microbes: SCP

Single-cell protein (SCP) is a crude, or a refined or edible protein extracted from pure microbial cultures, dead, or dried cell biomass. They can be used as a protein supplement for both humans or animal feed. Microorganisms like algae, fungi, yeast, and bacteria have very high protein content in their biomass. These microbes can be grown using inexpensive substrates like waste sulfite liquor from paper industries, waste from sugar industries, agricultural waste viz. wood shavings, sawdust, corn cobs etc. and even human and animal waste. A list of the microorganisms used for the production of Single Cell Protein is as follows:

Fungi

1. *Aspergillus fumigatus*
2. *Aspergillus niger*
3. *Rhizopus cyclopean*

Yeast

1. *Saccharomyces cerevisiae*
2. *Candida tropicalis*
3. *Candida utilis*

Algae

1. *Spirulina (sp)*
2. *Chlorella pyrenoidosa*
3. *Chondrus crispus*

Bacteria

1. *Pseudomonas fluorescens*
2. *Lactobacillus*
3. *Bacillus megaterium*

SCP is produced by a number of microbial species, however when considering human consumption microbial species are sparse. The range of sources for SCP used in animal feed is broader than that approved for human consumption and is expanding. SCP for human consumption cannot be produced is generally produced from inexpensive waste materials from the food and beverage processing industries, as well as directly from forestry and agricultural sources it has to be produced from food grade substrates, and therefore SCP production for human consumption is an expensive process.

SCP from Algae:

Microalgae which are produced for human or animal consumption typically have high protein content (e.g., 60–70%). They also provide fats (with ω-3 fatty acids and carotenoids being of particular interest), vitamins A, B, C, and E, mineral salts, and chlorophyll. They have relatively low nucleic acid content (3–8%).

SCP from Fungi:

A wide range of fungi have been considered for the production of SCP, such as from*Saccharomyces, Fusarium*, and *Torulopsis*. Fungi grown as SCP contains 30–50% protein.

In addition to protein, it provide vitamins primarily from the B-complex group (thiamine, riboflavin, biotin, niacin, pantothenic acid, pyridoxine, glutathione, folic acid, and p-amino benzoic acid). The cell walls of fungi are rich in glucans, which contribute fibre to the diet. Fungi have a moderate nucleic acid content (7–10%) however, it is too high for human consumption and requires processing to reduce it.

SCP from Bacteria:

Bacteria also have a long history of use as SCP, particularly in animal feed.Many bacteria such as *Bacillus cereus, Bacillus licheniformis, Bacillus pumilus, Bacillus subtilis, Corynebacterium glutamicum, E. coli, Methylococcus capsulatus, Methylomonas sp.* are commercially used for SCP production. Bacterial SCP generally contains 50–80% protein on a dry weight basis and the essential amino acid content is comparable or higher than the FAO recommendations. The Methionine content (3.0%) is higher than obtained from algal or fungal SCP. Similar amino acid composition is shown with methanol or methane grown bacteria. The bacterial SCP has high nucleic acid content (8–12%), especially RNA, and thus requires processing prior to usage as food/feed. In addition to protein and nucleic acid, bacterial SCP provides some lipids and vitamins from the B group of vitamins.

Imperial Chemical Industries developed a SCP (Pruteen) for animal feed from methanol, using the bacterium *Methylophilus methylotrophus*. Pruteen contained up to 70% protein and was used in pig feed.

Processing of SCP

Depending on the substrate material and intended food/feed application, various processing steps are required prior to formulation of the final SCP product. In the following section we review the most relevant processing needs for SCP.

a. Cell Wall Degradation in Single Cell Protein Products:

Some SCP are used as whole cell preparations, while in others the cell wall may be broken down to make the protein more accessible. SCP, such as Quorn™, may be consumed without degradation of the cell wall, in which case chitin and glucan from fungal cell walls contribute fibre to the diet. SCP derived from *Euglena* does not require disruption since the cells have proteinaceous pellicles, rather than cell walls, making it more readily digestible.

Various methods have been used to disrupt the cell wall, including mechanical forces (crushing, crumbling, grinding, pressure homogenization, or ultra-sonication), hydrolytic enzymes (endogenous or exogenous), chemical disruption with detergents, or combinations of these methods. Cell disruption may affect the quality and quantity of protein and other components in the SCP. Products such as Marmite® and Vegemite® are cell extracts, generated by heating the cells to 45–50 °C long enough for intracellular enzymes to partially hydrolysed the cell wall; the proteins are also reduced to smaller peptides.

b. Nucleic Acid Removal in Single Cell Protein Products:

Although algae generally have low nucleic acid content, the rapidly proliferating bacterial and fungal species have high nucleic acid (RNA) content. RNA content and degradation are affected by growth conditions, growth rate, and the carbon-nitrogen ratio. When SCP is produced for human consumption, high nucleic acid content is a problem because ingestion of purine

compounds derived from RNA breakdown increases uric acid concentrations in plasma, which can cause gout and kidney stones.

Different methods have been developed to decrease the RNA content in SCP. Endogenous RNA degrading enzymes (ribonucleases) can be used in degradation of RNA, after activation with heat treatment (60–70 °C) as used in the production of Quorn™. Ribonucleases can also be added to the process or used as immobilized enzymes. Alkaline hydrolysis and chemical extraction methods have also been studied. An alkali treatment to reduce RNA in *P. varioti* biomass, used in for Pekilo-process, below 2%. Treatment at 65 °C, pH 7.5–8.5, to activate endogenous ribonuclease, also reduced the RNA content to <2%, while the protein content stayed at 50%.

c. Safety of SCPs:

As for any food or feed product, SCP needs to be safe to produce and use. Regulations exist in most regions to ensure that food or feed are safe for consumption. Typically these distinguish not only between food (for humans) and feed (for animals), but also between food (providing nutrition and potentially taste and aroma) and food additives (preservatives, colourants, texture modifiers, etc.), or feed and feed additives. The international standards, regulated through the Joint FAO/WHO Expert Committee on Food Additives are to be followed, (WHO, 2017). Regulations differ depending on the intended purpose of the product, and although SCP is expected to be either food or feed (providing nutrition), some products may enter the market as additives (e.g., providing colour), rather than as SCP, even though protein is present in the product, limiting the extent to which they are added and their value as SCP.

Bacteria may also produce toxins which limit their use as SCP. Toxins may be extracellular (exotoxins) or cell bound (endotoxins). For example, both *Pseudomonas* spp. and *Methylomonas methanica* produce high levels of protein and have been assessed for use as SCP. Both also produce endotoxins that cause febrile reactions. These can be destroyed by heating. Further, a study on immunogenicity of SCP from *M. capsulatus* showed that the cell-free preparation (i.e., the cell wall is removed) did not cause immune responses in mice, although whole cell preparations did.

Production of SCP:

The production of single cell protein is usually carried out in a fermentation process. This is done by selected strains of microorganisms which are multiplied on suitable raw materials in a technical cultivation process directed to the growth of the culture and the cell mass followed by separation processes. Process development begins with microbial screening of high yielding strains, in which suitable production strains are obtained from samples of soil, water, air or from swabs of inorganic or biological materials and are subsequently optimized by selection, mutation, or other genetic methods. Then the optimal parameters of cultivation for the strains are optimized and all metabolic pathways and cell structures determined. Besides, process engineering and technical aspects of the process are designed to make the production ready for use on a large scale. The economic factors (energy, cost) are taken into consideration. Safety demands and environmental protection are also considered in the production of SCP both in relation to the process and to the product. Finally, safety and the protection of innovation throw

up legal and controlled aspects, namely operating licenses, product authorizations for particular applications and the legal protection of new processes and strains of microorganisms.

Fermentation process:

The fermentation process requires a pure culture of the microorganism that is in the correct physiological state, sterilization of the growth medium for the mass production of organism, a production fermenter used for drawing the culture medium in the steady state, cell separation, collection of cell free supernatant, product purification and effluent treatment.

A bioreactor is different from a fermenter as it is used for the mass culture of microorganisms. The chemical compounds synthesised by these cultured cells such as therapeutic agents can be extracted easily from the cell biomass. The design engineering and operational parameters of both fermenters and bioreactors are identical. Fermenters and bioreactors are also equipped with an aerator, which supplies oxygen to aerobic processes; a stirrer is used to keep the concentration of the medium the same. A thermostat is used to regulate temperature and a pH detector and some other control devices, which keep all the different parameters needed for growth constant.

For the production and harvesting of microbial proteins, cost is a major problem. Such a production even at a high rate causes dilute solutions usually less than 5% solids. There are many methods available for concentrating the solutions like filtration, precipitation, centrifugation and the use of semi-permeable membranes. The equipment used for these methods of dewatering is expensive and so would not be suitable for small scale productions and operations. The removal of the amount of water that is necessary to make the material stable for mass storage is not economically viable. Single cell proteins need to be dried to 10% moisture or they can be condensed and denatured to prevent spoilage. The physiological features of microbial organisms recommend the control of the carbon source concentrations, as a limiting substrate, as well as an adequate supply of oxygen for the maintenance of balanced growth under an oxidative metabolic pattern. However, since microbial growth is a time dependent process, it exerts continuous modifications on all process parameters which influence physiology, but most dramatically, over substrate concentration. Therefore, an adequate technology which maintains appropriate growth conditions for a prolonged period of time must be implemented specifically for the purpose of obtaining high yield and productivity values.

