Microbiology
Laboratory Manual

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MICROBIOLOGY LABORATORY: BASIC RULES AND REQUIREMENTS

Microbiology Lab Practices and Safety Rules

- Wash your hands with disinfectants when you arrive at the lab and again before you leave.
- Wear laboratory coats in the lab. Students with long hair must put up the hair.
- At the start and end of each laboratory session, students should clean their assigned bench-top area with a disinfectant solution provided. That space should then be kept neat, clean, and uncluttered throughout each laboratory period.
- Eating or drinking in the laboratory is not permitted. No mouth pipetting.
- Label everything clearly. Sterilize equipment and materials.
- Avoid loose fitting items of clothing. Wear appropriate shoes in the laboratory.
- Report any breakage of equipment to the instructor.
- Report any personal accidents such as cuts to the instructor at once.
- Turn off Bunsen burner when not in use.
- Discard all cultures and used glassware into the container labeled CONTAMINATED. (This container will later be sterilized.) Plastic or other disposable items should be discarded separately from glassware in containers to be sterilized.
- Never place contaminated pipettes on the bench top.
- When you flame sterilize with alcohol, be sure that you do not have any papers under you.
- Before beginning your laboratory exercise, wash off the bench top with the disinfectant provided. When exercises are completed, wash off the bench top again. Always wash your hands with soap and water before leaving the laboratory.
- Before leaving the laboratory, see that all the equipments are in the proper location and gas and water turned off.
- Purchase a fine point, waterproof marker and small roll of masking tape. Use them to clearly label your cultures.
- If you should spill or drop a culture or if any type of accident occurs, call the instructor immediately. Place a paper towel over any spill and pour disinfectant over the towel. Let the disinfectant stand for 15 minutes and then clean the spill with fresh paper towels. Remember to discard the paper towels in the proper receptacle and wash your hands carefully.
- Disinfect work areas before and after use with 70% alcohol or fresh 10% bleach. Laboratory equipment and work surfaces should be decontaminated with an appropriate disinfectant on a routine basis and especially after spills, splashes or other contamination.
- Replace caps on reagents, solution bottles and bacterial cultures. Do not open petri dishes in the lab unless absolutely necessary.
- Cultures are not to be removed from the laboratory unless the instructor gives permission.
- Always place culture tubes (broth and slants) in the upright position in a rack or basket for incubation or disposal.
- Dispose off all solid waste materials in a biohazard bag and autoclave it before discarding in the regular trash.
- Treat all cultures as potentially pathogenic, *i.e*., flood areas with disinfectant if cultures are spilled, wash hands after contact and notify your instructor at once.
- Read the instructions carefully before beginning an exercise. Also, make sure you have all the materials needed for the exercise at hand before you commence the experiment. Ask the instructor for clarification of any points about which you are in doubt.

- Flame the inoculating loop or needle immediately before and after use. If viscous material is present on the loop or needle, dry it at the side of the flame before placing it directly in the flame.
- Laboratory note books must be kept up-to-date. Illustrations should be done when requested.
- Make sure you consult the instructor to dispose of the cultures that are not needed any longer. Remove all labels and markings from the tubes before disposing of them; do not discard anything into the sinks.
- Please inform your instructor if you have any medical condition that could potentially affect your safety in the laboratory (eg: diabetes, epilepsy, immunosuppression etc.). This information will help the instructor to deal with any emergency that would arise. The information will be treated confidentially and it will not affect their ability to participate in the laboratory activities.
- Be systematic and logical. Keep a faithful record of all the experiments and observations. Update it regularly and submit it for evaluation at the end of each exercise.
- Work either using laminar air flow chamber or light the burner at least five minutes prior to making any inoculations and work near the burner.

Basic Record and Field Book/Lab Book

They are the permanent record of your work and hence should contain all the works related to the project. Basic Record is the whole systematic record of the work/study/project in full detail. Field book/lab book is for your daily use in the lab/field and for rough works, calculations, plan schedules, memoirs, etc. You should record your work in these books systematically and regularly. All the experiments conducted in the lab must be recorded in these books. It is a compilation of whole work done by the researcher, so it must be well maintained. Also it can be a good reference book for those who come along. These are the property of the research station and hence you are not supposed to keep those books in your home. When you resign from the job you should submit them up-to-date to the lab in charge without any delay.
You should note the following points while dealing with field book.

1. Keep the book neat and tidy.
2. Utilize the book efficiently preserving the legibility of your writing.
3. Name of the experiment should be entered along with the date of carrying out that experiment.
4. Next you mention the requirements for the experiment.
5. Summarize the theory and principle. This should be followed by the procedure.
6. Mention the general calculations for the experiment. It should contain all the related works of the project for which it is meant to.

The following points are to be taken care of:

1. Do not tear pages from the field book. Number the pages of field book.
2. Do not over write if a mistake has been committed in recording, put a line over it and write the correct word again.
3. Complete the index, indicating the experiment, its serial number, page number on which it is written.
4. The notebook should always be up to date and may be collected by the lab in charge at any time.
5. You have to submit the field book and basic record at the end of every month on the date assigned.

Mandatory Details Required the Basic Record

1. Index: An index containing the title of each experiment with page number and Sl. No.
2. Brief title of the experiment and date: Every experiment should have a descriptive title.
3. Aim/Objective: A clear objective should be there.
4. Technical Programme: This section should include any materials required, reagent composition, protocol and formulae. Procedure in the form of flow charts is helpful if it involves several parts. If an experiment is a repeat of an earlier experiment, you do not have to write down each step, but can refer to

the earlier experiment by page or experiment number. If you make any changes, note the changes and reasons why.

5. Observations: Periodical or quantitative or qualitative observations
6. Results: This section should include the final result of the experiment in accordance with the aim, organized in statistically valid tables and figures and discussed logically and justifiably. All raw data, including gel photographs, printouts, graphs, autoradiographs, etc if present are to be included.
7. Inference: The results obtained should be interpreted in accordance with the principle of the experiment.
8. Future Line: This section includes any suggestions from the protocol done, any refinements required etc.

It is mandatory to have clear and accurate records of all experiments conducted in the laboratory.

Basic requirements of a microbiology laboratory

A microbiology laboratory requires well-built rooms equipped with glassware, tools and equipments. Some of the most important items of equipment are the following.

I) Common Glassware

The most important glassware used in a microbiological laboratory are test tubes, culture tubes, Petri dishes, Measuring cylinder, pipettes, glass spreader, Flasks, screw-capped glass bottles, haemocytometer etc.

1) Test Tubes, Culture Tubes and Screw-Capped Tubes

- These are made up of glass, one end of which is closed and other end is open.
- If the side wall of the open end is slightly curved outside, it is called *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 *screw-capped tube*.
- The test tubes are used for testing the chemicals such as pH etc., culture tubes are used for preparation of agar slants and purification of microorganisms. The open end is plugged with non-absorbent cotton plug.
- Sometimes the microorganisms are purified and preserved in screw-capped tubes.

2) Petri dish

- R. J. Petri, a student of the most renowned bacteriologist Robert Koch devised this dish, hence called "Petri dish".
- It consists of two shallow glass dishes, the upper half or lid and the lower half.
- For the isolation and cultivation of different types of microorganisms these dishes are used in all microbiological laboratories.
- According to the requirement, its diameter varies.
- Molten agar medium is aseptically poured on the lower half of the sterilized Petri dish and then covered with the upper half.
- The petri dishes are sterilized by putting them in a Petri dish container and in turn in an oven or autoclave.

3) Pipette

- It is a cylindrical and graduated glass apparatus.
- Its one end (lower side) tapers, while the other end (mouth piece) is normal. The middle portion is wider or of the same size as mouth end.
- It is graduated with numbers 1, 2 . . . n. . . . . 10.
- It has different measuring capacity such as 0.1, 0.5, 1, 5, 10 ml etc. Hence measures different quantities.
- It is used for transferring appropriate amount of liquid into other containers.

- It should be sterilized in an oven or autoclave before use by keeping in pipette container after being plugged with cotton.
- For safety point, liquid should be sucked by attaching pipette-sucker at the normal end of the pipette.
- Pipettes should be sterilized by keeping them first in a steel can and is sterilized at 121°C for 30 minutes.

4) Glass Spreader

- It is made by bending a glass rod and making an L-shaped structure.
- It is used to spread evenly the microorganisms on agar surface present in liquid medium.
- The long arm is held in hand and the small arm is flame-sterilized and put on agar surface.
- It is brought forth and back so that microorganisms present in liquid may be dissociated and evenly spread on the entire surface of agar.

5) Haemocytometer

- This is a device used to measure the blood cells.
- This is also used for counting other cells viz., spores, bacteria etc.
- It consists of a number of chambers. Each big chamber has 1 X 1 X 0.1 mm = 0.1mm^3 volume with an area of 1 X 1mm = 1mm^2. The depth of chamber is 0.1mm. (1 X 1 X 0.1 mm = 0.1mm^3 = 0.0001cm^3 = 10^{-4}cm^3 = 10^{-4} ml) Hence, the bacterial cell count in the large chamber will be multiplied by 10^4 to give an estimate of bacterial cell number/ml.
- Each large chamber has 9 medium-sized chambers with 0.2 mm length, 0.2 mm width and 0.1mm depth with a volume of 0.004 mm^3.
- Each medium sized chamber is divided into 25 small chambers with 0.04 mm length, 0.04 mm width and 0.1mm depth with a volume of 0.00016 mm^3.

6) Cleaning of Glass wares. Care should be taken that the glassware used in microbiology laboratory are neat and clean. After receiving and before start of work, the glassware must be chemically cleaned so that there should not be chemical deposits on its surface. Moreover, dirty form, then by soaking overnight in chromic acid. If the latter is not effective, a mixture of concentrated nitric acid and sulphuric acid can be applied. All traces of the cleaner are then removed by repeated rinsing in tap water followed by distilled water. Sometimes, new glassware may contain some bacterial/fungal spores that come with packing materials. The glassware from factory also contains some amount of alkali, hence to remove the alkali, 2-3 % HCl is applied for 24 hours for neutralization.

After the use of glassware the medium is removed and the former is treated with 3% commercially available Lysol solution followed by washing with boiling water. After drying, it can be kept in oven for 3-6 hours at 140-180°C. The used pipettes, slides, cover slips, petri dishes, etc. are also cleaned by this method.

Chromic acid is widely used as cleaning agent for glassware. It is a mixture of sodium dichromate and concentrated sulfuric acid and possesses powerful oxidizing and solvent properties.

Preparation of Chromic acid

(i) First Method**:** Weigh 5g of sodium potassium dichromate and dissolve in 5ml distilled water in a beaker (250ml). Add 100ml concentrated H_2SO_4 slowly and stirring it constantly. The mixture is allowed to cool at about 40°C and then stored in a dry glass stoppered bottle.

(ii) Second Method**:** Weigh 1g of $K_2Cr_2O_7$ or $Na_2Cr_2O_7$ and mix with 100ml of concentrated H_2SO_4. After stirring several times (preventing from cake formation) allow the mixture to cool at 40°C and store in a clean, dry stoppered bottle.

II) Tools in Microbiology Laboratory

The most common equipment are inoculation needle, inoculation/transfer loop, Bunsen burner, autoclave (or pressure cooker), incubator, hot air oven, refrigerator, centrifuge, spectrophotometer, magnetic stirrer, orbital shaker, hot plate, Distillation water still, UV- lamp, water-bath, carbon dioxide cylinder, single-pan balance with weights (for rough use), chemical balance, pH meter, colony counter, laminar air flow, electrophoretic apparatus, microscopes etc.

1) Inoculation Needle & Inoculation Loop

- These are the most commonly used tools.
- Inoculation needle/loop is made up of a long platinum wire fixed into a metallic rod.
- A wire loop has a handle with steel screw shaft in which nichrome or platinum wire is to be fitted.
- The straight wire needle is used for transferring culture from solid medium. Even smaller amount of liquid culture can be manipulated by using straight needle.
- 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 to avoid contamination.

2) Bunsen Burner

- Sterilization of tools by using spirit lamp is called incineration.
- Gas enters the burner at the base, and its supply is regulated externally by the gas cock.
- The amount of air can be controlled by rotating a sleeve that fits over the holes in the body of the burner.
- To keep the flame from blowing out special tips are frequently used to fit over the top end of the barrel.
- 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 colour.

3) Water Bath

- Water bath is an instrument that is used to provide constant temperature to a sample.
- It consists of an insulating box made up of steel fitted with electrode heating coil.
- The temperature is controlled through a thermostat.
- In some of the water baths, plate form rotates, then it is called water bath shaker. It is more useful to microbiologists because it provides a uniform heat to the sample material meant for incubation.
- The main use of water bath is the incubation of samples at a desired and constant temperature.

4) Laminar Air Flow Chamber

- Laminar air flow is an apparatus consists 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 0.3 mm.
- In front of the blower, there lies a mechanism through which air blown from the blower produces air velocity along parallel flow lines.
- The laminar air flow 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 in to the system.
- Inside the chamber one fluorescent tube and another UV tube are fitted. Two switches for these tubes and a separate switch for regulation of the air flow are fitted outside the LAF. Due to uniform velocity

and parallel flow of air current, pouring of media, plating, slant preparations, streaking etc. are performed without any kind of contamination.

- Initially, dust particles are removed from the surface of the laminar air flow with the help of smooth cloth containing alcohol. 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.
- 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 / bring contaminants.
- Sit properly in front of the chamber and 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 microbiology laboratory, horizontal type of laminar air flow is used to supply the air through filter.

Precautions: Put off the shoes before entering to operate the apparatus. Wash the hands with detergents 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.

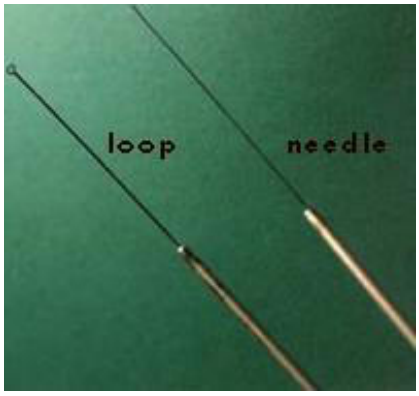
5. *Incubator*

- An incubator is an instrument that consists of copper/steel chamber around which warm water or air is circulated by electric 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 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 condition. A thermometer is inserted from the top for recording the temperature.
- Temperature greatly influences the microbial growth. Therefore, instrument is generally designed that can allow the desired microorganism to grow at a particular temperature.
- 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.
- 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.

Precautions: the door of the incubator should be opened only when necessary. 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 plug should be pushed inside the neck of the tube. The tube should be covered by a rubber cap so as to cover the plug. If the petriplates are to be incubated for a long time, they may be placed in moist chamber with a damp sterile cotton wool at the bottom.

6. *Colony Counter*

- It is a device used for counting small or closely growing colonies on the surface of media.
- For accuracy, the lens fitted or mounted in it helps to see the colonies.
- The lens is movable on the box and can be adjusted to see the colonies.
- The petriplate is kept on a slanting platform meant for it and illuminated with the help of light source from beneath.
- The numbers of colonies are read out with the support of digital reading meter.



Inoculation Loop & Needle

Bunsen Burner

Water Bath

Laminar Air Flow

Incubator

Colony Counter

Fig. 1. Tools in microbiology laboratory

The Microscope

A good microscope is an essential tool for any microbiology laboratory. There are many kinds of microscopes but the type most useful in diagnostic work is the compound microscope. By means of a series of lenses and a source of bright light, it magnifies and illuminates minute objects such as bacteria and other microorganisms that would otherwise be invisible to the eye. This type of microscope will be used throughout your laboratory course. As you gain experience using it, you will realize how precise it is and how valuable for studying microorganisms present in clinical specimens and in cultures. Even though you may not use a microscope in your profession, a firsthand knowledge of how to use it is important. Your laboratory experience with the microscope will give you a lasting impression of living forms that are too small to be seen unless they are highly magnified. As you learn about these "invisible" microorganisms, you should be better able to understand their role in transmission of infection.

Instructions

- Observe that a flat platform, or stage as it is called, extends between the upper lens system and the lower set of devices for providing light. The stage has a hole in the center that permits light from below to pass upward into the lenses above. The object to be viewed is positioned on the stage over this opening so that it is brightly illuminated from below. Note the adjustment knobs at the side of the stage, which are used to move the slide in vertical and horizontal directions on the stage. This type of stage is referred to as a mechanical stage.
- A built-in illuminator at the base is the source of light. Light is directed upward through the abbe condenser. The condenser contains lenses that collect and concentrate the light, directing it upward through any object on the stage. It also has a shutter or iris diaphragm which can be used to adjust the amount of light admitted. A lever is provided on the condenser for operating the diaphragm.
- The condenser can be lowered or raised by an adjustment knob. Lowering the condenser decreases the amount of light that reaches the object. This is usually a disadvantage in microbiological work. It is best to keep the condenser fully raised and to adjust light intensity with the iris diaphragm.

Fig. 2. Microscope

- Above the stage, attached to the arm, a tube holds the magnifying lenses through which the object is viewed. The lower end of the tube is fitted with a rotating nosepiece holding three or four objective lenses. As the nosepiece is rotated any one of the objectives can be brought into position above the stage opening. The upper end of the tube holds the ocular lens, or eyepiece (a monocular scope has one; a binocular scope permits viewing with both eyes through two oculars).
- Depending on the brand of microscope used, either the rotating nosepiece or the stage can be raised or lowered by coarse and fine adjustment knobs. These are located either above or below the stage. On some microscopes they are mounted as two separate knobs; on others they may be placed in tandem with the smaller fine adjustment extending from the larger coarse wheel. Locate the coarse adjustment on your microscope and rotate it gently, noting the upward or downward movement of the nosepiece or stage. The coarse adjustment is used to bring the objective down into position over any object on the stage, while looking at it from the side to avoid striking the object and thus damaging the expensive objective lens. The fine adjustment knob moves the tube to such a slight degree that movement cannot be observed from the side. It is used when one is viewing the object through the lenses to make the small adjustments necessary for a sharp, clear image.

Care and Handling of the Microscope

- Always use both hands to carry the microscope, one holding the arm, other under the base.
- Before each use, examine the microscope carefully and report any unusual condition or damage.
- Keep the oculars, objectives, and condenser lens clean. Use dry lens paper only.
- At the end of each laboratory period in which the microscope is used, remove the slide from the stage, wipe away the oil on the oil-immersion objective, and place the low-power objective in vertical position.
- Replace the dust cover, if available, and return the microscope to its box.

Handling and Examining Cultures

Microscopic examination of microorganisms provides important information about their morphology but does not tell us much about their biological characteristics. To obtain such information, we need to observe microorganisms in culture. If we are to cultivate them successfully in the laboratory, we must provide them with suitable nutrients, such as protein components, carbohydrates, minerals, vitamins, and moisture in the right composition. This mixture is called a culture medium. It may be prepared in liquid form, as a broth, or solidified with agar, a nonnutritive solidifying agent extracted from seaweed. Agar media may be used in tubes as a solid column or as slants, which have a greater surface area. They are also commonly used in petri dishes, or plates.

Solid media are essential for isolating and separating bacteria growing together in a specimen collected from a patient, for example, urine or sputum. When a mixture of bacteria is streaked across the surface of an agar plate, it is diluted out so that single bacterial cells are deposited at certain areas on the plate. These single cells multiply at those sites until a visible aggregate called a colony is formed. Each colony represents the growth of one bacterial species. A single, separated colony can be transferred to another medium, where it will grow as a pure culture. Colonies of several different species are regularly present on the same agar plate when certain patient specimens are inoculated onto them. Working with pure cultures permits the microbiologist to study the properties of individual species without interference from other species.

The appearance of colonial growth on agar media can be very distinctive for individual species. Observation of the noticeable, gross features of colonies, that is, of their colonial morphology, is therefore very important. The colour, density, consistency, surface texture, shape, and size of colonies all should be observed, for these features can provide clues as to the identity of an organism, although final identification cannot be made by morphology alone.

In liquid media, some bacteria grow diffusely, producing uniform clouding, whereas others look very granular. Layering of growth at the top, center, or bottom of a broth tube reveals something of the organisms' oxygen requirements. Sometimes colonial aggregates are formed and the bacterial growth appears as small puff balls floating in the broth. Observation of such features can also be helpful in recognizing types of organisms.

Disposal of Laboratory Wastes and Cultures

During laboratory practices, it has been noticed that the untreated waste is generally disposed off by the laboratory staff. It happens due to their unskilled work culture. Most of the laboratories situated in the rural area used discarded hospital material for land filling purposes. Any material which contains microorganisms should be treated first and thereafter, with the proper treatment should be thrown properly.

The treatment is necessary due to the reasons:

a) If it contains pathogenic microorganisms, the disease may transmit or spread to the healthy persons.
b) It may contaminate soil and causes soil, water and air-pollution. Hence, to check from such hazards, proper treatment is required to kill microorganisms.

The infected material is generally the solid or liquid culture media used for cultivation of microorganisms, or it may also contain cotton plugs, paper, cotton or cotton swabs, gloves, pins, PCR tubes, gel material etc.

Some of the materials such as cotton plugs, paper, napkins, swabs etc. should be autoclaved first and then it is incinerated. But the microbial contaminants containing materials should be treated with some disinfectant and thereafter autoclaved by putting them in suitable containers. The molten material should be discarded.

Sometimes, HCl is also added to hydrolyze the agar, if present in the medium. This is added before their safe disposal. All such laboratory materials should be disposed of after autoclaving.

2

METHODS IN MICROBIOLOGY

Sterilization

Sterilization is the process of destroying or physically removing all forms of microbial life including vegetative cells, spores and viruses from a surface, a medium or an article. The principal reasons for controlling microorganisms are:

1. To prevent transmission of disease and infection
2. To prevent contamination by undesirable microorganisms
3. To prevent deterioration and spoilage of materials by microorganisms

The methods of sterilization employed depend on the purpose for which sterilization is carried out, the material which has to be sterilized and the nature of the microorganisms that are to be removed or destroyed. The various agents used in sterilization can be grouped into physical and chemical agents

Physical Agents (Physical Methods)

- *Sunlight*

Direct sunlight has an active germicidal effect due to the combined effect of the ultraviolet radiation and heat. This is a natural method of sterilization.

- *Drying*

Moisture is essential for the growth of bacteria. Drying in air has therefore a deleterious effect on many bacteria. But spores are unaffected by drying. Hence this is very unreliable method.

- *Heat*

Heat has a killing effect on microorganisms and is one of the most popular reliable methods to destroy. Microorganisms has a minimum, optimum and maximum growth temperatures. Temperature below the minimum usually produces static (inhibition of metabolism).

Temperature above the maximum, generally kill microorganisms. This is because biochemical changes in the cells organic molecules result in its death. These changes arise from alterations in enzyme molecules or chemical break down of structural molecules especially in the cell membranes. Heat also drives off water and this loss of water may be lethal to the organisms.

The killing rate of heat may be expressed as a function of time and temperature. Each microbial species has a Thermal Death Time (TDT). It's the minimum time required to kill a population of microorganism in a microbial suspension at a given temperature and under defined condition. Each species also has a Thermal Death Point (TDP), the temperature at which it dies in a given time.

In determining the time and temperature for microbial destruction with heat, the following factors are considered.

1. Type of organism to be killed
2. Type of material to the treated
3. Presence of organic matter
4. Acidic or basic nature of the material

Nature of heat:

a) Dry heat

Many objects are best sterilized in the absence of water by dry heat sterilization; killing by dry heat is due to protein denaturation, oxidative damage and toxic effect of elevated levels of electrolytes.

Methods of Sterilization by Dry Heat

1. *Flaming*

Inoculating loops and points of forceps may be heated in the Bunsen flame, till they are red-hot. Articles such as mouth of the culture tubes, cotton wool plugs, glass slides etc. are passed over the flame without allowing it to become red hot.

2. *Incineration*

This is an excellent method for rapidly destroying, animal carcasses, pathological material and disposables.

3. *Hot Air Oven*

This is the most widely adopted method of sterilization by dry heat. The hot air oven utilizes radiating dry heat for sterilization. This type of energy does not penetrate materials easily and thus, long periods of exposure to high temperature are necessary. For example, at a temperature of 160°C, a period of two hours is required for the destruction of bacterial spores. Hot air oven is used to sterilize glassware, forceps, scissors, scalpels, glass syringes, liquid paraffin, dusting powder etc. A holding period of 160°C for an hour is used. The oven is usually heated by electricity, with heating elements in the wall of the chamber and it must be filled with a fan to ensure even distribution of hot air and elimination of air pockets. The materials should be arranged in a manner which allows free circulation of hot air in between the objects. It should not be over-loaded. Glass wares should be perfectly dry before being placed in the oven. Test tubes, flasks etc. should be wrapped in craft paper. Oven must be allowed to cool slowly for about 2 hours before the door is opened, since the glasswares may get cracked by sudden or uneven cooling.

Fig. 3. Hot Air Oven

Sterilization control: The spores of a non – toxigenic strain of *Clostridium tetani* are used as a microbiological test of dry heat efficiency. Paper stripes impregnated with 10^6 spores are placed in envelop and inserted into suitable packs. After sterilization is over, the strips are removed and inoculated into thioglycollate or cooked meat media and incubated for sterility test under strict anaerobic conditions for five days at 37°C.

b) Moist heat

Moist heat kills microorganisms by coagulating their proteins and is much more rapid and effective than dry heat because water molecules conduct heat better than air. Lower temperature and less time of exposure are therefore required than for dry heat. Moist heat readily kills viruses, bacteria, fungi etc.

a) Temperature below 100°C

i. Pasteurization of milk

For pasteurization of milk, there are two methods

- *Holding Method or Low Temperature Holding Method (LTH)*

  In this method, the milk is exposed to a temperature of 63°C (145°F) for 30 minutes in an appropriately designed equipment. This is followed by sudden cooling to 13°C or below.

- *Flash Process or High Temperature Short Time (HTST)*

  In this method, the milk is exposed to a temperature of 72°C for 15 seconds in the equipment. This is followed by sudden cooling to 13°C or below. The finished product should be stored at a low temperature to retard growth of microorganisms and pasteurization removes the pathogenic bacteria in milk. By these

processes all non-sporing pathogens such as mycobacteria, salmonellae and brucella are destroyed '*Coxiella bumetic'* is relatively heat resistant and may survive the holder method.

ii. *Vaccine bath*

It's used for killing non-sporing bacteria which may be present in vaccine. In vaccine bath, the vaccine is treated with moist heat for one hour at 60°C.

iii. Serum containing coagulable proteins can be sterilized by heating for one hour at 56°C in a water bath for several successive days.

b) Temperature at 100°C

i. Boiling

Most of the vegetative forms of bacteria, fungi etc. are killed almost immediately at 90-100°C, but sporing bacteria required considerable periods of boiling. Boiling water is not considered as a sterilizing agent because destruction of bacterial spores and inactivation of viruses cannot always be assured. Under ordinary circumstances, most species of microbes can be killed within 10 minutes. However, spores of bacteria and fungi, protozoa cysts and large concentrations of Hepatitis A viruses requires up to 30 minutes exposure or more because inadequate information exists on the heat tolerance of many microorganisms, boiling water is not reliable for sterilization purpose especially the sterilization of instruments and for surgical procedures.

In cases where boiling is considered adequate, the material should be immersed in water and boiled for a period of 10-30 minutes. The lid of the sterilizer should not be opened during that period. Addition of little acid, alkali or washing soda will increase the efficiency of the process.

ii. Steam under atmospheric pressure (100°C)

Steam under atmospheric pressure is used to sterilize culture media which may decompose if subjected to higher temperature. A Koch or Arnold sterilizer is an instrument that generates free floating steam.