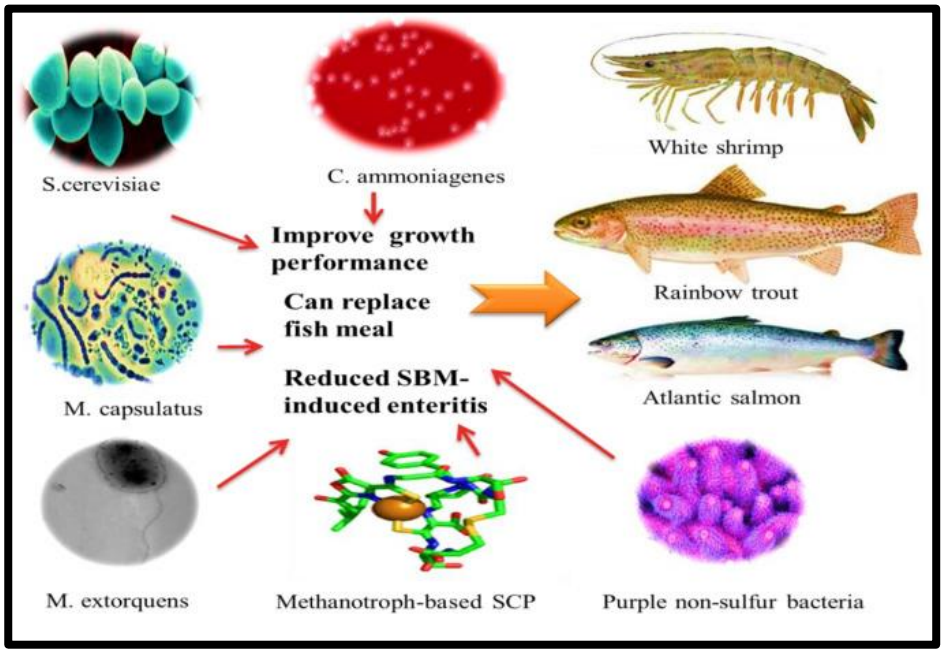
Batch fermentations are not inadequate for the purpose of biomass production, since the conditions in the reaction medium change with time. Fed-batch fermentations are better suited for the purpose of biomass production, since they involve the control of the carbon source supply through feeding rates. However, as the biomass concentration increases, the oxygen demand of the culture reaches a level which cannot be met in engineering or economic terms. The most commonly used principle has been the chemostat: a perfectly mixed suspension of biomass into which medium is fed at a constant rate and the culture is harvested at the same rate so that the culture volume remains constant. A common problem of industrial fermentations is the profuse appearance of foam on the head space of the reactor, causing reactor pressurization, spillages and contamination hazard. Among the various designs Air-lift has enjoyed the greatest success as the configuration of choice for continuous SCP production. This is presently used in the production of myco-protein which is the basis for Quorn™ products. The control of key process variables is

a critical element of SCP production, from oxygen transfer, substrate and product concentration, to the appearance of minimal amounts of toxic compounds through undesired metabolic processes, which may compromise the quality of the final product.

The biomass from yeast fermentation processes is harvested normally by continuous centrifugation. Filamentous fungi are harvested by filtration. The biomass is then treated for RNA reduction and dried in steam drums of spray dryers. Drying is expensive, but results in a stabilized product with shelf lives of years.

Solid State Fermentation (SSF) is growth of microorganisms on predominantly insoluble substrates where there is no free liquid. Generally, under combined conditions of low water activity and presence of intractable solid substrate, fungi show luxuriant growth. Hence, proper growth of fungi in SSF gives much higher concentration of the biomass and higher yield when compared to submerged fermentation. The advantage in the SSF process is the unique possibility of efficient utilization of waste as the substrate to produce commercially viable products. The process does not need elaborate pre-arrangements for media preparation. The process of SSF initially concentrated on enzyme production. But presently, there is worldwide interest for (SCP) production due to the dwindling conventional food resources.

In such a continuous operation for SCP production the economics of this production must be strongly taken into account. The economic factors that should be taken into account during this fermentation period are: Investment, Energy, Operating costs, Waste, Safety and the Global market.



SCP and its uses

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Chapter - 22
Cultivation of economically important microbes: Mushrooms

Pleurotus sp. are commercially important edible mushrooms commonly known as the oyster mushroom. Cultivation of oyster mushrooms has recently increased tremendously throughout the world because of their abilities to grow at a wide range of agro-based residues. *Pleurotus sajor-caju* is one of the most successfully cultivated species of these mushrooms and it is considered to be very delicious. Mushrooms have long been used in medicine and food. *Pleurotus* species contain high potassium to sodium ratio, which makes mushrooms an ideal food for patients suffering from hypertension and heart diseases. Agriculture residues are the major source of lignocellulosic materials, which is the best substrate for solid state fermentation of edible fungi such as *P. sajor-caju*. The mushroom production gives additional income to farmers by utilizing byproducts or co-products from other crops. In addition, mushrooms are an excellent source of food to address the problem of malnutrition in developing countries like India. Cultivation of *Pleurotus sajor-caju* is carried out by Solid state fermentation (SSF) process.

SSF promoted higher yields and better product characteristics than cultivation in the liquid medium. In addition, costs are much lower due to the efficient utilization and value addition of wastes. The main drawback of this type of cultivation concerns the scale-up of the process, largely due to heat transfer and culture homogeneity problems. SSF processes generally employ a natural raw material as carbon and energy source; the nature of the solid substrate is one of the most important factor affecting SSF processes and its selection depends on several factors mainly related factors cost and availability. The substrates normally used are cellulose, sugars, and starch material nitrogen sources (ammonia, urea, peptides) and mineral salts. Paddy straw is lignocellulosic waste material and the major constituents of lignocellulose are cellulose, hemicelluloses, and lignin. Lignocellulosic waste material yields a variety of products *viz.*, enzymes, flavors, organic acids, single-cell protein, and bioactive compounds.

Experiment 57

Aim:

Solid-state fermentation using cellulose waste material (paddy straw) for SCP(Mushroom) production using *Pleurotus sajor-caju.*

Principle:

Pleurotus sajor-caju is a variety of mushroom usually grown on paddy straw. Paddy straw is a lignocellulosic material comprising roughly about 35-55% cellulose, 20-40% hemicellulose and 10-25% lignin. This agricultural by-product, rich in cellulose, can be effectively utilized for the production of value-added products by the action of certain microorganisms possessing the enzyme cellulase. One good example is the fermentation of cellulosic materials present in paddy straw by the fungus *Pleurotus sajar-caju* resulting in the production of proteinrich mushroom.

Requirements:

Pure culture of the mushroom fungus, *Pleurotus* sp.
Potato Dextrose Agar medium (PDA)
Sorghum or wheat grains (for spawn)
High density polypropylene bags (30x15cm size and 150 gauge thick) / saline bottles (for spawn production)
Calcium carbonate
Non-absorbent cotton
Polythene bags (60x30cm or 75x45cm)
Processed paddy straw

Procedure:

Spawn production

1. Select healthy and whole sorghum grains, soak the grains in water and remove the chaffy and damaged grains which float on the water.
2. Half cook the grains for 30 minutes in boiling water to soften them. Grains should not be sticky but should slightly break.
3. Drain the excess water, spread the grains over a clean cloth to dry them (approx. 30 min).
4. For every one kg of grains, mix 20g of calcium carbonate thoroughly (to adjust the pH of the grains, to remove excess moisture and to prevent the grains from sticking together).
5. Fill the grains in clean glucose/saline bottles or in poly bags (250-300g), plug them withnon-absorbent cotton, cover with aluminum foil.
6. Autoclave the bottles/bags at 1.42 kg/cm^2 pressure for 90 minutes.
7. Cool them to room temperature and inoculate with a pure culture of *Pleurotus* fungus aseptically.
8. Incubate the bags for 10-15 days to obtain complete growth of white mycelium covering the entire spawn bag. This is known as the **Mother Spawn.**
9. Use the mother spawn within 25-35 days for making further generations known as the**Bed Spawn**.
10. From each mother spawn bag (18-30 days old),25-30 bed spawn bags can be produced by transferring 10g of spawn from mother spawn bag to bed spawn bag.

Preparation of mushroom beds:

1. Processing of substrate:

1. Cut the raw paddy straw into bits of 3-5cm, soak them in water for 4-6 hours, drainthe water and boil them at 80^0c for one hour (or) treat the straw with steam at 1.2kg/cm^2 for 30-45 min.
2. Drain the water and spread the straw on a clean sac previously soaked in Carbendazim solution or potassium permanganate solution (1g/litre).
3. Shade dry to 65-70% moisture (water should not drip when squeezed by hand). 1 kg of straw when boiled and shade dried weighs about 4-5 Kg.