The container and the medium are simultaneously sterilized and evaporation from the medium is prevented one exposure of 90 minutes usually ensures complete sterilization of the medium. This is an inexpensive method.

iii. Sterilization above 100°C (steam under pressure)

Heat in the form of saturated steam under pressure is the most practical and dependable agent for

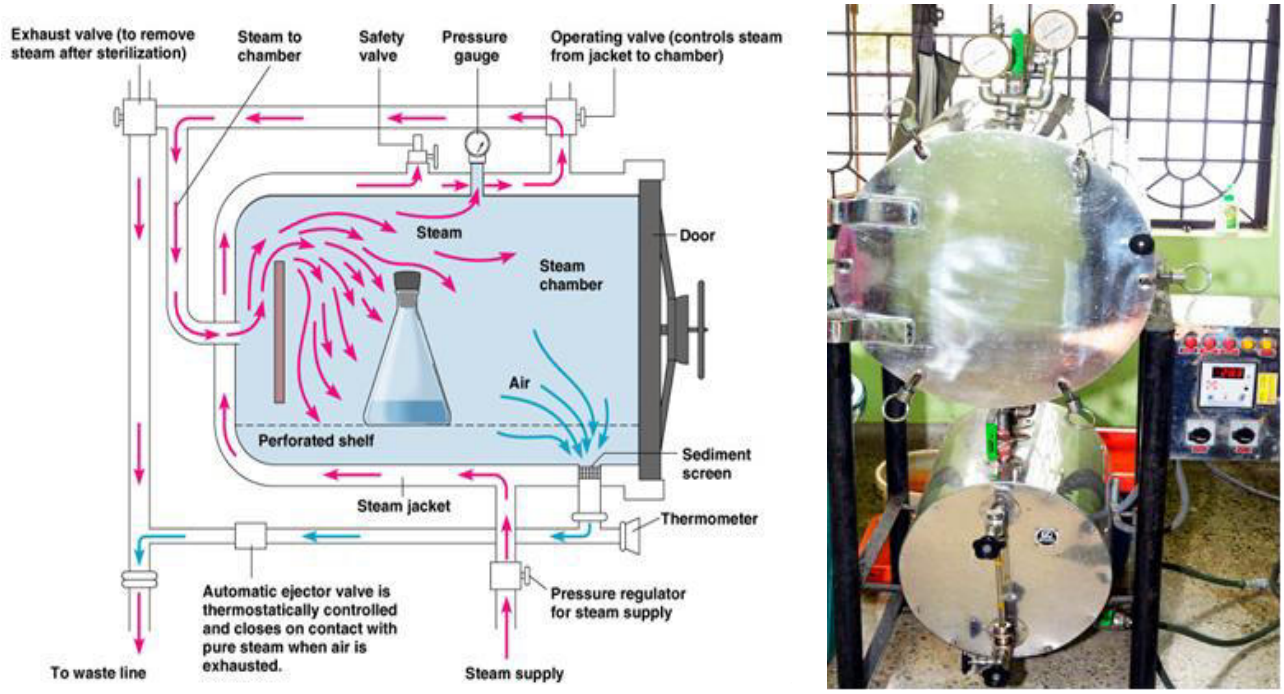


Fig. 4. Autoclave

sterilization. Steam under pressure provides temperature above those obtainable by boiling. Moreover, it has advantages of rapid heating, penetration and moisture in abundance, which facilitates the coagulation of the protein of microorganisms, resulting in complete destruction of all forms of microbial life, including bacterial endospores. It is important to note that the sterilizing agent is the moist heat not the pressure. The laboratory apparatus designed to use steam under regulated pressure is called an autoclave. It is essentially a double jacketed steam chamber equipped with devices which permit the chamber to be filled with saturated steam and maintained at a designed temperature and pressure for any period of time The articles to be sterilized are placed in the sterilizing chamber and steam is maintained in the steam jacket into the sterilizing chamber, cool air is forced out and a special valve increases the pressure to 15 pounds/square inch above normal atmospheric pressure. The temperature rises to 121.5°C and the superheated water molecules rapidly conduct heat into microorganisms and will be killed. The time for destruction of the most resistant bacterial spore is reduced to 15 minutes. For denser objects, up to 30 minutes of exposure may be required.

Autoclave is an essential equipment in every microbiology laboratory. It's used to sterilize many media, solutions, discarded cultures, glass wares, metal wares etc.

- *Filtration*

  Filtration is the process of removal of microorganisms from liquid or gases using a mechanical device known as filter. This is an excellent way to reduce the microbial population in solution of thermo labile materials such as sera, antibiotic solutions, intravenous solutions, carbohydrates solutions used in the preparation of culture media etc. As fluid passes through the filter, microorganisms are trapped in the pores of the filtering material. The solution that drips through the filter is collected in a previously sterilized container. Porosity, electric charges of the filter, electric charge carried by the organisms, nature of the fluid being filtered etc. can influence efficiency of filtration.

  Types of filters: Seltz Filter, Berkefeld Filter, Membrane Filter, High Efficiency Particulate Air (HEPA) filter

- *Irradiation*

  The process of exposing organisms to anyone of the radiation such as UV-rays, X-rays, gamma rays etc. is known as irradiation. Irradiation is an effective method of sterilization. Two types of radiations are used for sterilization.

  a) Non ionizing radiation
     - UV radiation
     - Infrared radiation
  b) Ionizing radiation
     - X rays
     - Gamma rays

Chemical Methods

The physical agents for controlling microorganisms are generally intended to achieve sterilization. Instead, they are expected only to destroy the pathogenic organisms on or in or removal of pathogenic microorganisms is called disinfection. An agent, usually a chemical that kills the pathogenic microorganisms on/in animate objects is known as a disinfectant. A disinfectant does not necessarily sterilize an object because a few microorganisms and viable spores may remain. The chemical agents that are applied to the body to prevent infection or species are called antiseptic. The antiseptic prevents the growth or action of microorganisms either by destroying them or by inhibiting their growth and metabolism.

An antimicrobial agent is generally called as a germicide. A disinfectant or antiseptic can be particularly effective against a specific group called as bactericides, fungicides or algaecides. Some chemicals do not kill microorganisms, but they temporarily prevent from multiplication. Many different chemicals are available for use as disinfectants and each has its own advantages and disadvantages.

Characteristics of a desirable disinfectant

The disinfectant must be effective against a wide variety of infections/agents, at high dilutions and in the presence of organic matter. The chemical must be toxic for infection agents but it should not be toxic to people or corrosive for common upon storage, colourless with a pleasant odors, soluble in water and lipids for penetration into microbes and have a low surface tension so that it can cracks in surface. If possible, the disinfectant should be relatively inexpensive.

The factor influencing the effectiveness of chemical disinfectants:

1. Size of the microbial population
2. Nature of microbes present
3. Concentration and nature of the disinfectant
4. Duration of exposure
5. Temperature
6. Local environment

The main modes of action of disinfectant:

1. Protein coagulation
2. Disruption of cell membrane, thus resulting in exposure of the contents of the cell to the adverse environment and loss of the constituents of the cell and changes in the composition of the contained cytoplasm. These cause death of cell.
3. Removal of free sulfhydryl groups which are essential for the functioning of the enzymes and thus the life of the cells.
4. Inhibition of respiration of catabolic/anabolic reactions.
5. Loss of membrane integrity resulting in leakage of essential constituents such as potassium cations, inorganic phosphate, pentose, nucleotides and nucleosides and proteins.

Major Group of Chemical Agents: Phenol and Phenol compounds, Alcohols, Heavy metals and their compounds, Dyes, Soaps and Detergents, Aldehydes, Gaseous agents

3

CULTURAL CHARACTERISTICS OF MICROORGANISMS

Aim

To determine the cultural characteristics of microorganisms in identifying and classifying organisms into taxonomic groups

Principle

When grown on a variety of media, microorganisms will exhibit the difference in the microscopic appearance of their broth. These differences are called cultural characteristics and are used as a basis for separating microorganism into taxonomic groups. The cultural characteristics for all taxon microorganisms are contained in Bergey's Manual of systematic bacteriology.

The following characters of colony are noted:

1. Size: in millimeter
2. Shape: circular / irregular
3. Surface : smooth, rough, granular
4. Elevation : flat, low convex, high convex, raised, umbonate, umbulate
5. Edge: entire, undulate, lobate, crenated, fimbricate, ciliate
6. Opacity : opaque, translucent, transparent
7. Colour of colony
8. Consistency : mucoid, friable
9. Other properties : hemolysis, pigmentation, swarming

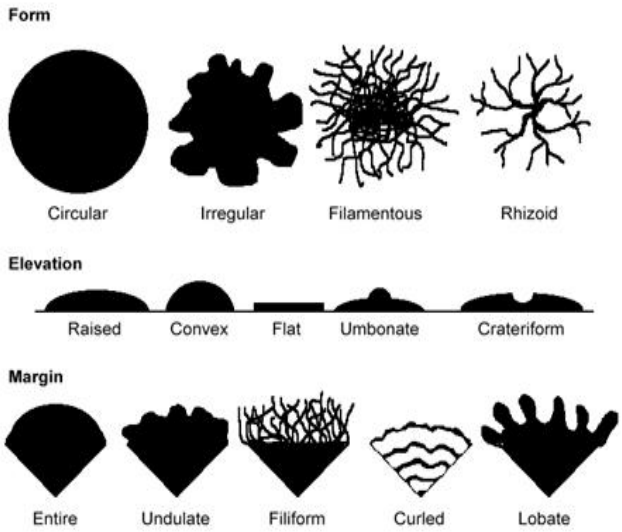


Fig. 5. Growth on Solid Media

Morphology on nutrient agar slants

The isolated bacteria can be identified based on their colony characteristics in the following manner.

1. Degree of growth : scanty, moderate, abundant
2. Surface : smooth, rough, granular
3. Elevation : convex, flat, raised
4. Edge : entire, undulate, crenate
5. Opacity : opaque, translucent, transparent
6. Consistency : firm, butyrosis, powdery, mucoid, membranous
7. Colour of Colony : creamy white, lemon yellow, bluish green
8. Form : filiform, echinulated, beaded, effuse, rhizoid
9. Changes in Medium : changes in colour, pitting of agar

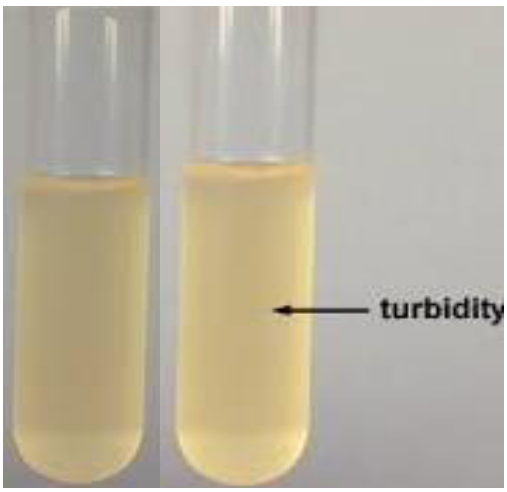


Fig. 6. Growth on Liquid Media

Morphology on Nutrient Agar Plates

These demonstrate well isolated colonies and are evaluated in the following manner.

1. *Size*: pinpoint, moderate, small or large
2. *Colour of the colony*
   a) Form: the shape of the colony:
   b) circular: unbroken, peripheral edge
   c) irregular: intended, peripheral edge
   d) rhizoid: root like, spreading growth
3. *Margin*: The appearance of the outer edge of the colony is described as follows
   a) entire: sharp
   b) lobate: marked indentations
   c) undulate: wavy indentations
   d) serrate: tooth like appearance
   e) filamentous: thread like, spreading edge
4. *Elevation*: the degree to which the colony growth is raised on the surface is described as follows:
   a) flat: elevation not discernible
   b) raised: slightly elevated
   c) convex: dome shaped elevation
   d) umbonate : raised with elevated convex central region

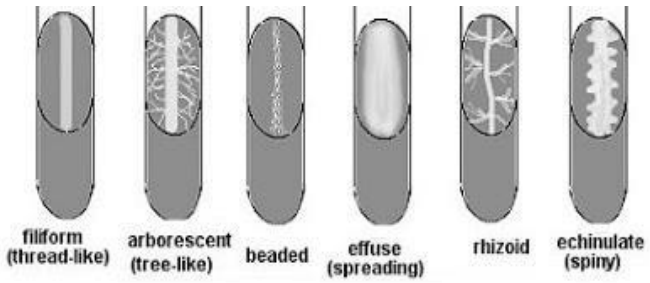


Fig. 7. Growth on Nutrient Agar Slant

Growth in Liquid Media

The liquid medium (nutrient broth, peptone water and other liquid media) the following characteristics are noted:

1) the degree of growth : scanty, moderate, abundant
2) presence of turbidity and its nature (uniform turbidity)
3) presence of deposits, pellicle formation on surface & its quality

Morphology on Nutrient Broth Cultures

These are evaluated for the distribution & appearance of the growth as follows:

1) uniform time turbidity : finely dispersed throughout
2) flocculent: flank aggregates, dispersed throughout
3) pellicle: thick, pad like growth on the surface
4) sediment: concentration of growth at the bottom of broth cultures may be granules

Fig. 8. Growth on Plates

4

CULTURE MEDIUM

The food materials on which the organism is grown is known as culture medium and the growth of organism is known as culture. Different microorganisms require different nutrient materials. Thus, culture media vary in form and composition, depending upon the species to be cultivated. It must contain all the ingredients required by the organism and in certain proportions. Basically there should be an energy source, various macro and micronutrients, vitamins etc. it must have a suitable pH. Moreover, it must be sterile so that the organism cultivated may form a pure culture.

A culture is an *in vitro* technique of growing or cultivating microorganisms or only other cells in a suitable nutrients medium called culture medium in the laboratory. The primary aim of constructing a culture medium for any microorganism is to provide a balanced mixture of required nutrients, at concentrations that will permit good growth. Culture media give artificial environment simulating natural conditions necessary for growth.

Characteristics of an Ideal Culture Medium

- Satisfactory growth for small inoculum – even for single cell.
- Rapid growth
- Easy to prepare
- Cheap
- Easily producible
- Demonstrate all the characteristics in which we are interested

Basic Requirements of Culture Medium

- Energy source
- Carbon source
- Nitrogen source
- Salts
- pH
- Adequate oxidation
- Growth factors

Common Ingredients of Culture Media

1. Water
   It is essential for existence of living cells. They act as source of hydrogen and oxygen.
2. Peptone
   Golden granular hygroscopic powder which are obtained from meat, casein fibrin or soya bean flour.
   Function: nitrogen source, carbon source, buffers
3. Meat Extract
   It contains protein degradation products, carbohydrates, inorganic salts, enzymes, excites and growth factors that are rich in vitamin B complex.
   Function: Source of growth factors, inorganic salts etc.
4. Yeast Extract
   It contains proteins, amino acids, growth factors (Vitamin B), Carbohydrates and inorganic salts like potassium and phosphates.

Functions: Source of growth factors and hence excellent stimulators of growth. It can be used as suitable for meat extract.

5) Electrolyte
Mainly used are sodium chloride or other electrolytes.
Functions: Essential to maintain the osmotic pressure

6) Agar
Dried mucilaginous substance obtained from gelidium species and other algae available as long shield or in powder form; contains mainly long chain polysaccharides, protein like material and inorganic salts.
Functions: it melts at 98°C and solidifies at 42°C, hence used as solidifying agent.

7) Fermentable Compounds
Mainly used are sugars, alcohols etc.
Function: Act as source of energy, fermentation reactions are helpful in the identification and classification of organisms.

8) Buffers
Carbonates and phosphates are used as buffer.
Function: To resist change in pH of the medium.

Types of Media

- Liquid Media
- Semi Solid Media
- Solid Media

a) Liquid Media or Broth

No solidifying agents (eg: agar) is added while preparing the medium. The most commonly used non-synthetic liquid media are nutrient broth, peptone solution, milk, blood, serum etc. broth is a clear transparent straw coloured fluid prepared from meat extract or peptone. Beef infusions are rich in minerals, organic micronutrients, protein, protein derivatives and carbohydrates. They are often supplemented with 1% peptone or yeast extract culture fluids made from beef infusion are commonly called infusion broth, where as those made from beef extract are called extract broth.

Advantages of Liquid Media

- For obtaining bacterial growth from blood or water when large volumes have to be tested.
- For preparing bulk cultures for preparation of antigens or vaccines.
- It's used to study growth rate and the sedimentation rate of bacterial cells.

Disadvantages of Liquid Media

- It's difficult to isolate different types of bacteria from mixed population.
- It's difficult to study colony characteristics.

b) Semi-Solid Media

The semi-solid medium remains in the semi-solid condition. It is prepared by adding small amount of agar (0.5%) or gelatin. Agar is a complex carbohydrates prepared from algae like gelidium and gracillaria. Agar forms a colloidal solution in hot water and sets in the form of a jelly when cool. Gelatin is an animal extract prepared by hydrolysis of collagen with boiling water. When dissolved in water, it's in the form of a liquid when warm and sets in the form as it cools. Many bacteria, when grown on a gelatin medium, produce a digestive enzyme gelatinase, which liquefies gelatin. This feature is important in the identification and classification of bacteria.

The semi-solid medium may be selective which promotes the growth of one organism and retards the growth of another organism. This type of medium can be used to study bacterial motility (semisolid media are useful for cultivation of microaerophlic bacteria).

c) Solid Media

The solid medium is solid in consistency. It is prepared by adding 2% or 1% gelatin; agar or silica gel is sometimes an inorganic solidifying agent for autotrophic bacteria. It's used for colony characterization, colony identification, etc.

Based on composition, culture media can be classified into:

- Simple Media
- Complex Media
- Synthetic or defined Media
- Semi Solid Media
- Special Media

a) Simple Media

It's also called basal media. eg: Nutrient Broth. It consists of meat extract, peptone, Sodium Chloride and water. Nutrient agar made by adding 2% agar to nutrient broth is the simplest and commonest medium in routine diagnostic laboratories.

b) Complex Media

These have added ingredients for special purpose or for bringing out certain characteristics or providing special nutrient required for the growth of certain organisms.

c) Synthetic or defined Media

These media are prepared from pure chemical substances and the exact composition of the medium is known. They are used for research purpose.

d) Semi Solid Media

The nutritional requirements of some microorganisms include some additional ingredients of unknown chemical composition such as peptone, meat extract, yeast extract, etc. Chemical composition is not fully known. They are called semi solid media.

e) Special Media

No single medium or set of conditions can support the growth of all the different types of organisms that occur in nature. To cultivate, recognize, enumerate and isolate certain types of microorganisms many special purpose media are needed on the basis of their application or function, these special media can be classified into different types.

- Enriched Media
- Enrichment Media
- Selective Media
- Indicator Media
- Differential Media
- Selective Media
- Sugar Media
- Transport Media

i. Enriched Media

In these media, substances like blood, serum or egg are added to a basal medium

eg. Blood Agar, Chocolate Agar, Egg Media etc

ii. Enrichment Media

Some substances are added to liquid media with the result that wanted organism grows more in number than unwanted organism. Such media are used in mixture cultures

eg. Tetrathionate broth (inhibit coliforms and allow typhoid paratyphoid bacilli to grow freely)

iii. Selective Media

It favors the growth of particular microorganism. This is like enrichment media with the difference that inhibiting substance is added to solid medium.

eg. Desoxycholate citrate medium for dysentery bacilli

iv. *Indicator Media*

These media contain an indicator which changes colour when a bacterium grows in them.

eg. Incorporation of sulphite in Wilson and Blair medium *Salmonella typhi* reduces sulphite to sulphide in Wilson and Blair medium and the colonies of *S. typhi* have a black metallic sheen.

v. *Differential Media*

Media that distinguish between different groups of bacteria and even permit to identification of microorganisms based on their biological characteristics. A medium which has substances incorporated into it, enabling it to bring out differing characteristics of bacteria and thus helping to distinguish between them. Such media are called differential media eg. Mac conkey medium consists of peptone, lactose, agar, neutral red and taurocholate. It shows lactose fermenters, are colourless or pale (this may also be termed indicator medium).

Blood agar is an enriched medium, but also differentiates between hemolytic organisms and non- hemolytic organisms. So it also acts as a differential medium.

vi. *Sugar Media*

The usual sugar media consist of 1% sugar concerned. In peptone water along with appropriate indicator, a small tube (Durham's tube) is kept inverted in sugar tube to detect gas production.

vii. *Transport Media*

Delicate organisms like gonococci which may not survive the time taken which may not survive the time taken for transporting the specimen to the laboratory or may be overgrown by non-pathogens (dysentery or cholera organisms) require a special medium called transport medium.

eg. Stuart Medium for gonococci.

viii. *Anaerobic Media*

These media are used to grow anaerobic organisms.

eg. Robertson's Cooked Meat Media

5

MEDIA PREPARATION

1. *Peptone Broth*

Peptone	: 10g
NaCl	: 5g
Distilled water	: 1000ml

2. *Nutrient Agar*

Peptone	: 5g
NaCl	: 5g
Beef extracts	: 3g
Agar	: 20g
Distilled water	: 1000ml

The ingredients are dissolved in warm water and pH adjusted to 7.2-7.6. Autoclaved at 121°C, 15 lbs for 15 minutes.

3. *Nutrient Broth*

Peptone	: 5g
NaCl	: 5g
Beef extracts	: 3g
Distilled water	: 1000ml

The ingredients are dissolved in warm water and pH adjusted to 7.2-7.6. Autoclaved at 121°C, 15 lbs for 15 minutes.

4. *Mac Conkey Agar*

Peptone	: 20g
NaCl	: 5g
Bile salt	: 1.5g
Lactose	: 10g
Neutral red solution	: 10ml
Crystal violet	: 0.001g
Agar	: 13.5g
Distilled water	: 1000ml

5. *Sabouraud's Dextrose Agar (SDA)*

Peptone	: 10g
Dextrose	: 40g
Chloramphenicol	: 0.05g
Agar	: 15g
Distilled water	: 1000ml

6. *Sabouraud's Dextrose Broth*

Peptone	: 10g
Dextrose	: 40g
Chloramphenicol	: 0.05g
Distilled water	: 1000ml

7. *Mueller – Hinton Agar*

Beef infusion form	: 300g
Acid hydrolysate of casein	: 17.5g
Agar	: 17g
Starch	: 1.5g

8. *Lactose Broth*

Peptone	: 5g
Beef extract	: 3g
Lactose	: 5g
Distilled water	: 1000ml

9. *EMB (Eosin Methylene Blue) Agar*

Peptone	: 10g
Lactose	: 5g
Sucrose	: 5g
Dipotassium hydrogen phosphate	: 2g
Eosin Y	: 0.40g
Methylene blue	: 0.065g
Agar	: 13.50g
Distilled water	: 1000ml

10. *Methylene Blue Solution (1:25,000)*

Methylene blue dye	: 1mg
Distilled water	: 25ml

Dissolved the methylene blue in distilled water and was dispensed into regular staining bottles.

11. *Carbohydrate Fermentation*

Peptone	: 1g
Carbohydrates	: 10g
NaCl	: 5g
Phenol red indicator	: 10ml (0.1g in 10ml ethanol)
Distilled water	: 1000ml

Mix all the ingredients, except phenol red indicator. Adjust pH to 7. Then add phenol red indicator. Dispense the medium in 8ml test tubes containing the Durham's tubes. Sterilize the medium at 10lbs for 20 minutes.

12. *Oxidation – Fermentation*

Peptone	: 20g
Dipotassium hydrogen phosphate	: 2g
NaCl	: 5g
Bromothymol blue	: 3ml (1% aqueous solution)
Agar	: 13.50g
Distilled water	: 1000ml

Mix all the ingredients, expect Bromothymol blue indicator. Adjust pH to 7.1. Then add Bromothymol blue indicator. The medium is poured into the tube to a depth of about 4cm. sterilized by autoclaving at 121°C for 20 minutes at 10 lbs. it was then allowed to set.

13. *Voges – Proskauer*

Reagents: Barrett's A

α – naphthol	: 5g
Ethanol	: 100ml

Dissolve α – naphthol in small amount of alcohol and then add remaining alcohol to 100ml. Store in brown bottle at 4°C.

Barrett's B

Potassium hydroxide	: 40g
Distilled water	: 100ml

Cool the volumetric flask in cold water with 80ml water, add KOH crystals, dissolve and make up to100ml. Store in polyethene bottles at 4°C.

14. Citrate Utilization

$MgSo_4$	: 0.2g
Ammonium dihydrogen phosphate	: 1g
Dipotassium phosphate	: 1g
Sodium citrate	: 2g
Sodium chloride	: 0.5g
Bromothymol blue	: 0.08g
Agar	: 15g
Distilled water	: 1000ml

Dissolve the ingredients in 1000ml distilled water. Dispense in tubes and sterilize by autoclaving at 121°C for 20 minutes at 10 lbs.

15. Nitrate Broth

Beef extract	: 3g
Peptone	: 5g
Potassium Nitrate	: 1g
Distilled water	: 1000ml

Dissolve all the ingredients and sterilize by autoclaving at 121°C for 20 minutes at 15 lbs.

Reagents: Sulphanlic acid

Dissolve 8g of sulphanlic acid in 1 l of acetic acid.

α- Naphthol amines

Dissolve 5g of α-Naphthol amines in 1 l of acetic acid. Immediately before use, mix equal volumes of Sulphanlic acid and α- Naphthol amines to give the test reagent.

16. Urease Test

Peptone	: 1g
Phenol red	: 0.012g
Dextrose	: 1g
NaCl	: 5g
Disodium phosphate	: 1.2g
Mono potassium phosphate	: 0.8g
Agar	: 15g
Distilled water	: 1000ml

Dissolve ingredients in 950ml distilled water. Sterilize by autoclaving at 10lbs for 20 minutes. Cool to 58°C and aseptically add 50ml of 40% urea. Sterilize the urea solution by autoclaving at 10lbs for 15 minutes, mix well and add the Phenol red indicator. Dispense into sterilized test tubes and allow to set in a slanting position.

17. Mannitol Motility Test

Peptone	: 20g
NaCl	: 5g
Potassium Nitrate	: 2g
Mannitol	: 64g
Agar	: 6g
Distilled water	: 1000ml
Phenol red	: 4ml (1g in 100ml ethanol)

Mix all the ingredients, expect phenol red indicator. Adjust pH to 7. Then add phenol red indicator. Dispense in tubes. Sterilize the medium at 10lbs for 20 minutes.

18. Triple Sugar Iron Agar Test

Peptone	: 20g
Yeast extract	: 3g

Beef extract	: 3g
Lactose	: 10g
Sucrose	: 10g
Glucose	: 10g
Sodium chloride	: 5g
Ferrous sulphates	: 0.2g
Sodium thiosulphate	: 0.3g
Agar	: 12g
Distilled water	: 1000ml
Phenol red	: 0.024g

Mix all the ingredients, expect phenol red indicator. Adjust pH to 7. Then add phenol red indicator. Sterilize by autoclaving at 121°C for 20 minutes. Allow the medium to set in slopped form with a butt about 1 inch long. The medium is used in the form a butt and slant.

6
PLATING TECHNIQUES

The common plating techniques employed in microbiology are Streak Plate Method, Spread Plate Method and Pour Plate Method.

1) *Streak Plate Method*

This method was developed by two bacteriologists, Leoffler and Gaffkey in the laboratory of Robert Koch. This method is routinely employed for the isolation of bacteria in pure culture. In this method a sterilized inoculating loop or transfer needle is dipped into a suitable diluted suspension of microorganisms which is then streaked on the surface of an already solidified agar plate to make a series of parallel, non-overlapping streaks. The process is known as streaking and the plate so prepared is called a streak plate. The main objective of the streak plate method is to produce well separated colonies of bacteria from concentrated suspensions of cells.

A sterilized inoculating needle with a loop made up of either platinum or nichrome wire is used for streaking. One loopful of specimen is transferred onto the surface of the agar plate in a sterile petridish and streaked across the surface in the form of a zig-zag line. This process is repeated thrice to streak out the bacteria on the agar plate so that some individual bacteria are separated from each other. The first streak will contain more organisms than the second and the second more than the third and so on. The last streaks should thin so on. The last streaks should thin out the culture sufficiently to give isolate colonies. The successful isolation depends on spatial separation of single cells. Each colony usually represents the growth from a single organism when such a plate is incubated colonies will appear on the surface of the medium. Because of the high concentration of water in agar, some water of condensation forms in petriplate during incubation. Moisture is likely to drip from the cover to the surface of the agar and spread out, resulting in a confluent mass of growth and running individual colony formation. To avoid this, petriplates are routinely incubated bottom side up. Pure colonies can be obtained from well isolated colonies by transferring a small portion of each to separate culture media.

2) *Spread Plate Method*

The spread plate technique is used for the separation of a dilute, mixed population of the microorganisms so that individual colonies can be isolated. In this technique, a small volume of dilute microbial mixture is transferred to the center of an agar plate and spread evenly over the surface with a sterile L-shaped bent glass rod, while the petridish is spun, at some stage, single cells will be deposited with the bent glass rod on the agar surface. Incubate the agar plate at 37ºC for 24 hours, in the inverted position. The dispersed cells will develop into isolated colonies. Because the number of colonies will be equal to the number of viable organisms in the sample spread plates can be used to count the microbial population.

3) *Pour Plate Method*

In pour plate method, successive dilutions of the inoculum (serially diluting the original specimen) are added into sterile petriplate to which is poured melted and cooled (42ºC - 45ºC) agar medium and thoroughly mixed by rotating the plates which is then allowed to solidify. After incubation, the plates are examined for the presence of individual colonies. The pure colonies may be isolated and transferred into test tube culture media for making pure cultures. This technique is employed to estimate the viable bacterial count in a suspension.