2. Spawning:

1. Take a polythene bag of the given specification, tie it at the bottom to provide a flat circularbottom.
2. Divide the spawn present in the spawn bag into four equal portions by spreading them evenly in a clean disinfected plastic tray.
3. Spread the paddy straw bits uniformly into the bag to a height of 5cm. Sprinkle one portion of spawn over this, evenly covering the entire surface. Gently press with fingers.
4. Spread a second layer of straw to a height of about 10cm and sprinkle the second portion of the spawn evenly. Give a mild pressing.
5. Similarly, spread the third layer of straw to 10cm height and sprinkle the third portion of spawn.
6. Spread the fourth layer of straw to 10cm height and sprinkle the fourth portion of spawn.
7. Finally, spread paddy straw to 5cm height. Gently press with fingers and tie the bag with a thread or rope.
8. For aeration, make 2-4 vents of one cm diameter at different places of the bag.

Filling mother spawn Cellulosic paddy straw Mushroom fruiting bodies under SSF

3. Spawn running and cropping:

1. After preparation, arrange the cylindrical beds in racks in spawn running room maintained at a humidity of around 80%.
2. Mycelium from the spawn slowly grows and covers the entire bed surface within 15- 25 days depending upon the species used.
3. Until this stage, the water spray is not needed. At the end of spawn run, small pinheads appear through the vents which is the indication of fruiting.
4. Now transfer the beds to the cropping room with more ventilation and aeration. Here open the beds carefully either fully or partly or in splits.
5. Water spraying daily as fine mist is necessary or the growing beds to develop into fruiting bodies (18–45days). The buds turn into mature forms within 24-48 hours.
6. Harvest the mushrooms before the margins roll upward or downwards.
7. The average yield of oyster mushrooms would be approx. 635g/bed (500g of paddy straw) with a good shelf life of up to 72 hours.

Observation:

Observe the bed for the emergence of fruiting bodies, calculate the average yield per bed and estimate the production cost.

Cultivation of Mushrooms: *Pleurotus sajor-caju*

Chapter - 23
Cultivation of economically important microbes: Organic acids

Organic acids are important value-added products that have diverse applications in food, pharmaceutical, and chemical industries, and metabolic engineering and synthetic biology. Citric, lactic, gluconic, and itaconic acid are currently produced by fermentation. Microbial production of acetic acid is important for food applications, in the form of vinegar. Propionic, succinic, and pyruvic acid are not currently being produced commercially by fermentation, but have significant potential for future manufacture by fermentation process.

Citric acid (C6H8O7, 2 - hydroxy - 1,2,3 - propane tricarboxylic acid) is a natural constituent and common metabolite of plants and animals. It is the most versatile and widely used organic acid. The food industry is the largest consumer of citric acid, using almost 70% of the total production followed by about 12% for the pharmaceutical industry and 18% for other applications. This acid was first produced commercially by John and Edmund Storage Company in the UK in the year 1826.

Although methods were developed to synthesize citric acid using chemical means, better successes were achieved using microbial fermentations, and over the period of time, this technique has become the method of ultimate choice for its commercial production. As a result of which much attention has been paid on research to improve the microbial strains, operational parameters and to maintain their production capacity.

Microorganisms used for citric acid production:

A large number of micro-organisms including bacteria, fungi and yeasts have been employed to produce citric acid (table below). Most of them, however, are not able to produce commercially acceptable yields. Among these, only *Aspergillus nige*r and certain yeasts such as *Saccharomycopsis sp*. are employed for commercial production. However, the fungus *A. niger*has remained the organism of choice for commercial production. The main advantages of using this microorganism are:

1. its ease of handling,
2. its ability to ferment a variety of cheap raw materials, and
3. high yields.

For citric acid production by yeast is carried out using carbohydrates and n- alkanes as substrates. Carbohydrates such as beet molasses, cane molasses, sucrose, commercial glucose, starch hydrolysate etc. are used in fermentation medium. The starchy raw material is diluted to obtain 20- 25% sugar concentration and mixed with a nitrogen source (ammonium salts or urea) and other salts. The pH of the fermentation medium is kept around pH 5 when molasses are used and at pH 3 when sucrose is used.

The fermentation is carried out by any of the following processes:

1. **Submerged fermentation process:** It is the process in which the fungal mycelium grows throughout a solution in a deep tank.
2. **Liquid surface culture process/Surface fermentation:** In this case, *A. niger* floats on the surface of a solution.

3. **Kofi process or solid state fermentation:** It is a Japanese process in which special strains of *Aspergillus niger* are used with the solid substrate such as sweet potato starch.

1. Submerged fermentation process:

The submerged fermentation (SmF) process is the commonly employed technique for citric acid production. It is estimated that about 80% of world production is obtained by SmF. Several advantages such as higher yields and productivity and low labour cost are the main reasons for this process.

In SmF, fermentation media are employed to utilize sugars and starch based media (brewery wastes, beet molasses, cane molasses, wood hemicellulose, corn starch, palm oil, coconut oil, soybean oil, glycerol, n-paraffin). Molasses and other raw materials demand pre-treatment (Generally, cane molasses contains calcium, magnesium, manganese, iron and zinc, which have a retarding effect on the synthesis of citric acid), addition of nutrients and sterilization. Inoculum addition is either by adding a suspension of spores, or of pre-cultivated mycelia. When spores are used, a surfactant is added in order to disperse them in the medium. For pre-cultivated mycelia, an inoculum size of 10% of fresh medium is generally required. Normally, submerged fermentation is concluded in 5 to 10 days depending on the process conditions. It can be carried out in batch, continuous or fed batch systems, although the batch mode is the choice.

2. Liquid surface culture process/Surface fermentation:

The liquid surface culture (LSC) was introduced in 1919 by Société des Produits Organiques in Belgium, and in 1923 by Chas Pfizer & Co. in the US. This technique is more sophisticated and the surface method requires less effort in operation, installation and energy cost. The culture solution is held in shallow trays (capacity of 50-100 L) and the fungus develops as a mycelial mat on the surface of the medium. The trays are made of high purity aluminium or special grade steel and are mounted one over another in stable racks. The fermentation chambers are provided with an effective air circulation in order to control temperature and humidity. Fermentation chambers are always in aseptic conditions, which might be conserved principally during the first two days when spores germinate. Refined or crude sucrose, cane syrup or beet molasses are generally used as sources of carbon. When applied, molasses is diluted to 15-20% and is treated with hexacyanoferrate (HFC).

3. Kofi process or solid state fermentation:

Citric acid production by SSF (the Koji process) was first developed in Japan and is the simplest method for its production. SSF can be carried out using several raw materials (sweet potato fibrous residues, rice, and wheat bran, apple pomace, grape pomace, orange peels, kiwifruit peel, coffee husk, carrot waste, soy residue etc). Generally, the substrate is moistened to about 70% moisture depending on the substrate's absorption capacity. The initial pH is normally adjusted to 4.5-6.0 and the temperature of incubation may vary from 28 to 30 °C. The most common organism is *Aspergillus niger*. However, the yeast are also employed using these substrates. The important advantages of the SSF process is trace elements may not affect citric acid production as it does in SmF therefore, substrate pretreatment is not required in Koji fermentations.

Experiment 58

Aim:

Citric acid production by submerged fermentation using spores of *Aspergillus niger.*

Principle:

Different fermentation processes have been developed for the different strains and carbon sources as described above for commercial production of citric acid using *A. niger*. Surface and submerged methods have been used with *A. niger* and either carbohydrates or n-alkanes.In order to achieve abundant excretion of citric acid, the growth of the strain must be restricted. High sugar concentrations favor high glycolytic flux, but limitations in either nitrogen or phosphorus are required as well as very low levels of certain heavy metals, including iron and manganese.

Various media have been described in the literature, and the general requirements for production with *A. niger* can be summarized as follows:

Carbon source:	60 - 240g
Nitrogen source:	2-3g
Phosphate source:	1.5-3.0g
Manganese:	<0.000001g
Iron:	<0.0013g
Zinc:	0.00025g
Distilled water:	1000ml
pH:	2 or 3, high aeration

Either nitrogen or phosphate limitations favor citric acid production. Nitrogen sources such as ammonium sulfate and ammonium nitrate are commonly used in concentrations between 1 to 3g l^{-1}. The type and concentration of the nitrogen source affect cell growth and citric acid formationInitial pH values are between 2.5 to 3.0; fermentation temperature is between 28 and 35°C; inoculation is performed with spores or with preculture mycelia in a 1:10 ratio. The characteristics of the inoculum and of the medium influence the culture morphology, which impacts citric acid yield. The fermentation process requires 5 to 10 days of incubation depending on the process.