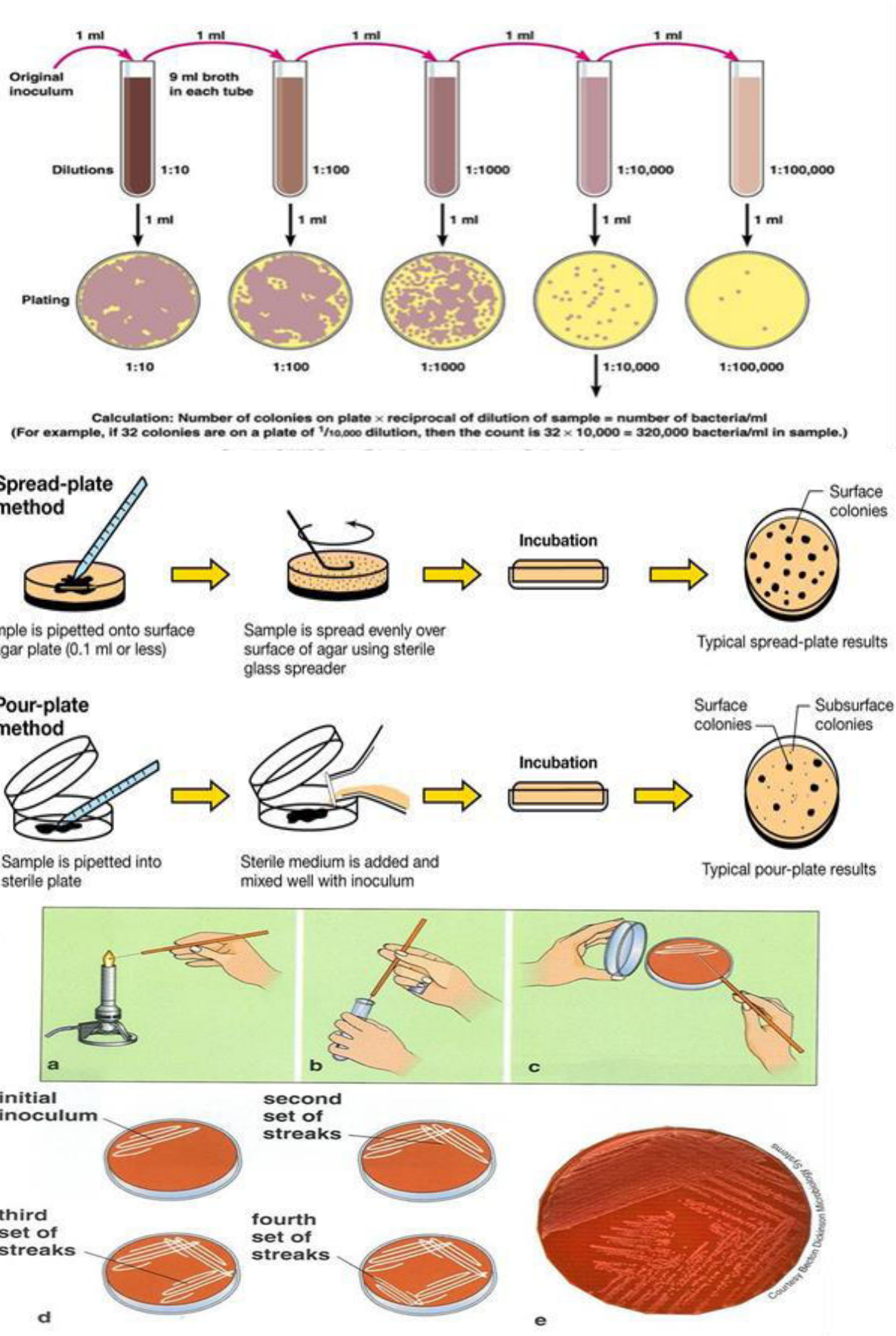


Fig. 9. Plating Techniques

7
METHODS OF ISOLATION OF PURE CULTURE

A culture that contains only one kind of microorganisms is called a pure culture. A culture which contains more than one kind of microorganisms is called mixed culture. Most of the cultures obtained in nature are mixed cultures. Pure cultures are essential to study the cultural, morphological and physiological characters of an individual species. There are different methods for obtaining pure cultures from mixed cultures.

Streak Plate Method
Spread Plate Method
Pour Plate Method
} Follow the procedures as discussed in chapter 6 Plating techniques

Micromanipulator Method

- In this technique, a microscope is used to pick out a single bacterial cell with the help of a device known as micromanipulator. A single viable cell may be transferred on the culture medium to develop turbidity.
- Enrichment, selective and indicator media are widely used for the isolation of pathogens from specimens such as faeces with varied flora.
- Pure culture may be obtained by pre-treatment of specimens with appropriate bactericidal substances which destroy the unwanted bacteria. This method is the standard practice for the isolation of tubercle bacilli from sputum and other clinical specimen.
- Obligate aerobes and anaerobes may be separated by cultivation under aerobic or anaerobic conditions.
- Microorganisms can also be violated by controlling physical environment especially temperature. Bacteria with different optimum growth temperature can be separated by incubating at different temperature. Only thermophiles bacteria grow to 60ºC. A mixture containing vegetative and spore forming bacteria can be separated by heating at 80ºC. In this method, the bacteria in the vegetative state will be eliminated. This method is useful for the isolation of tetanus bacilli from dust and similar sources.
- Separation between motile and non-motile bacteria can be effected using Craigie's tube. This consists of a tube of semisolid agar with a narrow tube open at both ends placed in the center of the medium in such a way that it projects above the level of the medium. The mixture is inoculated into the central tube, the motile bacteria alone transverse the agar and appear at the top of the medium outside the central tube.

8
CULTURE PRESERVATION TECHNIQUES

Microbiologist or laboratories concerned with microbial studies preserve cultures for a short period or many years conserving all the characteristics of the organisms. These preserved cultures may be made available in future for various purposes such as:

- Use in the laboratory classes
- Research work
- Use as test agents for particular procedure

Some methods used for culture preservation include refrigeration, deep freezing, freezing under liquid nitrogen and lyophilization.

1. Refrigeration

Live cultures on a culture medium can be successfully stored in refrigerators or cold rooms maintained at 4ºC. Generally, the metabolic activities of the microorganisms will be greatly slowed down at this temperature. Storing cultures in a refrigerator at a temperature of 4ºC, slows growing protects from damage due to evaporation of medium and preserve the culture. Thus growth will occur slowly, nutrients will be utilized and waste products produced, which will eventually kill the microorganisms. So subculturing of refrigerated cultures is to be carried out at regular intervals. In the case of bacteria, subculturing should be done at intervals of 2-3 weeks. In the case of fungi, regular subculturing is necessary at intervals of 3-4 months.

2. Deep Freezing

Cultures can be preserved for several years in glycerol at 40ºC in a deep freezer. In this method approximately 2 ml of the glycerol solution is added onto the agar slope culture by shaking. The culture suspension is transferred into each ampoule which is placed in a mixture of industrial methylated spirit and CO_2 and is freezed rapidly to -70ºC. Ampoules are removed from the mixture and placed directly into a deep freezer at 40ºC. During transfer from these stock cultures, tubes are placed to water bath at 45ºC for a few seconds or until the suspensions melt and are aseptically streaked onto agar plates.

3. Freezing Under Liquid Nitrogen

Freezing in liquid nitrogen at temperature of -196ºC also suspends metabolism of cells and these survive unchanged for long periods. In this method, cell suspension in the presence of a stabilizing agent such as glycerol or dimethyl sulfoxide, that prevents the formation of ice crystals which may kill frozen cells, is sealed into small ampoules and stored in liquid nitrogen refrigerator. Most species of bacteria can remain viable for 10-30 years or even more without undergoing change in their characteristics. The liquid nitrogen method has been successful with many species that cannot be preserved by lyophilization.

4. Lyophilization

Lyophilization or freeze drying is the rapid dehydration of organisms while they are in a frozen state. Most of the microbes are protected from the damage caused with water loss by this method. Because metabolism requires water, the organisms are in a dormant state and can retain viability for over 30 years unchanged in their characteristics. In this technique, the culture is rapidly frozen at -70ºC and then dehydrated by vacuum and the tubes containing freeze dried cultures are sealed and stored in the dark at 4ºC in refrigerators.

It is the most satisfactory method of long term preservation of microorganisms. It's universally used for the preservation of bacteria, viruses, fungi etc. Lyophilized cultures are revived by opening the vials adding liquid medium and transferring the culture to a suitable growth medium.

Refrigerator

Deep Freezer

Lyophilizer

Fig. 10. Culture Preservation Techniques

9

BACTERIAL IDENTIFICATION

Simple Staining

Aim

To compare the morphological shapes and arrangements of bacterial cells

Principle

In simple staining, bacterial smear is stained with a single reagent, which produces a distinctive contrast between the organism and its background. A simple stain that stains the bacteria is the direct stain. The purpose of simple staining technique is to determine cell shape, size arrangement of bacterial cells. Simple staining is performed by using basic stains which have different exposure time (Crystal Violet 20-60 s, Carbol fuschin 15-30 s and Methylene blue 1-2 miutes).

Procedure

- Clean glass slide was taken and was washed and dried.
- Bacterial smears were prepared from the bacterial cultures.
- The slide was kept on the staining tray and 5 drops of stain was added for a designed period.
- The extra stain was poured off and the smear was washed gently under slow running tap water.
- The slide was then blot dried using blotting paper.
- The slide was then examined under 10X, 45X and oil immersions objects respectively.

Observation

On the basis of microscopic observation, bacteria appeared blue, violet and red respectively depending on the stain taken.

Differential staining

Differential staining requires the use of at least 3 chemical reagents that are applied sequentially to a heat fixed smear. Its function is to impart its colour to all cells. In order to establish a colour contrast, the second reagent used is the decolorizing agent. Based on the chemical composition of cellular components the decolorizing agent may or may not remove the primary stain from the entire cell or from any cell structure. The final reagent is the counter stain. Following discoloration, if the primary stain is not washed out, the counter stain cannot be absorbed and neither the cell nor its components will retain the colour of the primary stain. If the primary stain is removed, the decolorized cellular components will accept and assume the contrasting colour of the counter stain. In this way, cell type or their structure can be distinguished from each other. On the basis of the stain that is retained the most important differential stain used in bacteriology is the Gram stain.

Gram Staining

Aim

To differentiate two principal groups of bacteria

Principle

The Gram stain, a differential stain was developed by Hans Christian Gram, a Danish physician, in 1884. Gram staining classifies bacteria into 2 major groups, Gram positive and Gram negative bacteria. The Gram stain reaction is based on the difference in the chemical and physical composition of bacterial cell wall. Gram positive cells have a thick peptidoglycan layer, whereas peptidoglycan layer in Gram negative cells is much thinner and surrounded by outer lipid containing layer.

In Gram negative, the higher amount of lipid in the formation of large pores thus facilitating the leakage of crystal violet-iodine complex and resulting in the decolonization of the bacterium which later takes this complex counter stain. In contrast, the Gram positive cell wall are thick and composed mainly of proteins and cross linked mucopeptide, when treated with alcohol it causes dehydration and closure of the cell wall pores thereby not allowing the loss of complex and cell retains primary stain. The bacteria which retain the primary stain appear dark blue or violet and not decolorized when stained with Gram's method are called Gram positive, where as those that lose the crystal violet used counter stain, saffranin appear red are called as Gram negative.

The Gram stain uses different reagents in the order, crystal violet, iodine solution, alcohol and saffranin.

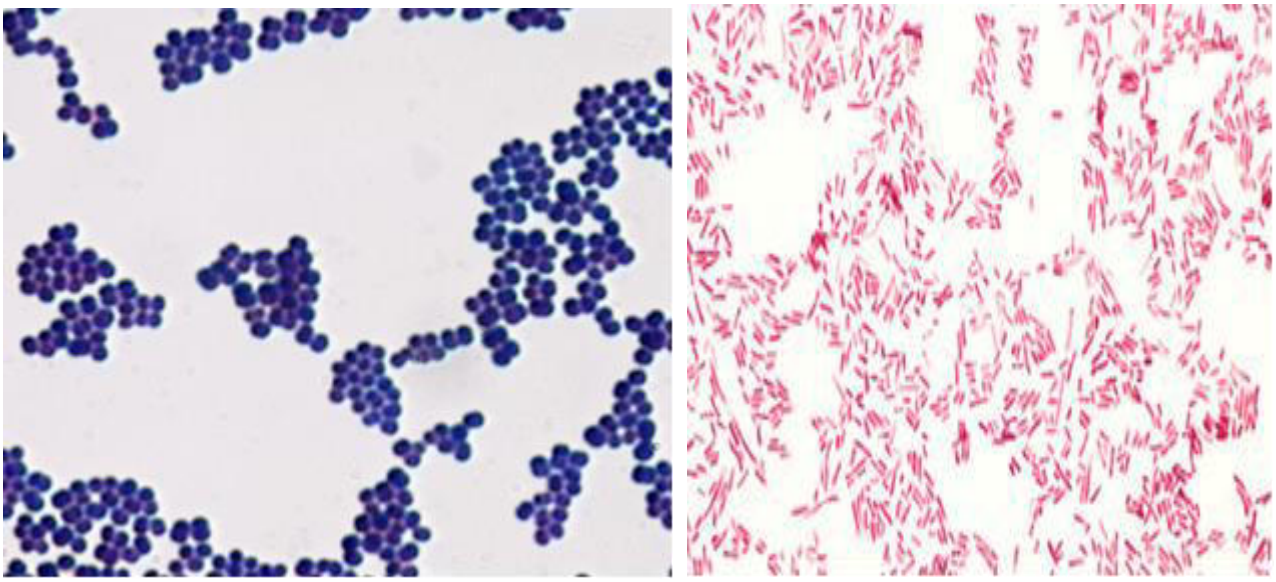
Procedure

- Thin smear was prepared of the given bacterial species on a clean glass slide.
- Let the smear dry.
- Heat fixed smear.
- Hold the smear using the slide rack.
- Covered each smear with crystal violet for 1 minute.
- Washed each slide with distilled water for few seconds using wash bottles.
- Covered each smear with Gram's iodine solution for 1 minute.
- Gently washed with distilled water.
- Decolorized with 95%.
- Washed the slide with distilled water and drained.
- Counter stain was applied saffranin for 30 seconds.
- Washed with distilled water and blot dried with absorbent paper.
- The stained slides were air dried and observed under the microscope.

Observation

- Examined the slides microscopically using oil immersion objective.
- Identified the Gram reaction of the given cultures and classified it and described the morphology and arrangement of cells.

Those bacteria that appear blue are referred to as Gram positive and these appearing pink are described as Gram negative.



Gram Positive **Gram Negative**

Fig. 11. Gram Staining

Hanging-Drop and Wet-Mount Preparations

Procedure

- Take a cover glass and clean it thoroughly, making certain it is free of grease (the drop to be placed on it will not hang from a greasy surface). It may be dipped in alcohol and polished dry with tissue, or washed in soap and water, rinsed completely and wiped dry.
- Take one hollow-ground slide and clean the well with a piece of dry tissue. Place a thin film of petroleum jelly around (not in) the concave well on the slide.
- Gently shake the broth culture of Proteus until it is evenly suspended. Using good aseptic technique, sterilize the wire loop, remove the cap of the tube, and take up a loopful of culture. Be certain the loop has cooled to room temperature before inserting it into the broth or it may cause the broth to "sputter" and create a dangerous aerosol. Close and return the tube to the rack.
- Place the loopful of culture in the center of the cover glass (do not spread it around). Sterilize the loop and put it down.
- Hold the hollow-ground slide inverted with the well down over the cover glass, and then press it down gently so that the petroleum jelly adheres to the cover glass. Now turn the slide over. You should have a sealed wet mount, with the drop of culture hanging in the well.
- Place the slide on the microscope stage, cover glass up. Start your examination with the low-power objective to find the focus. It is helpful to focus first on one edge of the drop, which will appear as a dark line. The light should be reduced with the iris diaphragm and, if necessary, by lowering the condenser. You should be able to focus easily on the yeast cells in the suspension. If you have trouble with the focus, ask the instructor for help.
- Continue your examination with the high-dry and oil-immersion objectives (be very careful not to break the cover glass with the latter). Although the yeast cells will be obvious because of their larger size, look around them to observe the bacterial cells.
- Make a hanging-drop preparation of the Staphylococcus culture following the same procedures just described.

- Record your observations of the size, shape, cell groups, and motility of the two bacterial organisms in comparison to the yeast cells.
- Discard your slides in a container with disinfectant solution.

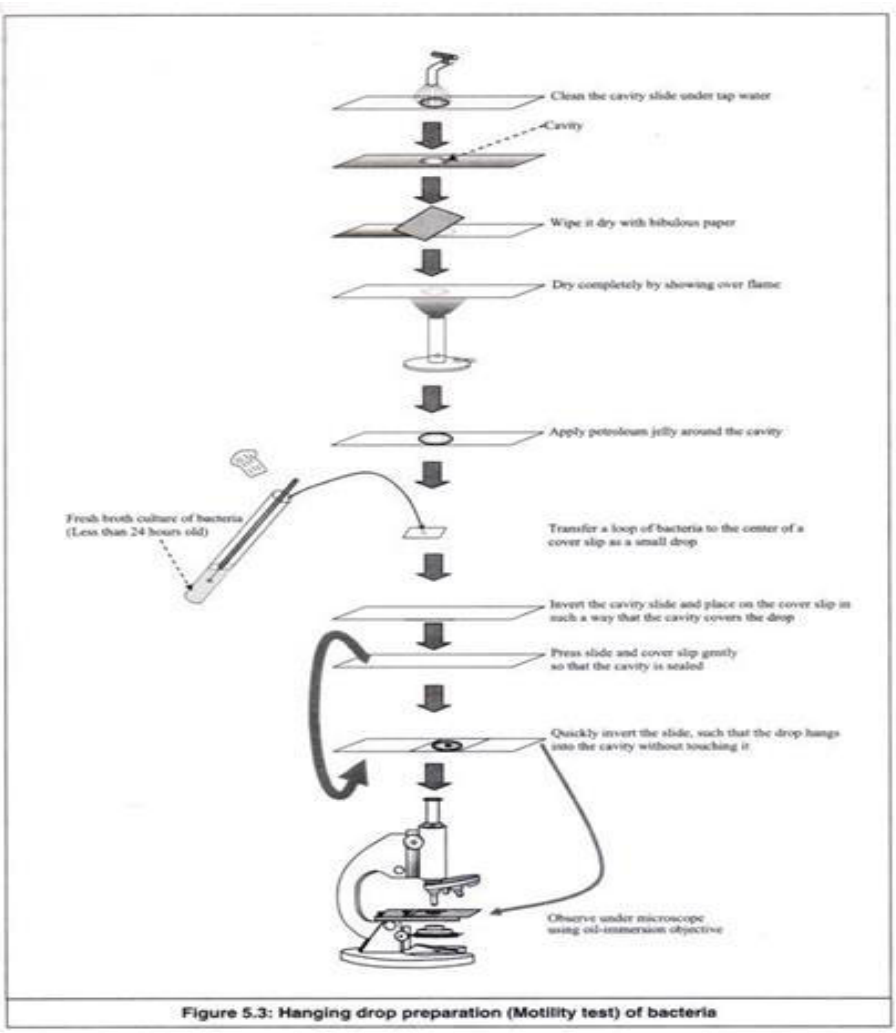


Figure 5.3: Hanging drop preparation (Motility test) of bacteria

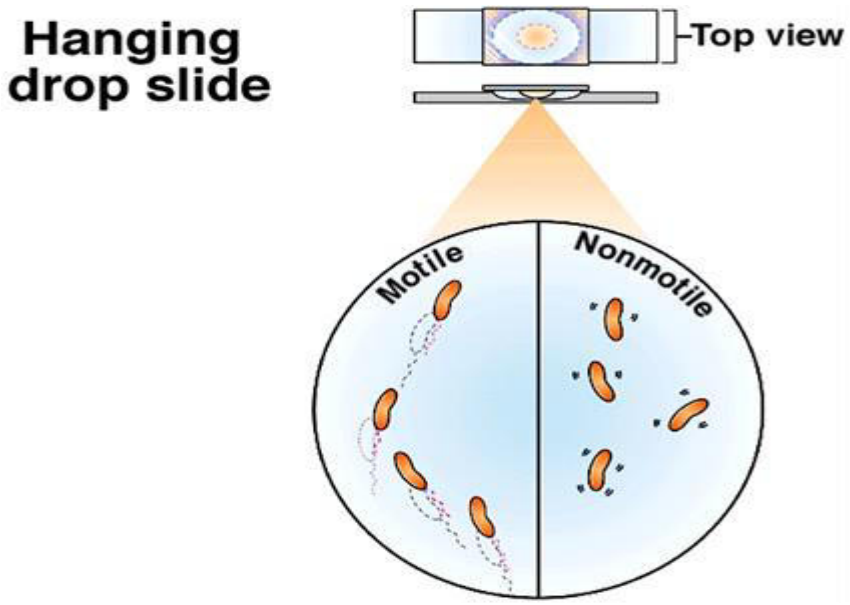


Fig. 12. Hanging Drop Method

Some Special Features of Common Microorganisms in Laboratory

1. *Escherichia coli*

Section : Facultative, Gram negative rods
Family : Enterobacteriaceace
Genus : *Escherichia*
Species : *coli*

- Common coliform bacterium used in laboratory practices
- The bacterium is rod shaped generally 1.3 x 2.5 µm in size
- Gram negative, facultatively anaerobe, motile having peritrichous flagella
- It causes diarrhea due to the presence of enterotoxins.
- It is catalase positive and oxidase negative

Colony Characteristics

a) On Nutrient Agar
Small, regular, circular, translucent colonies

b) On Mac conkey Agar
Small, regular, circular, lactose fermenting colonies

Biochemical Tests

Table 1. Biochemical tests of *E.coli*

1	Sugar Fermentation Tests	
a	Glucose	Fermented with acid & gas production
b	Lactose	Fermented with acid & gas production
c	Sucrose	Fermented with acid & gas production
2	Oxidation – Fermentation test	Fermentative
3	Mannitol Motility Test	Motile with diffused growth
4	Indole Production	+
5	Methyl Red	+
6	Voges – Proskauer	-
7	Citrate Utilization	-
8	Nitrate Reduction	+
9	Urease test	-
10	Triple Sugar Iron Agar	A/A with gas production & no H_2S production

2. *Klebsiella*

Section : Facultative, Gram negative rods
Family : Enterobacteriaceace
Genus: *Klebsiella*

- No specific growth requirements and grow well on standard laboratory media
- Grows best between 35 and 37ºC and at pH 7.2
- The bacterium is rod shaped, non-motile
- Gram negative, facultatively anaerobe
- It is catalase positive and oxidase negative

Colony Characteristics

a) *On Nutrient Agar*
Large, regular, convex, opaque, mucoid colonies

b) *On Mac conkey Agar*
Large, regular, convex, opaque, mucoid, lactose fermenting colonies

Biochemical Tests

Table 2. Biochemical tests of *Klebsiella*

1	Sugar Fermentation Tests	
a	Glucose	Fermented with acid & gas production
b	Lactose	Fermented with acid & gas production
c	Sucrose	Fermented with acid & gas production
2	Oxidation – Fermentation test	Fermentative with gas production
3	Mannitol Motility Test	Motile , growth only on stab line
4	Indole Production	-
5	Methyl Red	-
6	Voges – Proskauer	+
7	Citrate Utilization	+
8	Nitrate Reduction	+
9	Urease test	+
10	Triple Sugar Iron Agar	A/A with gas production & no H_2S production

3. *Pseudomonas*

Section : Facultative, gram negative rods
Family : Enterobacteriaceace
Genus: *Pseudomonas*

- The cells are straight or slightly curved of 1.5 – 5.0 x 0.5-1.0 µm in size
- Aerobic and motile
- Some species are pathogenic to human, animals and plants
- They are catalase and oxidase positive

Colony Characteristics

a) On Nutrient Agar

Medium, regular, flat, translucent colonies with greenish pigmentation

b) On Mac conkey Agar

Small, irregular, flat, translucent, non-lactose fermenting colonies

Biochemical Tests

Table 3. Biochemical tests of *Pseudomonas*

1	Sugar Fermentation Tests	
a	Glucose	Fermented with acid & no gas production
b	Lactose	Non-fermentative
c	Sucrose	Non-fermentative
2	Oxidation – Fermentation test	Oxidative
3	Mannitol Motility Test	Fermented with diffused growth
4	Indole Production	-
5	Methyl Red	-
6	Voges – Proskauer	-
7	Citrate Utilization	+
8	Nitrate Reduction	+
9	Urease test	-
10	Triple Sugar Iron Agar	K/K with gas production & H_2S production

4. *Staphylococcus aureus*

Section : Facultative, gram positive cocci
Family : Staphylococcaceae
Genus : *Staphylococcus*
Species : *aureus*

- They appear round (cocci) and form in grape-like clusters.
- Non-Motile
- It is a common cause of skin infections, respiratory disease and food poisoning.
- It is catalase and coagulase positive

Colony Characteristics

a) On Nutrient Agar

Small, regular, circular, entire, smooth, convex, opaque, golden yellow colonies

b) On Mac conkey Agar

Small, regular, circular, entire, smooth, convex, opaque, lactose fermenting colonies

Biochemical Tests

Table 4. Biochemical tests of *S. aureus*		
1	Sugar Fermentation Tests	
a	Glucose	Fermented with acid only
b	Lactose	Fermented with acid only
c	Sucrose	Fermented with acid only
2	Oxidation – Fermentation test	Fermentative
3	Mannitol Motility Test	Fermentative and non - motile
4	Indole Production	-
5	Methyl Red	+
6	Voges – Proskauer	+
7	Citrate Utilization	-
8	Nitrate Reduction	+
9	Urease test	+
10	Triple Sugar Iron Agar	A/A without gas production & H_2S production

10

BIOCHEMICAL TESTS FOR IDENTIFICATION OF BACTERIA

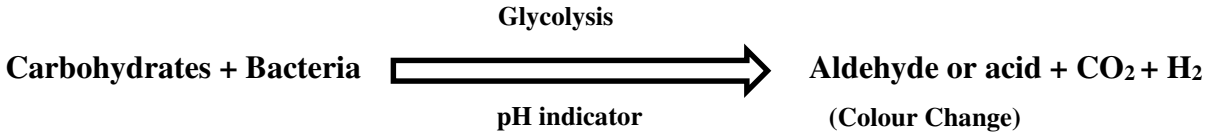
1) Carbohydrate Fermentation

Aim

To determine the ability of microorganisms to degrade and ferment carbohydrate with the production of acid and gas

Principle

Most microorganisms use carbohydrate differently depending on their enzymes components. In fermentation, substrate and alcohols undergo anaerobic dissimilation and produce an organic acid (For example lactic acid, formic acid or acetic acid). The pH indicator Phenol Red is used to detect the production of acid, which is red at a neutral pH 7 and changes to yellow at a slightly acidic pH of 6.8. This indicates a positive reaction.



In some cases, acid production is accompanied by the evaluation of gas such as Hydrogen or Carbon dioxide. To detect the presence of gas produced or Durham's tube (an inverted inner vial) is placed in the fermentation broth, in which the evaluation of gas will be visible as a bubble.

Cultures that are not capable of fermenting any carbohydrate and not producing concomitant evolution of gas are noted. This is a negative reaction.

Materials Required

8 ml Test Tube, Durham's Tube, Phenol Red Indicator, Sugar (Glucose, Lactose, Sucrose)

Procedure

- Using sterile technique, culture was inoculated into its appropriately labeled medium by means of loop inoculation.
- Care was taken during this step not to shake the fermentation tube.
- 1 tube of each fermentation broth was kept uninoculated as a comparative control.
- All the tubes were incubated at 37°C for 24 hours and the reaction was observed.

Observation

All carbohydrate broth cultures were observed for colour and presence or absence of gas bubble by comparing with the uninoculated tube (control).