Product Recovery:

Citric acid cannot be recovered directly from the fermented liquor by crystallization because of excess impurities. In the classic protocol, citric acid is precipitated as calcium citrate by the addition of lime. The washed precipitate is treated in aqueous suspension with H_2SO_4, yielding gypsum as a by-product. The citric acid solution is concentrated by vacuum evaporation and crystallized at low temperatures. Solvent extraction is usually carried out to avoid the formation of gypsum. Different solvents can be used; the extraction step is usually carried out at low temperature and the solvent is stripped with hot water. A mixture of n-octyl alcohol and tridodecylamine has been recommended for citric acid used in food and drug applications.

Estimation of citric acid:

a. ***Quantitative test:***

1. 1 ml of pyridine and 5 ml of acetic anhydride is added to 1 ml filtrate containing citric acid. Incubated for 30 min in an ice bath.

2. The standard citric acid stock solution (100 mg/100 ml Distilled Water DW) and different dilution (0.2 to 1ml) in 5 test tubes.
3. The volume is made up to 1 ml with DW. 1 ml of pyridine and 5 ml of acetic anhydride are added.
4. The blank is prepared by taking 1ml of DW in the place of citric acid.
5. It is then kept for incubation in an ice bath for 30 min.
6. The absorbance is read at 420 nm. Concentration of citric acid is found by plotting a graph of absorbance versus concentration.

b. Titration Method:

1. 10 ml of the citric acid sample is taken in a conical flask.
2. 0.1 N NaOH is taken in a clean burette and filled upto mark zero.
3. 2–3 drops of phenolphthalein indicator were added to the sample.
4. The sample was titrated with 0.1 N NaOH until permanent faint pink color persists and percentage of citric acid can be calculated as:

1 ml of 0.1 (N) NaOH = 7.0 mg of citric acid

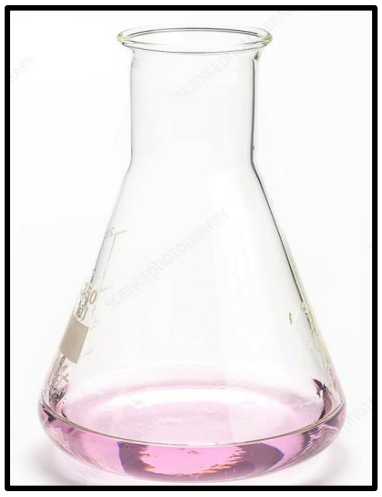
Observation:

Observe the end point of the titration as a faint pink colour, record the volume of NaOh required to get the end point and calculate the concentration of citric acid present in the filtrate.

From this calculate the yield of citric acid in μg/ml as follows:

If the titrate volume is 0.4ml (burette reading), then citric acid = 0.4 x 0.7/0.1 = 2.8 mg/10ml (sample volume)

Therefore, 2.8mg is converted to μg/ml as 2.8 x 1000 /10 = 280 μg/ml citric acid yield is obtained.



Citric acid titration: End point (faint pink)

Chapter - 24
Cultivation of economically important microbes: Biofertilizers

Fuentes-Ramirez and Caballero-Mellado in 2005, defined a biofertilizer as 'A product that contains living microorganisms, which exert direct or indirect beneficial effects on plant growth and crop field through different mechanisms'. From the science point of view biofertilizer is an individual microorganism used for plant growth promotion, but in the agricultural context this term refers to the product composed of beneficial strain(s), which are useful for nutrient mobilization, included in a carrier, possessing features that allow its storage at the time specified by the producer, and ready to effective application to the soil or plant. The main role of biofertilizers application is promoting plant growth without deleterious side effects for the environment and increasing harvest yields.

Several microorganisms are commonly used as biofertilizers including nitrogen-fixing soil bacteria (*Rhizobium, Azotobacter, Azospirillum, Gluconacetobacter diazotrophicus* and *methylobacterium* sp), nitrogen-fixing cyanobacteria (*Anabaena azollae, Nostoc, Spirulina, Oscillatoria, Microcoleus, Microcystis*), phosphate-solubilizing bacteria and fungi (*Pseudomonas striata, Bacillus megaterium var. phosphaticum, Bacillus subtilis, Penicillium digitatum and Aspergillus awamori*), and Arbuscular Mycorrhizal fungi (*Glomus, Gigaspora, Acaulospora, Sclerocysts).* Similarly, phytohormone (auxin)-producing bacteria and cellulolytic microorganisms are also used as biofertilizer formulations.

Bio-fertilizers play an important role in improving fertility of the soil. In addition, their application to soil improves the structure and texture of the soil and minimizes the sole use of chemical fertilizers.

Their mode of action differs and can be applied alone or in combination:

1. Biofertilizers fix atmospheric nitrogen in the soil and root nodules of legume crops and make it available to the plant.
2. They solubilise the insoluble forms of phosphates like tricalcium, iron and aluminium phosphates into soluble forms.
3. They scavenge phosphate from soil layers.
4. They produce hormones and anti metabolites which promote root growth.
5. They decompose organic matter and help in mineralization in soil and restore the soil's natural, nutrient cycle.
6. THEY ENHANCE PLANT TOLERANCE TO ENVIRONMENTAL STRESS AND PROVIDE PROTECTION AGAINST DROUGHT.
7. When applied to seed or soil, biofertilizers increase the availability of nutrients and improve the yield by 10 to 25% without adversely affecting the soil and environment.

Bio-fertilizers are usually amended with carrier material to increase effectiveness of the bio-fertilizers. It also increases water retention capacity. A carrier is a material, such as peat, lignite powder, vermiculite, clay, talc, rice bran, seed, charcoal, soil, rock phosphate pellet, paddy straw compost, wheat bran, or a mixture of such materials, etc. which provides better shelf life to biofertilizer formulation. Recently, immobilization of Biofertilizer cultures in alginate and

other polysaccharides have been developed by the producers to effectively maintain the viability of the organisms considering time required to reach the destined location.

Bio-fertilizer are mostly cultured and multiplied in the laboratory. However, blue green algae and *Azolla* (water fern) can be mass-multiplied in the field.

Experiment 59

Aim:

Isolation, Identification and Production of biofertilizer using symbiotic nitrogen fixing microorganism (*Bradyrhizobiumjaponicum*) from root nodules of *Glycine max* (L.)Merr. (soybean).

Principle:

Rhizobia are diazotrophic bacteria that fix nitrogen by establishing themselves inside the root nodules of legumes. These symbiotic associations are biological contributors of fixed nitrogen in soil based ecosystems. Rhizobium is the microsymbiont, which infects the roots of legume and nodulates. Rhizobium is a common name of the nodulating microsymbiont which consists of six genera as *Rhizobium, Bradyrhizobium, Mesorhizobium, Sinorhizobium, Allorhizobium, Azorhizobium* with about 36 species.

Rhizobia are aerobic, gram-negative, non-sporulating rod shaped bacteria which form specialized structures on roots called 'nodule'. The size and morphology of the nodules vary with the plant species. The nodules on clover are relatively small and round or oval shaped. On the other hand, cowpea, common bean, and soybeans, the nodules are relatively large, round, and firmly attached to the root. On alfalfa, peas and vetch, the nodules are usually longer and finger-like projections.

Requirements:

Well developed legume nodule obtained from soybean root
Mercuric chloride (0.1%) and Alcohol (70%)
Forceps, Sterile glass rod , Petri dishes and sterile distilled water blanks
Congo red Yeast extract mannitol agar medium (CRYEMA)
Inoculation loop
Incubator

Composition of CRYEMA: HiMedia

Mannitol:	10.0g
Dipotassium phosphate:	0.5g
Magnesium sulphate:	0.2g
Yeast extract:	1.0g
Sodium chloride:	0.1g
Congo Red:	0.025g
Agar:	20.0g
Distilled water:	1000ml
Final pH (at 25°C):	6.8±0.2

Suspend 31.8 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and

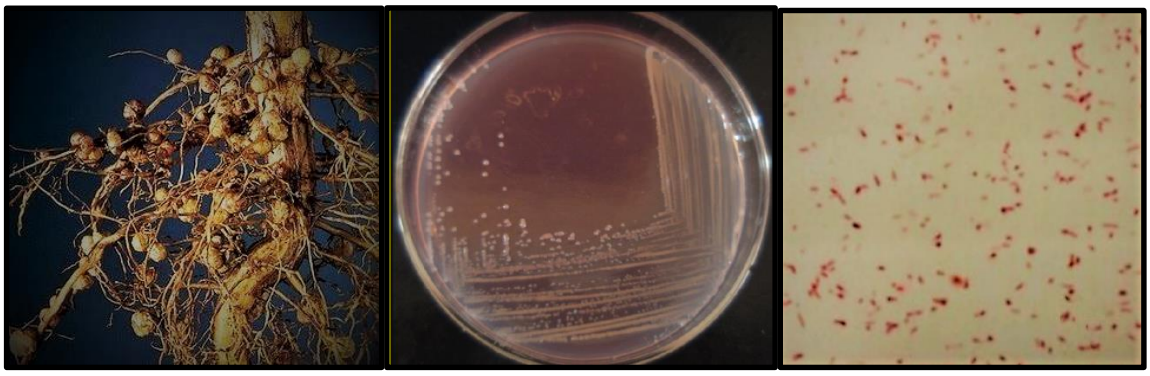
pour into sterile Petri plates.