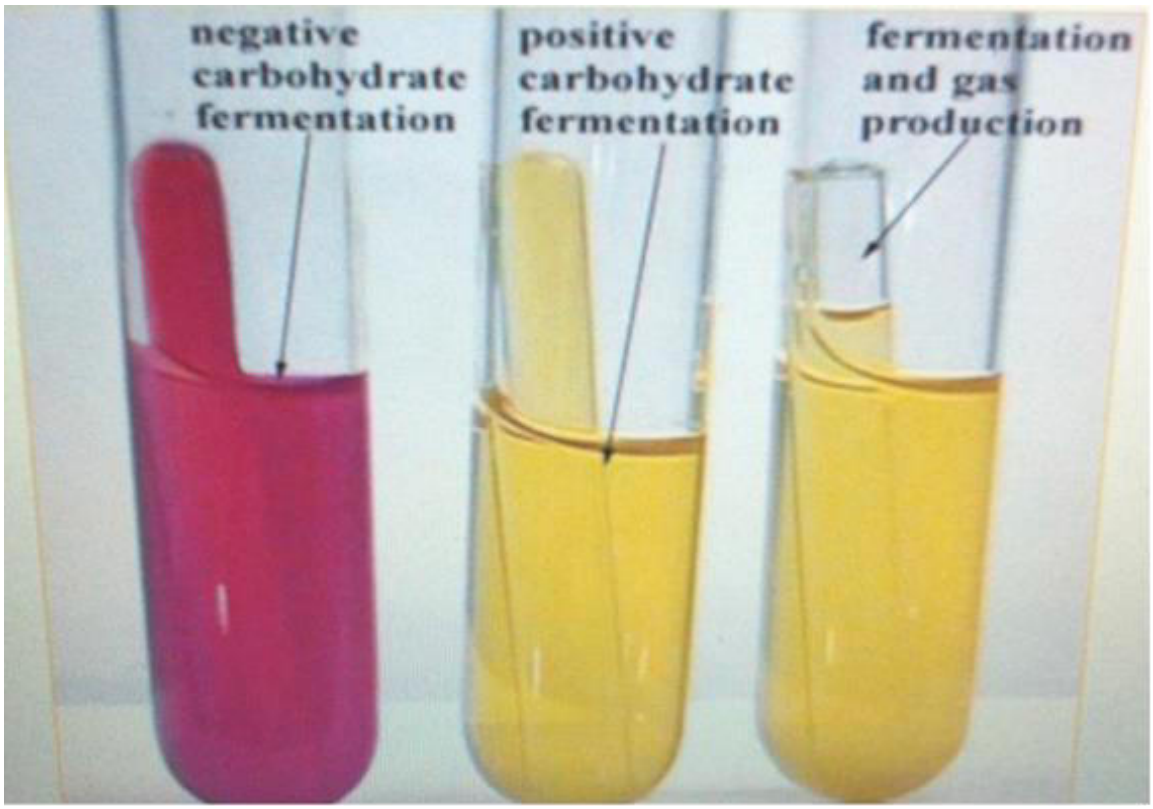


Fig. 13. Carbohydrate Fermentation Test

Note

A – Only acid is formed; the broth has turned yellow

AG – Acid & Gas formed, the broth turned Yellow and gas bubble is trapped

-ve – No change

Sugar fermentation test

Table 5. Carbohydrate – Fermentation Test

Glucose	Lactose	Sucrose
Fermented with acid production only eg. *S. aureus*	Fermented with acid production only Eg: *S. aureus*	Fermented with acid production only Eg: *S. aureus*
Fermented with acid and gas production eg. *E. coli, Klebsiella*	Fermented with acid and gas production Eg: *E. coli, Klebsiella*	Fermented with acid and gas production Eg: *E. coli, Klebsiella*
Non- Fermenting eg. *Acinoetobacter*	Non- Fermenting Eg: *S. typhi* *S. paratyphi* *Pseudomonas sp.*	Non- Fermenting Eg: *S. typhi* *S. paratyphi* *Pseudomonas sp.*

2) Oxidation – Fermentation Test

Aim

To determine the oxidation fermentation characteristics of microorganisms

Principle

This method depends upon the use of semisolid tube medium containing the carbohydrate (Glucose) together with a pH indicator. The acid is produced only at the surface of medium where conditions are aerobic the attack on the medium where conditions are aerobic the attack on the sugar is oxidative. If acid is produced throughout the medium including lower layers and where the conditions are aerobic breakdown is fermentative.

Fermenting organism (*Enterobacteriaceace, Vibrio*) produce an acidic reaction throughout the medium in the covered (anaerobic) as well as open (aerobic) tube. Oxidizing organisms (*Pseudomonas)* produce an acidic reaction only in the open tube. Organisms that cannot breakdown carbohydrate aerobically/anaerobically (alkali genes faecalis) produce an alkaline reaction in the open tube and no change in the covered tube. This medium may be used for detecting gas production and motility.

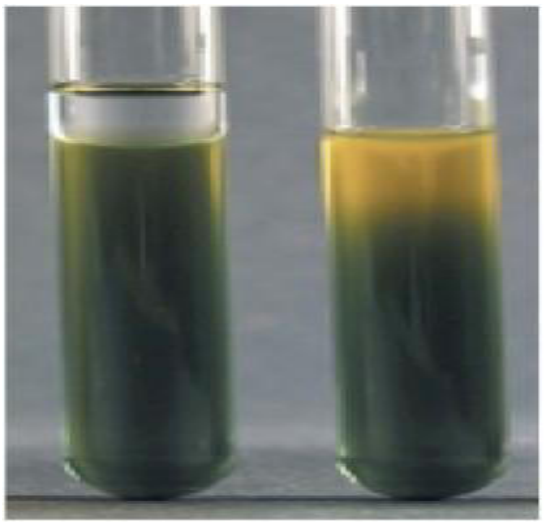


Fig. 14. Oxidation Fermentation Test

Materials Required

Bacterial broth culture, D-F medium, liquid paraffinaol

Procedure

- Using sterile technique, two tubes of medium were inoculated by stabbing with sterile urine.
- Two inoculated tubes were used as control.
- Liquid paraffin was poured over the medium to form a layer about 1cm in depth into one of the tube of each pair.
- The tubes were incubated at 37°C for 24-48hrs was observed.

Observation

The tubes were observed for the colour of the medium and the type of metabolism was recorded.

Table 6. Oxidation – Fermentation Test

OXIDATIVE	FERMENTATIVE
Eg. *Pseudomonas*	Eg. *Klebsiella* *S. typhi* *S. paratyphi A* *E. coli*

3) Indole Production Test

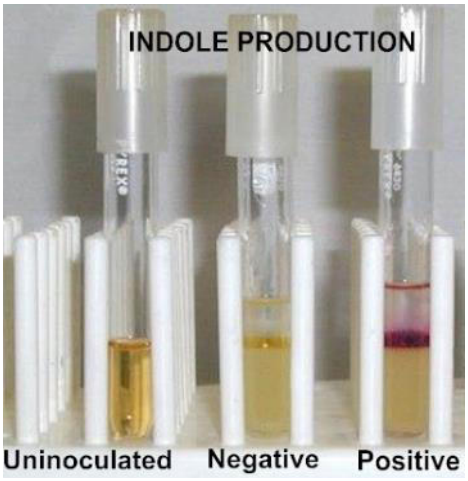


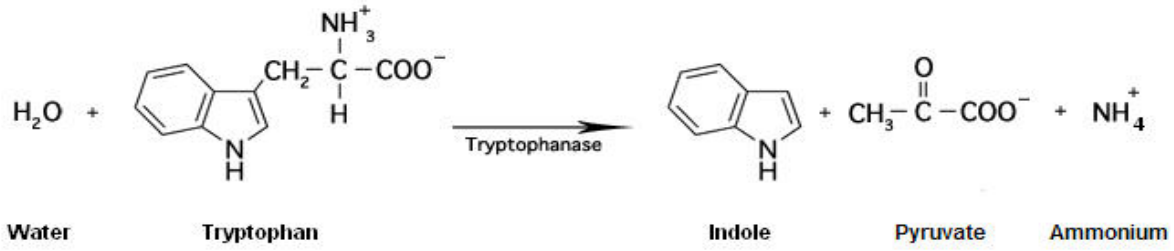
Fig. 15. Indole Production Test

Aim

To determine the ability of microorganisms to decompose the amino acid tryptophan to indole

Principle

Tryptophan an essential amino acid oxidized by some bacteria by the enzyme *tryptophanase* resulting in the formation of indole, pyruvic acid and ammonia. In this experiment, the medium contains the substrate tryptophan which is utilized by the microorganisms.



Enzymatic Degradation of Tryptophan

This ability to hydrolyse tryptophan with the production of indole is not a characteristic of all microorganisms and therefore serves as a biochemical mask. The presence of indole is detected by adding Kovac's reagent, which produces a cherry red reagent layers. This colour is produced by the reagent which is composed of Paradimethyl aminobenzaldehyde yielding the cherry red colour

Indole Reaction with Kovac's Reagent

Culture producing a red reagent layers following addition of the Kovac's reagent are indole positive. The absence of red colouration demonstrates that the substrate tryptophan was not hydrolyzed and indicating indole negative reaction.

Another reagent used is Ehrlisch's reagent. It's believed to be more sensitive than Kovac's reagent and is recommend for the detection of indole production by anaerobic and non-fermentative Gram negative organism Kovac's reagent was used usually initially to classify the members of *Enterobacteriaceace* family.

Materials Required

15 ml test tubes, bacterial culture, peptone water, Kovac's reagent

Procedure

- The peptone water tubes were inoculated with bacterial broth culture using sterile needle technique.
- An uninoculated tube was kept as control.
- Both tubes were incubated at 37°C for 24-48 hours.
- After proper incubation, 1 ml of Kovac's reagent was added to both tubes including the control.
- The tubes were shaken gently after an interval for 10 – 15 minutes.

Observation

The tubes were observed for the colour in the top reagent layer.

Note

Development of cherry red colour in the top layer of the tube is a positive test. Absence of red colouration is indole negative.

Examples

Positive: *E. coli, Proteus vulgaris*

Negative: *Klebsiella sp., Proteus mirabilis*

4) Methyl Red Test

Aim

To determine the ability of microorganism to oxidize glucose with the production and stabilization of high concentrations of acid end products

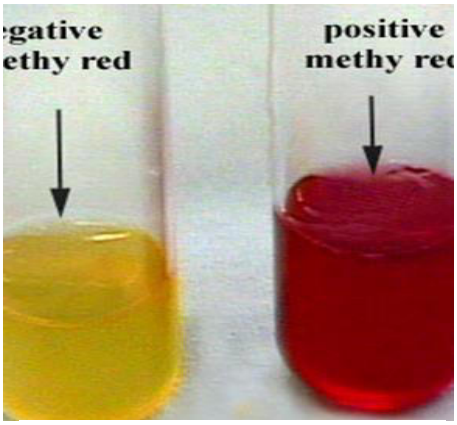


Fig. 16. Methyl Red Test

Principle

All enteric organisms oxidize glucose for energy production and the end products of this process will vary depending on the specific enzymatic pathway present in the bacteria. In this test, the pH indicator methyl red detects the presence of large concentrations of acidic red detects the presence of large concentrations of acidic products. The test can be used in differentiating *Escherichia coli* and *Enterobacter aerogenes* (both coliform bacteria) that are used as indicator of the sanitary quality of water, foods etc.

Both of these organisms initially produce organic acid end products during the early incubation period. The low acid end products produce acidic pH (4) which is stabilized and maintained by *E. coli* at the end of incubation. During the later incubation period *Enterobacter aerogenes* enzymatically converts these acids into nonacid end products such as 2,3 butanedial and acetyl methyl carbinol (pH 6).

Glucose + H_2O ⟹ **Lactic Acid, Acetic Acid, Formic Acid** **+ CO_2 + H_2 (pH 4)** **(MR Indicator)**

At a pH of 4, Methyl red indicator will turn red throughout the tube, which is indicating of a positive test. At pH 6, still indicating the presence of acid but with a lower hydrogen ion concentration, the indicators turn Yellow, which is indicating the negative test.

Materials Required

MR broth, 24 hours broth cultures, Methyl red indicator, inoculating loop

Procedure

- Using sterile technique experimental organisms were inoculated into appropriately labeled tubes containing MR broth by means of loop inoculation.
- Uninoculated tube was kept as control
- Both tubes were incubated at 37°C for 24-48 hours.
- After proper incubation 5 drops of MR indicator was added to both tubes including control.
- It was mixed well and colour was observed.

Observation

The tubes were observed for changes in the colour of Methyl Red.

Interpretation

The colour of MR reagents remaining red is a positive test and the colour turning to yellow is negative.

Examples

MR positive – *E. coli, Proteus* sp*; Salmonella* sp.

MR negative – *Klebsiella, Enterobacter sp.*

5) Voges – Proskauer Test

Aim

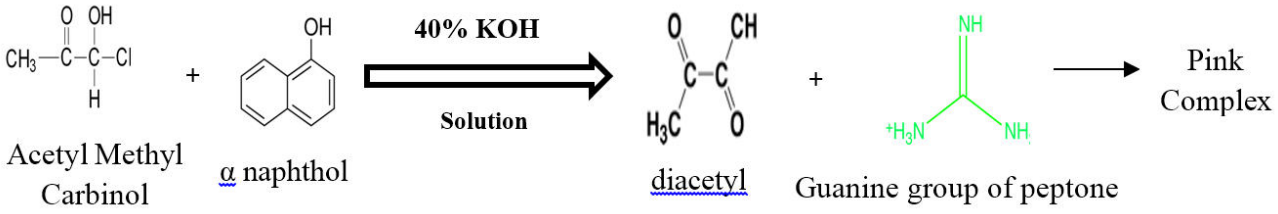
To determine the ability of many microorganisms to produce acetone (acetyl methyl carbinol) during fermentation of glucose

Principle

This determines the ability of many bacteria to ferment carbohydrates with the production of non- acidic / neutral end products, acetyl methyl carbinol or its reduction product, acetyl methyl carbinol or its reduction products, acetyl- methyl carbinol or its reduction product 2,3 Butylene glycol from the organic acids.

The reagent used in this test, Barrett's reagent, consists of a mixture of alcoholic α- naphthol and 40% potassium hydroxide solution. Detection of the acetyl methyl carbinol requires this end product to be oxidized to a diacetyl compound. This reaction will occur in the presence of α- naphthol catalyst and a guanidine group that is present in the peptone. At a result, a pink complex a guanidine group that is present in the peptone. As a result, a pink complex is complex is formed imparting a rose colour to the medium.

Acetyl Methyl Carbinol reaction with Barrett's reagent



Development of deep rose colour in culture with in a minute following the addition of Barrett's reagent is indicative of presence of the acetyl methyl carbinol and represents a positive result. The absence of rose colouration is a negative result.

Procedure

- Using sterile technique, the experimental organism was inoculated into VP broth by means of loop inoculation.
- One tube is kept uninoculated as control.
- The tube will be incubated at 37°C for 24-48 hours.
- After proper incubation, about 3 ml of Barrett's reagent A & 1 ml of Barrett's reagent B was added into both tubes including control.
- The tubes were shaken gently for 30 seconds with the caps off to expose the media to oxygen.
- The reaction was allowed to complete in 15 – 30 minutes and tubes were observed.

Observation

The tubes were observed for the development of crimson red colour.

Fig. 17. Voges Proskauer Test

Note: the colour may be more intense at the surface.

Interpretation

Red colour formation indicates a positive test and colour change is negative.

eg. Positive – *Klebsiella* sp., *Enterobacter*

Negative – *E. coli, Proteus* sp.

6) Citrate Utilization Test

Aim

To determine the ability of a microorganism to utilize citrate as the sole source of carbon and as energy source for the growth and ammonium salt as a sole source of nitrogen

Principle

Citrate test is used to differentiate among enteric bacteria on the basis of their ability to utilize / ferment citrate as the sole carbon source. In the absence of glucose or lactose some microorganisms utilize citrate as a carbon source. This ability depends on the presence of citrase enzyme that facilitates the transport of citrate in the cell. Citrate, the first major intermediate in Krebs's cycle is produced by the condensation of active acetyl CoA with oxalo acetic acid and acetate. These products are then enzymatically converted to pyruvic acid and carbon dioxide. During this reaction the medium becomes alkaline; CO_2 combines with sodium and water to form carbonate, an alkaline product. This changes the bromothymol blue indicator in the medium from green to Prussian blue.