Procedure:

1. Uproot the soybean plant and wash the roots gently and thoroughly under running tap water to remove soil particles.
2. Remove the nodules along with the root portion without damaging it.
3. Immerse intact, undamaged nodules for 5 – 10 seconds in 70 % ethanol.
4. Rinse the nodules in sterile distilled water 2-3 times.
5. Surface sterilize the nodules by soaking in 0.1% acidified mercuric chloride or 2.5 – 3.0 % sodium hypochlorite solution for 1-2 minutes.
6. Wash the nodules in 5-6 changes of sterile distilled water using sterile forceps.
7. Crush the sterilized nodules with a blunt ended sterile glass rod in a large drop of sterile water in a sterile test tube.
8. Under aseptic conditions, transfer one loopful of nodule suspension and streak it over the sterile solidified CRYEMA Petri plate.
9. Simultaneously, aliquots of serial dilutions prepared from the nodule suspension may be used for plating with CRYEMA either by spread plate method or pour plate method.
10. Incubate the plates at 28°C for 3-5 days.

Observation:

Appearance of circular, raised, smooth glistening whitish pink (faint) translucent colonies B*radyrhizobium japonicum*are observed on CRYEMA plates.



Root nodules, Colony morphology and Grams nature of *Bradyrhizobium japonicum from soybean*

Bradyrhizobium japonicum produced circular, whitish pink, convex, glistening translucent colonies with an entire margin. The bacteria was Gram Negative rod, aerobic, non-spore forming and motile. It showed positive reactions for citrate utilization, catalase and ammonia production and negative to methyl red and hydrolysis of casein.

Experiment 60

Aim:

Isolation, Identification and Production of non symbiotic nitrogen fixing bacteria (*Azotobacter* sp.) from soil samples.

Principle:

Azotobacter is the non-symbiotic, free living, aerobic nitrogen fixing bacteria. They are gram negative, polymorphic, form cyst and accumulate inclusions of poly hydroxy butyrate (PHB) and produce abundant gum. In addition to nitrogen fixation, they secrete plant growth hormones viz, indole acetic acid, gibberellic acid and growth factors such as thiamine, riboflavin etc. They are also capable of producing some antifungal antibiotics. In total six different species are identified based on their pigmentation. Among them, *A. chroococcum* is the dominant species found in tropical soils.

1. *A. chroococcum* - Produces black pigments (melanin)
2. *A. vinelandii* - Produces yellow pigments
3. A. beijerinckii - Produces green yellow fluorescent pigments
4. *A. insignis* - Produces yellow – brown pigments
5. *A. macrocytogenes* - Produces pink pigments
6. *A. paspali* - Produces pink to green fluorescent pigments

Beijerinck first isolated and described *Azotobacter*. Thesecells are found abundant in the rhizosphere region. Lack of organic matter in the soil is a limiting factor for the proliferation of *Azotobacter*. They depend on the energy derived from the degradation of plant residues.

Requirements:

Organic matter rich soil sample
Sterile distilled water tubes for preparation of dilutions
Petri plates
Waksman No. 77 medium

Composition of Waksman No. 77 medium

Mannitol:	10.0g
$CaCO_3$:	5.0g
K_2HPO_4:	0.5g
$MgSO_4.7H_2O$:	0.2g
NaCl:	0.2g
Ferric chloride:	Trace
$MnSO_4.4H_2O$:	Trace
N-free washed Agar:	15.0g
Final pH:	7.0
Distilled Water:	1000ml

Procedure:

1. Weigh one g of organic rich soil sample and put in the 100 ml water and mix thoroughly
2. Shake for 15 min for complete dispersion (Soil stock)

3. Transfer one ml of the suspension to 9 ml sterile distilled water tube 1 (10^{-1} dilution)
4. Prepare dilutions by transferring 1 ml from tube 1 (10^{-1}) to tube 2 (10^{-2}) and so on (from 10^{-1} To 10^{-6}).
5. Pour 1ml of dilutions 10^{-4}, 10^{-5}, 10^{-6} into the petri dishes appropriately labelled.
6. Maintain 3 replicates for each dilutions
7. Pour molten and cooled media (45 ^{o}C) of about 15 ml and mix well by gently shaking clockwise and anticlockwise direction for 3 or 4 times and allow it to solidify.
8. Incubate the plates in inverted position at room temperature for 3-4 days for appearance of *Azotobacter* colonies.

Observation:

Azotobacter produces raised, gummy colonies on agar surface and aged cultures show yellowish brown/black colouration due to pigment production.

Colony morphology and Grams nature of *Azotobacter chroococcum*

Azotobacter chroococcum produced circular, gummy, glistening transparent colonies with an entire margin. The bacteria was Gram negative rod, aerobic, and produced PHB.

Experiment 61

Aim:

Isolation, Identification and Production of phosphate solubilizing bacteria Phosphobacteria from soil sample.

Principle:

Phosphorus is one of the essential and vital nutrients for plant growth. Phosphorus nutrition is an essential component for the activities in food synthesis, cell reproduction, growth of shoot and root, flower formation, fruits and seed setting. The inorganic forms of the element in soil are compounds of calcium, iron and aluminum. The organic forms are compounds of myoinositol phytins, phospholipids and nucleic acids. A large portion of phosphatic fertilizer applied to soil is fixed in soil by conversion in the form of insoluble phosphates such as calcium, magnesium, aluminium and iron phosphates, which are not available to plants. Only 20 -25 % of phosphorus applied in the form of fertilizers is available to plants. These insoluble and

unavailable forms of phosphorus in soil remain unutilized by plants.

A different group of microorganisms such as bacteria and fungi, which convert insoluble inorganic phosphate compounds into soluble form, e.g., *Pseudomonas striata, Bacillus megaterium var. phosphaticum, Bacillus subtilis, Penicillium digitatum* and *Aspergillus awamori*.

These organisms can be isolated from soil by serial dilution and plating techniques.

Requirements:

Rhizosphere soil
Pikovskaya's
Sperber Agar plates
Sterile distilled water tubes for dilution preparation
Sterile Petri Plates
Sterile pipettes
Pikovskaya's Broth

Composition of Pikovskaya's Broth:

Glucose:	10.0g
$Ca_3(PO_4)_2$:	5.0g
$(NH_4)_2SO_4$:	0.5g
KCl:	0.2g
$MgSO_4$. $7H_2O$:	0.1g
$MnSO_4$:	Trace
$FeSO_4$:	Trace
Yeast Extract:	0.5g
Distilled Water:	1000ml

Composition of Sperber Agar

Glucose:	10.0g
Yeast extract:	0.5
$Ca_3(PO_4)_2$:	2.5
$CaCl_2$:	0.1
$MgSO_4 7H_2O$:	0.25
Agar:	15.0
Distilled water:	1000ml
Final pH at 25 ^oC:	7.2

Procedure:

1. Prepare soil or rhizosphere soil suspensions by taking 1g sample in 100 ml sterile distilled water.
2. Prepare serial dilution up to 10^{-4} by transferring 1 ml aliquot to 9ml sterile distilled water with a sterile pipette.
3. Transfer 1.0 ml aliquot from 10^{-3} and 10^{-4} to sterile petri dishes. Perform the experiment in triplicates for greater accuracy.
4. Pour approximately 15 to 20 ml molten and cooled medium (45 ^oC) to each Pikovskaya

petri dish and mix the inoculum by gently rotating the petri dish.

5. Incubate the plates at room temperature at 28 ^{o}C for 3-5 days.

Observation:

Observe and calculate the number of colonies per gram soil sample by applying the formula

No. of colony forming units (cfus) / g of sample = Mean no. of cfu x Dilution factor / Qty of sample on dry weight basis

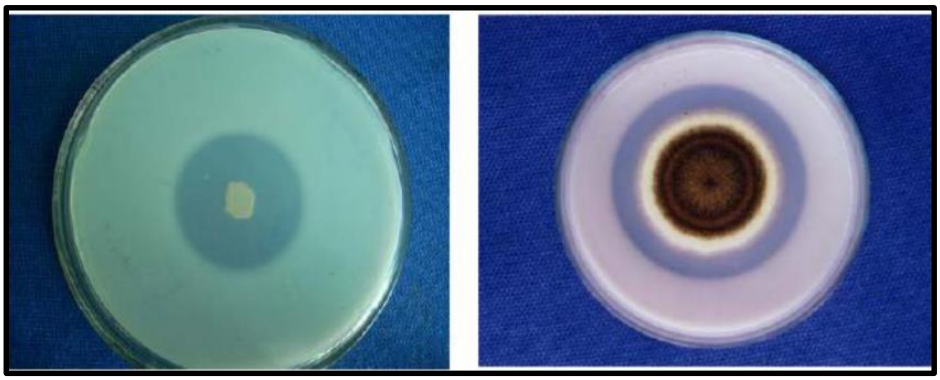
Observe a clear halo zone around bacterial colonies surrounded by turbid white background after 5-7 days of incubation at 28 ^{o}C on Sperber Agar plates.

Count the number of colonies with clear halo zone in each plate and arrive at the average number of phosphate solubilizing bacteria in each case and isolate the bacteria in its pure culture and maintain on nutrient agar at 4^{o}C for identification and production of biofertilizer.

Phosphate solubilization index:

The diameter of bacterial colony and halozone was measured and the values were used for calculating solubilization index by following the formula:

Solubilization index = Colony diameter + Halozone diameter / Colony diameter



Phosphate solubilizing Bacteria and fungi (halo zone): *Bacillus* and *Aspergillus*

Production of Biofertilizer:

Biofertilizers are formulated using microbial strain cultures properly mixed together with carrier based inoculants. The organic carrier materials are more effective for the preparation of bacterial inoculants. The solid inoculants carry more bacterial cells and support the survival of cells for longer periods of time. The mass production of carrier based bacterial biofertilizers involves three stages.

1. Mass culturing of microorganisms in fermentor
2. Processing of carrier material
3. Mixing of broth culture with the carrier and packing

1. Mass culturing of Microorganisms in Fermentor:

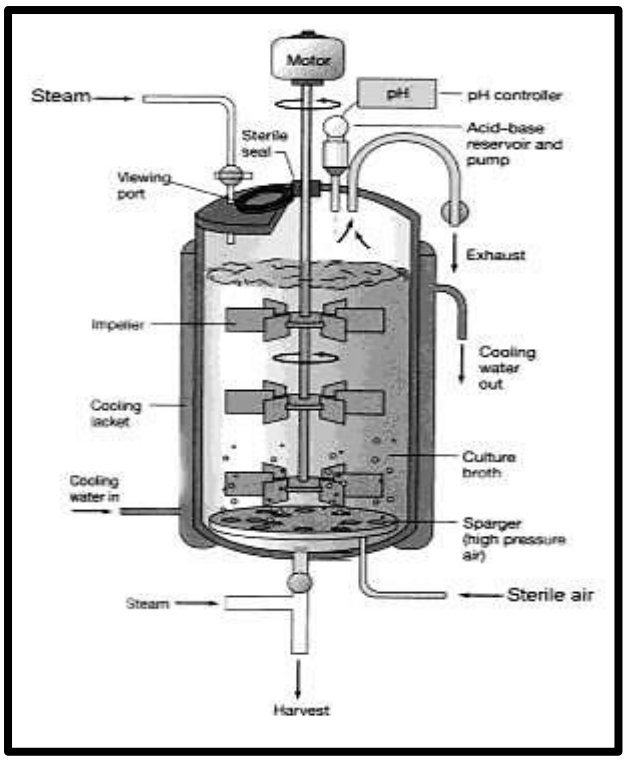
Although many bacteria can be used beneficially as a biofertilizer, the technique of mass production is standardized for *Rhizobium, Azospirillum, Azotobacter and phosphobacteria and Gluconacetobacter*. The media used for mass culturing are as follows: *Rhizobium*: Yeast extract

mannitol broth.
Azotobacter: Waksman No.77 broth.
Phosphobacteria: Nutrient broth

1. Prepare appropriate broth in 50 ml flasks and inoculate the pure culture into the flasks.
2. Grow the culture under shaking conditions at 30±2 ^{o}C until maximum cell population of 10^{10} to 10^{11} cfu/ml is obtained.
3. Under optimum conditions this population level could be attained within 4 to 5 days for *Rhizobium*; 2 to 3 days for Phosphobacteria and 6-7 days for *Azotobacter*. The culture obtained in the flask is called **starter culture**.
4. Use the starter to inoculate the broth in large size flasks of 250 ml, 500 ml, 3 liters and 5 liters and grow until required level of cell count is reached.
5. For large scale production of inoculant, inoculum from starter culture is transferred to large flasks/seed tank fermenters.

Fermenter:

Bacterial biofertilizers are normally mass cultured in fermenters. Fermenter is the huge stainless steel vessel which maintains the controlled environmental conditions for the growth of microorganisms and provides access for inoculation, sampling, aeration and cleaning. It should be made of stainless steel to withstand high pressure and also to resist corrosion. The fermenters come with a fermenting capacity ranging from litres to 100,000 gallons.



A Fermenter

Sterilization of growth medium in the Fermentor

1. Prepare required quantity of growth medium and adjust to the required pH

2. Pour the medium into the fermentor vessel after closing the sampling valve, keep the air outlet valve open
3. Bring the growth medium to boiling under maximum heat by using a steam generator.
4. Close the air outlet valve and allow the pressure to build up inside the vessel
5. Maintain a pressure of 15 1b / in^2 at 121°C for 20 minutes.
6. Switch off the fermentor and cool the medium by circulating cool water.

I. Mass production of carrier based biofertilizers:

Mass culturing in Fermentor

1. Spray the inoculation port with alcohol and flame thoroughly
2. Allow the port to cool, inoculate the media in the fermentor vessel with the log phase culture grown in 5 litre flask. Usually 1 -2 % inoculum is sufficient, however inoculation is done up to 5% depending on the growth of the culture in the larger flasks.
3. Turn on the air pump, open the air outlet valve and regulate the airflow to 3-10 litres of air per hour per litre of the medium. The sterile air provides aeration as well as agitation for the growth of culture.
4. Draw samples and analyze for growth, periodically if necessary.
5. Once the culture reaches full growth turn off the air supply and harvest the broth with the population load of 10^9 cells ml^{-1} after incubation period through the sampling port.
6. There should not be any fungal or any other bacterial contamination at 10^{-6} dilution level.
7. It is not advisable to store the broth after fermentation for periods longer than 24 hours. Even at 4 ^oC, the number of viable cells begins to decrease.

2. Processing of carrier material:

The use of ideal carrier material is necessary in the production of good quality biofertilizer. Peat soil, lignite, vermiculite, charcoal, press mud, farmyard manure and soil mixture can be used as carrier materials. The neutralized peat soil/lignite are found to be better carrier materials for biofertilizer production.

Characteristics of an Ideal carrier

1. Cheaper in cost
2. Should be locally available
3. High organic matter content
4. Should not be toxic
5. Water holding capacity of more than 50%
6. Easy to process, friability and vulnerability.
7. Amenable for mixing

Preparation of carrier:

1. Powder the carrier material (peat or lignite) to a fine powder so as to pass through 212 micron IS sieve.
2. Neutralize the pH of the carrier material with the help of calcium carbonate (1:10 ratio) ,

since the peat soil / lignite are acidic in nature (pH of 4 - 5).

3. Sterilize the neutralized carrier material in an autoclave to eliminate the contaminants. For large scale production gamma irradiation and sun drying method is followed.

3. Mixing of broth culture with the carrier and packing:

Add the bacterial culture drawn from the fermenter to the neutralized and sterilized carrier material to the moisture content of 35 to 45% on wet basis. The carrier and broth can be mixed either manually (by wearing sterile gloves) or mechanically. After mixing, pack the inoculants in 200g quantities in polythene bags, seal with electric sealer and allow for curing for 2 -3 days at room temperature (curing can be done by spreading the inoculant on a clean floor/polythene sheet/ by keeping in open shallow tubs/ trays with polythene covering for 2 -3 days at room temperature before packaging). Curing improves the cell count to 10^9 to 10^{10} cells/g. After curing it is then packed in low density polythene bags. The Inoculants may be allowed for curing even after packing for 3- 4days at room temperature.

Storage:

1. The packet should be stored in a cool place away from the heat or direct sunlight.
2. The packets may be stored at room temperature or in cold storage conditions in lots in plastic crates or polythene / gunny bags.

II. Mass production of liquid biofertilizers:

All the bacterial biofertilizers are produced in liquid formulations also. Liquid biofertilizers are produced through a three step process.

1. Preparation of starter culture and seed culture

Prepare the starter culture from the pure culture in the respective growth medium as given for carried based inoculants

Mass culturing in fermentor:

Follow the procedure of mass culturing similar to carrier based biofertilizer.

Harvest the broth once the population reaches the cell load of 10^{10} cell per ml broth

2. Preparation of liquid formulation

Fill the harvested culture in the sterile plastic container of one liter or 500 ml capacity, add Glycerol one ml per liter of broth to arrest the metabolic activities of the cell also to avoid bursting of the container under storage.

Seal the mouth with sterile caps and store under room temperature.

III. Methods of Application of different Biofertilizers:

Biofertilizers are prepared as both carried based and liquid inoculants. Liquid formulation of biofertilizers is suitable for drip fertigation wherever crop is grown under precision farming.

1. Method of application of carrier based bacterial biofertilizers:

Bacterial biofertilizers are applied as carrier based inoculants. Peat or lignite is used as carrier material. Carrier based bacterial inoculants are applied by the following methods.

1. Seed treatment or seed inoculation
2. Seedling root dip and

3. Main field application

a. Seed treatment:

Mix one kg of the inoculant with approximately one liter of rice gruel to make slurry. Treat the seeds required for one hectare with the slurry so as to have a uniform coating of the inoculant over the seeds and then shade dry for 30 minutes. The shade dried seeds should be sown within 24 hours.

b. Seedling root dip:

This method is used for transplanted crops. Mix one kg of the inoculant in 200 liters of water. Dip the root portion of the seedlings required for one hectare in the mixture for 15- 20 minutes before transplanting.

c. Main field application:

Mix two kg of the inoculant with 25 kg of dried, powdered farm yard manure and then broadcast in one hectare of the main field just before transplanting.

Biofertilizer production: A *Rhizobium* and B *Azotobacter*

Chapter - 25
Cultivation of economically important microbes: Biocontrol agent

The conventional chemical pesticides have not only enhanced the food production, but have adversely affected the environment and non-target organisms. Due to the side-effects of chemical pesticides, sustainable crop production through eco-friendly pest management is essentially required in recent scenarios. Among the several microorganisms viz., bacteria, fungi, virus, protozoans and entomopathogenic nematodes, a few have been systematically studied for their effective beneficial characteristics. Among these organisms, *Trichoderma viride* is a very promising biocontrol agent against soil borne plant parasitic fungi. *Trichoderma* is being used world wide for suitable management of various foliar and soil borne plant pathogens. Moreover, they are acclaimed as effective, eco friendly and cheap, nullifying the ill effects of chemicals.

Experiment 62

Aim:

Isolation, identification and mass production of *Trichoderma viride* as an effective biocontrol agent.

Principle:

Trichoderma viride is an effective biocontrol agent against fungal pathogens like *Fusarium oxysporum* infecting soybean plants. The root system of the soybean plant infected with *Fusarium oxysporum* exerts a negative influence on the nodulation and further growth phases of the plant. However, when *Trichoderma* is used as a biocontrol agent it also promotes enhanced growth of shoot and root systems.

Trichoderma viride was isolated from soil samples by using Potato dextrose agar (PDA) medium. Samples were inoculated over plates by serial dilution technique and the plates were incubated at 26 ^{o}C for 4 days. The fungal colonies which were picked up and purified by streaking and incubated at 26 ^{o}C for 7-8 days. Green conidia forming fungal bodies were selected and microscopic observation was carried out to identify it as *Trichoderma viride*.

The culture was maintained on PDA slants at 4 ^{o}C in the refrigerator for further studies. The pure culture of *Trichoderma viride* was used to test antifungal activity against *Fusarium oxysporum.*

Requirements:

Soil sample from agricultural field
Sterile distilled water tubes
Potato dextrose agar plates
Bunsen burner, inoculation loop
Incubator

Principle:

1. Collect soil samples in a clean petri dish from a soybean field.
2. Prepare serial dilutions as mentioned in earlier experiments 10^{-4} and 10^{-7}
3. Pour one ml of each dilution from 10^{-4} to 10^{-7} to the appropriately labelled petri plates

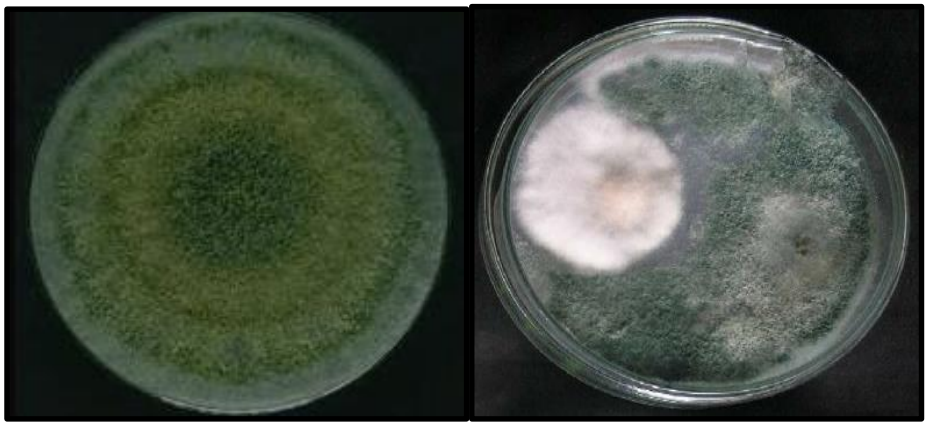
and add molten PDA and allow it to solidify.

4. Incubate all the petri plates at 26 ^{o}C for 7-8 days.
5. Observe and identify the growth of *Trichoderma viride* as green conidia forming fungi.
6. Isolate the fungi on a PDA plate in its pure culture form by taking single fungal hyphae with the help of sterile needle.

Antifungal activity of Trichoderma extracts against fusarium pathogens:

A disc diffusion method was used to detect the antifungal efficacy of *Trichoderma* extracts against *Fusarium oxysporum*. The dried extract of *Trichoderma* was dissolved in sterile distilled water to attain a final concentration of 10 mg/ml. Ten ml of PDA medium was poured into sterile Petri dishes as a basal medium, followed by the addition of 15 ml of seeded medium.

The seeded medium was prepared by mixing 1 ml of fungal spore suspension of *Fusarium* (10^6 spores/ml) with 100 ml of PDA medium. Sterile filter paper discs (8 mm in diameter) were loaded with the *Trichoderma viride* extract (10 mg/disc) and placed over the seeded plates. The plates were incubated at 28 ^{o}C for 5 days and the diameters of the inhibition zone were measured using a ruler.



Trichoderma viride* and its antifungal activity on *Fusarium oxysporum

Mass culturing of *Trichoderma viride:*

The Grains viz rice, wheat and pulses were used for estimating the biomass of *Trichoderma viride* at 26 ^{o}C. 20 g of each grain was washed well and boiled in distilled water for 1 hr. and then mesh properly and filter it, now makeup 1 liter with distilled water. Now these grain mediums were packed separately in individual 500 ml conical flasks. They were plugged with cotton wool and autoclaved at 121^{o}C at 15 psi for 1hour. After cooling, 1 gm of the fungal culture was inoculated into each flask, separately. All the experimental work was carried out in a laminar air flow chamber. The flasks were incubated in a BOD incubator at 26^{o}C for 3 weeks. To avoid clumping, after 7 days of inoculation, the flasks were shaken vigorously to separate the culture and to break the mycelial mat. After 14 days of incubation, the mycelial mat appeared in flasks. Now it has grown well for 21 days. The flasks were shaken in a mechanical shaker for 10 min. The suspension was filtered through double layered muslin cloth and then biomass was estimated in each grain medium.

Mass production of *Trichoderma viride:*

Commercial application of *Trichoderma viride* either to increase crop health or to

manage plant diseases depend on the development of commercial formulations with suitable carriers that support the survival of Trichoderma for a considerable length of time.

Talc based formulation:

In India, talc based formulations of *T. viride* was developed at Tamil Nadu Agricultural University, Coimbatore for seed treatment of pulse crops and rice. *Trichoderma* is grown in the liquid medium and is mixed with talc powder in the ratio of 1:2 and dried to 8% moisture under shade. The talc formulations of *Trichoderma* have a shelf life of 3 to 4 months. It has become quite popular in India for management of several soil-borne diseases of various crops through seed treatment at 4 to 5 g/kg seed. Several private industries produce large quantities of talc formulations in India for supply to the farmers. The annual requirement of Trichoderma has been estimated as 5,000 tonnes to cover 50 per cent area in India.

Delivery of *Trichoderma* for disease management:

For successful disease control, delivery and establishment of *Trichoderma* to the site of action is very important. The most common methods of application of Trichoderma are seed treatment, seed biopriming, seedling dip, soil application and wound dressing.

Seed treatment:

Seed coating with *Trichoderma* is one of the easy and effective methods of delivering the antagonist for the management of seed/soil-borne diseases. Seed is coated with dry powder/dusts of *Trichoderma* just before sowing. For commercial purposes, dry powder of antagonist is used at 3 to 10 g per kg seed based on seed size. Seed treatment with *Trichoderma* species inhibited the growth of oilseed-borne fungi like *Aspergillus flavus, Alternaria alternata, Curvularia lunata, Fusarium moniliforme, F. oxysporum, Rhizopus nigricans, Penicillium notatum and Penicillium chrysogenum* which affects oil seed crops like soybean, sesame and sunflower.

Seed biopriming:

Treating seeds with biocontrol agents and then incubating under warm and moist conditions until just prior to emergence of radical is referred to as **bioprimming**. This technique has potential advantages over simple coating of seeds as it results in rapid and uniform seedling emergence. *Trichoderma* conidia germinate on the seed surface and form a layer around bio primed seeds. Such seeds tolerate adverse soil conditions and perform better. Biopriming could also reduce the amount of biocontrol agents that is applied to the seed. Seed biopriming is successfully used in tomato, brinjal, soybean and chickpea in Tarai region of Uttaranchal.

Soil treatment:

Soil is the repertoire of both beneficial and pathogenic microbes. Delivering of *Trichoderma* sp. to soil will increase the population dynamics of augmented fungal antagonists and thereby would suppress the establishment of pathogenic microbes onto the infection court.

Root treatment:

Seedling roots can be treated with spore or cell suspension of antagonists either by drenching the *Trichoderma* in nursery beds or by dipping roots in *Trichoderma* suspension before transplanting. Root dipping in the antagonist's suspension not only reduces disease severity but also enhances seedling growth in rice, tomato, brinjal, chili and capsicum.

Foliar spraying/Wound dressing:

The efficacy of biocontrol agents for foliar diseases is greatly affected by fluctuation of microclimate. Phyllosphere is subjected to diurnal and nocturnal, cyclic and non-cyclic variation in temperature, relative humidity, dew, rain, wind and radiation. Hence, water potential of phylloplane microbes will vary constantly. It also varies between leaves or the periphery of the canopy and on sheltered leaves. Higher relative humidity could be observed in the shaded, dense region of the plant than that of peripheral leaves. The dew formation is greater in the centre and periphery. The concentration of nutrients like amino acid, organic acids and sugars exuded through stomata, lenticels, hydathodes and wounds varies highly.

Production of *Trichoderma viride* using Solid State fermentation:

Mass production is achieved through solid and liquid fermentation techniques. The commercial success of biocontrol agents requires:

1. Consistent and broad spectrum action
2. Safety and stability
3. Longer shelf life
4. Low capital costs
5. Easy availability of career materials
6. Economical and viable market demand

Solid-state fermentation (SSF) is an effective method for the mass production of fungal biopesticides since it provides micro propagules with higher conidia content. Various cheap cereal grains like sorghum, millets, and ragi are used as substrates. The grains are moistened, sterilized and inoculated with *Trichoderma* and incubated for 10 to 15 days for production of *Trichoderma.*This fermentation process produces dark green spore coating on the grains. These grains can be powdered finely and used as seed treatment or the grains can be used as it is for enriching soil.

Standards for Trichoderma formulations:

1. Colony Forming Units (CFUs) of Trichoderma sp. should be a minimum of $2x10^6$ CFU per ml or gm on selective medium.
2. Pathogenic contaminants such as *Salmonella, Shigella* or *Vibrio* should not be present. Other microbial contaminants need not exceed $1x10^4$ count ml/gm. iii.
3. Maximum moisture content should not be more than 8% for dry formulation of fungi.

***Trichoderma viride cultured* on rice grains; uninoculated and powdered**

Appendix-1

A standard solution is one of known concentration.
Following standard solutions are frequently required in microbiology laboratories:

1. Molar
2. Molal
3. Normal

Molarity (M) = Molarity is defined as the number of moles of solute contained in one litre. A molar (1.0 M) solution of sodium hydroxide contains 1.0 mole or 40 g of sodium hydroxide in one litre of solution because, NaOH (aq.) Na^+ + OH; 1 mole of Na+ = 23 g, 1 mole of oxygen = 16 g, 1 mole of hydrogen = 1 g.
Thus, 1 mole of NaOH = Total 40g
When 40g (I mole) of NaOH is dissolved in 1 litre of water it gives 1 molar solution.

Molality (m) = Molality expresses the concentration of a solution in which the quantity of solute is given in moles, and the quantity of solvent in kilograms. Molality is defined as the number of moles of solute dissolved in 1000 g (1 kg) of solvent. A one molal (1.0 m) solution of sodium hydroxide contains 1.0 mole or 40 g of NaOH dissolved in 1 kilogram of water.

Normality (N) = Normality expresses the concentration of a solution in which the quantity of solute is given in gram equivalent weights and the volume of solution in litres. Normality therefore represents the number of gram equivalent weights of solute dissolved in one litre of solution. That is, Gram equivalent weight of solutes are dependent on the total positive or negative charges in the formula of acid, base or salt.

PPM (Parts Per Million Solution) Gram of solution per million grams of solution or one g of solute dissolved in 1 million ml of solution. Therefore 1 ppm = 1 mg NaCl/100 ml or 1 mg NaCl/ml
The formula for ppm
1 ppm=1/1,000,000=0.0001.
Before calculating ppm, one must be sure they are measuring the same volume or mass of the substances in the equation. For example, to determine the proportion of 0.2 grams (g) of salt in 0.98 liters (L), both units must be converted to the same unit of mass. To do this, one must know that 1 L of water = 1 kg of mass, so in this formula, 0.98 L of water will be 1 kg of water. 0.2 g of salt will be 0.0002 kg of water.
To convert to ppm, 0.0002 kg will be multiplied by 1,000,000. The final result will be 220 ppm salt in 1 L of water. It is also important to note that a measure of mg/L of water is also considered a ppm amount by mass.

Appendix-2

Addresses for procuring chemicals, instruments, readymade media and cultures required for microbiology practicals

1. Readymade media:

1. Himedia Laboratories Pvt. Ltd. A-406, Bhaveshwar Plaza, 235 Marg, Mumbai-400 086, India.
2. LOBA Chemie Pvt. Ltd. 78/80, Babu Genu Road, P.B. No. 2042 Mumbai-400 002.

2. Chemicals:

1. Poona Chemical Laboratory 207, Magalwar Peth, Near Gadital Behind Modi Petrol pump, Pune - 411011.
2. S.D. Fine Chen Ltd. 3 15 H.O., T.V. Ind. Estate, 248 Worli Road, Post Box No. 19160 Mumbai-400 030.
3. Glaxo India Limited Dr. Annie Besant Road, Mumbai-400025.
4. Ranbaxy Fine Chemical Division 12th Floor, Devika Towers 6, Nehru Place New Delhi-110019.
5. Borosil Glass works Ltd. 4031404, Kaliandas Udyog Bhavan. Near Century Bazar, Worli Mumbai-400025
6. Qualigens Fine Chemicals Division. Glaxo SmithKline Pharmaceuticals Ltd. Dr.Annie Besant Road, Worli. Mumbai-400030.
7. Loba Chemie Pvt. Ltd. Jehangir Villa, 107 WodeHouse Road, Colaba, Mumbai - 400005 (India).
8. Asgi Enterprises. 10, Dadi Santuk Lane, Mumbai-2 Thomas Baker. (Chemicals) Pvt. Ltd, Head Office. 4/86, Bharat Mahal, Marine Drive, Mumbai 400002 (India).
9. Universal Laboratories Pvt. Ltd. Corporate office-507, Raheja Centre, 214, Nariman point, Mumbai-400021. (India).
10. Biotech Pvt Ltd. 18/1, Madhukunj Society, Panchavati, Off Pashan Road, Pune-411008.
11. Bangalore Genei. No. 6, 6 th Main, BDA. Industrial Suburb, Near SRS Road Peenya, Bangalore-560058. (India).

3. Instruments:

1. M/S Toshniwal Brothers Pvt. Ltd. 198, Church gate, Mumbai-400020.
2. M/S MICLAB Instruments Gulmohar 'B', Flat No. 5 428-43013, Gultekadi, Pune-411009.
3. M/s TEMPO Industrial Corporation I, lamington Chambers 394, Dr. Bhadkamkar Marg, Mumbai-400004.
4. M/S Opel Instruments Pvt. Ltd. 562, Sadashiv Peth, Laxmi Road, Bhanuvilas Chowk, Pune-411030.

5. Biotron Healthcare (India) P. Ltd. 30 1, Coral classic, 20h Road. Chembur, Mumbai 400071. Remi Equipments Ltd. 14, Shah Industrial Estate, Veera Desai Road. Andheri (W), Mumbai 400053.
6. SAKSHAM Technologies Pvt.,Ltd. 502, Niti Apartments, Underai Road, Near New Era. Cinema, Malad(W), Mumbai-400064.
7. Millipore (India) F'vt. Ltd. 50-A. I1 Phase, Ring Road, Peenya, Bangalore-560058, India.
8. Labindia Instruments Pvt. Ltd. 201, Nand chambers, L.B.S. Marg, Near Vandana cinema, Thane-400602.

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1. Fermentation Technology Laboratory, Indian Institute of Science, Bangalore-3 (Karnataka)
2. National Collection of Industrial Microorganisms National Chemical Laboratory, Council of Scientific and Industrial Research; Pashan Road, Pune (Maharashtra)
3. Microbial Type Culture Collections I Gene Bank (MTCC) Institute of Microbial Technology Post box No. 1304, Sector 39-A, Chandigarh-160014, India.

5. Fungal Cultures:

1. Herbarium Cryptogame India Orientails Division of Mycology and Plant Pathology Indian Agriculture research Institute, New Delhi- 12, India.
2. Microbial Type Culture Collections I Gene Bank (MTCC) Institute of Microbial Technology Post box No. 1304, Sector 39-A, Chandigarh-160014, India.
3. Agharkar Research Institute Bhandarkar Road, Pune.

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ISBN: 978-93-91768-69-0

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ISBN 978-93-91768-69-0
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