Citrate test is preferred / performed by inoculating the microorganisms in to an organic synthetic medium. Simmons citrate agar (solid) or Koser's citrate medium (liquid) in which sodium citrate is the only source of carbon and energy.

Bromothymol blue is green when acidic (pH 6.8 and below). When alkaline (pH 7.6 and above). Formation of blue colour constitutes a positive test. Citrate negative culture will show no growth and the medium will remain green.

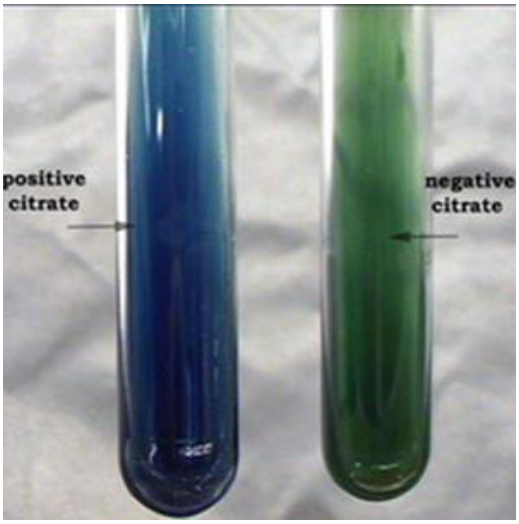


Fig. 18. Citrate Utilization

Materials Required

Bacterial broth, Simmons Citrate Agar Slants, Inoculation Loop

Procedure

- Using sterile technique Simmons citrate agar slant was inoculated with the test organism by means of a stab and streak inoculation.
- An uninoculated tube was kept as control.
- Both tubes were incubated at 37°C for 24 – 48 hours & was observed

Observation

The tubes were observed for growth and colouration of the medium.

Interpretation

Colour of the medium if turned blue, a positive result is indicated. Colour of the medium remains as green, indicates a negative result.

7) Nitrate Reduction Test

Aim

To determine the ability of bacteria to produce an enzyme nitrate reductase

Principle

The reduction of nitrate by some aerobic and facultative anaerobic microorganisms occur in the absence of molecular oxygen an anaerobic process whereby the cell uses in organic substances such as nitrates or sulphates to supply oxygen that is subsequently utilized as a final hydrogen acceptor during energy formation. The biochemical transformation may be utilized as follows:

$$NO_3^- + 2H^+ + 2e^- \xrightarrow{\text{Nitrate reductase}} NO_2 + H_2O$$

Some organisms possess the enzymatic capacity to act further on nitrates to reduce them to ammonia or molecular nitrogen. These reactions may be described as follows:

$$NO_2^- \longrightarrow NH_3^+$$

Nitrate reduction can be determined by cultivating organisms 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 additional of 0.1% agar. The semisolid impedes the diffusion of oxygen in to the medium, there by favoring the anaerobic requirement necessary for nitrate reduction. An organisms ability to reduce nitrate to nitrite is determined by the addition of two reagent solution A, which is sulphanlic acid followed by solution B, which is α-napthylamine followed reduction, the addition of solution A and B will produce an immediate cherry red colour.

$$NO_3^- \xrightarrow{\text{Nitrate Reductase}} NO_2^-$$

Cultures not producing a colour change suggest one of two possibilities

- Nitrates were not reduced by the organism
- The organism possessed such potent nitrate reductase enzymes that nitrate were rapidly reduced beyond nitrates to ammonia or even molecular nitrogen.

This test determines the production of an enzyme called nitrate reductase, resulting in the reduction of nitrate (NO_3). With this enzyme, nitrate is reduced to nitrite (NO_2). It then forms nitrous acid that reacts with the first reagent sulphanlic acid, and that reacts with the other reagent α-napthylamine to form a red colour. The development of red colour, therefore, verifies that nitrates were not reduced to nitrites by the organism. If nitrites were reduced a negative nitrate reduction had occurred. If the addition of zinc does not produce colour change, the nitrates in the medium were reduced beyond nitrites to ammonia or nitrogen gas. This is a positive reaction or result. Reduction of nitrate is generally an anaerobic respiration in which an organism derives its oxygen from nitrate.

Materials Required

Bacterial broth, Nitrate broth, Nitrate reagent and inoculation loop.

Procedure

- Using sterile technique the test organism was inoculated in to nitrate broth by means of loop inoculation.
- An uninoculated broth was kept as control.
- Both tubes were incubated at 37°C for 24-48 hours.

- After proper incubation equal amounts of nitrate reagent (solution A & B) were added to nitrate broth Cultures and to the control tube and the reaction was observed

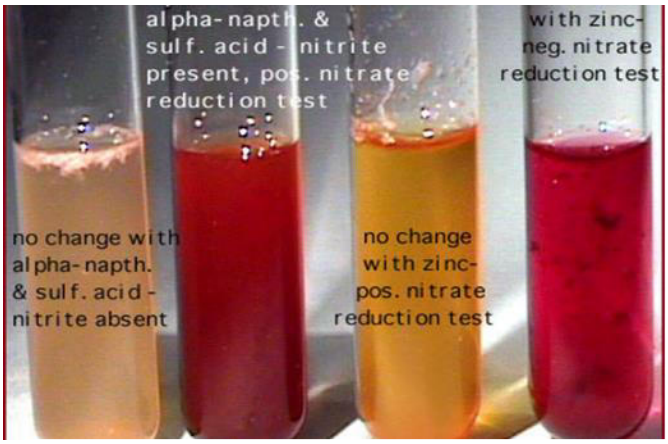


Fig. 19. Nitrate Reduction Test

Observation

The tubes were observed to see a red colour has been developed or not.

A minute quantity of zinc was added to cultures in which no red colour was developed and it was observed to see if red colour has been developed or not.

Interpretation

Development of red colour indicates nitrate positive and no colour change indicates a negative test.

Eg: Positive: all members of *Enterobacteriaceace*

Negative: *Haemophilus duceryi.*

8) Urease Test

Aim

To determine the ability of microorganism to degrade urea by means of the enzyme urease

Principle

Urease is a hydrolytic enzyme that attacks nitrogen and carbon bond in amide compounds such as urea and forms the alkaline end products ammonia. The presence of urease is detectable when the organisms are grown in a urea broth medium containing the pH indicator phenol red. As the substrate urea is split into its products, the phenol red to turn to a deep pink. This is a positive reaction for the presence of urease. Failure of deep pink colour to develop is evidence of negative reaction.

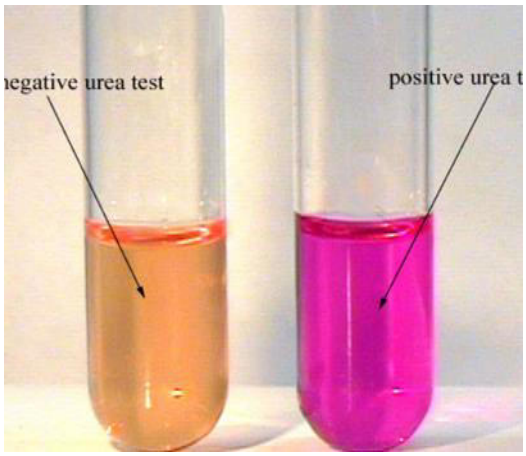
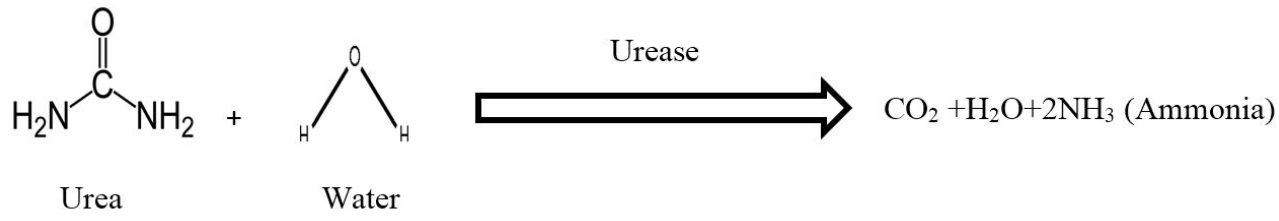


Fig. 20. Urease Test



Materials Required

Bacterial broth cultures, Christener's urea agar slant and the inoculation loop

Procedure

- Using sterile technique, the test organism was inoculated the media by means of loop of inoculation.

- An uninoculated tube was kept as control.
- The tubes were incubated at 37°C for 24-24 hours and the reaction was observed.

Observation

The tubes were observed to see if pink colour has developed or not

Interpretation

Development of pink colour indicator a positive test and no colour change shows a negative test,
Eg Urease Positive – *Klebsiella* sp., *Proteus* sp.
Urease Negative – *E. coli*, *Salmonella* sp.

9) Mannitol Motility Test

Aim

To detect whether the given organism is motile and also mannitol is fermenting or not

Principle

Mannitol motility test medium is an example of semisolid agar media; motile bacteria swarm and give a diffused spreading growth that is easily recognized by the naked eye. The final sterile medium should be quite clear and transparent. After incubating the stabbed culture, non-motile bacteria generally give growth that are confined to stab line and have sharply defined margins leaving the surrounding medium clearly transparent. Motile bacteria typically give diffused, hazy growth that spreads throughout the medium rendering it slightly opaque. This test also helps to identify whether the microorganisms ferment Mannitol or not. It produces acidic end products which in turn change the red colour of phenol red indicator to yellow.

Materials Required

Bacterial culture broth, mannitol fermentation media (semisolid) and inoculation loop

Procedure

- Using sterile technique the test organism was inoculated in to the medium using stab inoculation method.
- An uninoculated tube was kept as control.
- Both tubes were incubated at 37°C for 24-48 hours and the reaction was observed.

Observation

The tubes were observed for motility and also for colour changes from red to yellow.

Interpretation

Diffused growth – Motile bacteria eg: *Pseudomonas* sp.

Growth at stab line only – Non-motile bacteria eg: *Staphylococcus aureus* only

Red colour – Mannitol non-fermenting eg: *Bacillus cereus*

Yellow colour - Mannitol fermenting eg: *E. coli*

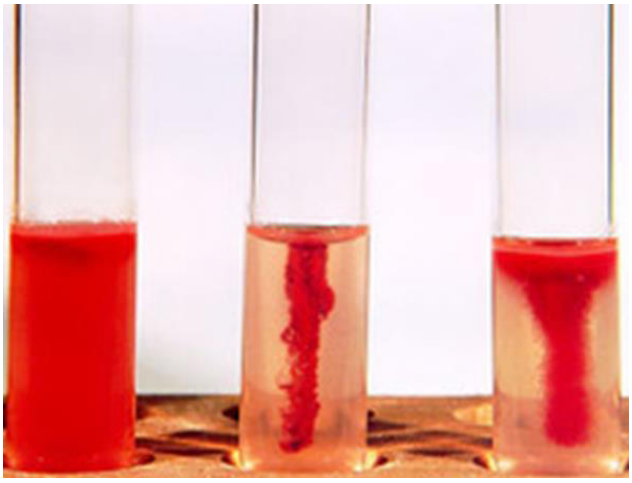


Fig. 21. Mannitol Motility Test

10) Triple Sugar Iron Agar Test

Aim

To identify the microorganisms based on the ability to ferment the carbohydrates (Glucose, Sucrose and Lactose)

Principle

The triple sugar- iron agar test is designed to differentiate among the different groups or genera of the *Enterobacteriaceace,* which are all Gram negative bacilli capable of fermenting glucose with the production of acid and to distinguish them from other gram negative intestinal bacilli. This differentiation is based on the differences in carbohydrate fermentation patterns and hydrogen sulfide production by the various groups of intestinal organisms. Carbohydrate fermentation is indicated by the presence of gas and a visible colour change of the pH indicator, phenol red. The production of hydrogen sulphide in the medium is indicated by the formation of a black precipitate that will blacken the medium in the butt of the tube.

To facilitate the observation of carbohydrate utilization patterns, 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 colour of the carbohydrate medium from orange red to yellow in the presence of acids. In case of oxidative decarboxylation of peptone, alkaline products are produced 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 colour in the butt of the tube.

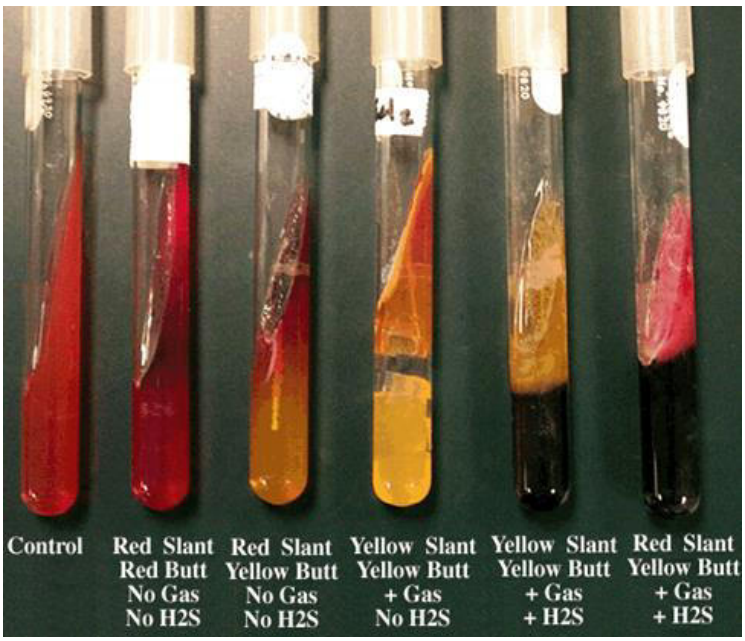


Fig. 22.Triple Sugar Iron Agar Test

Carbohydrate fermentation is indicated by the production of gas and a change in the colour of the pH indicator from red to yellow. To facilitate the detection of organisms that only ferment glucose, the glucose concentration is one-tenth the concentration of lactose or sucrose. The 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. If the tube is tightly closed, an acid reaction (caused solely by glucose fermentation) will also involve the slant.

Materials Required

Bacterial broth cultures, TSI agar slants, Inoculation Loop.

Procedure

- Using sterile technique, the test organism was inoculated into the media by means of stab and streak inoculation.
- An uninoculated tube was kept as control
- Both tubes were incubated at 37°C for 24 hours and the reaction was observed

Observation

The tubes were observed for the colour of both the butt and slant and also gas production by means of cracks or bubble or blackness of butt.

Observation	Interference	Examples
A/A without gas and H_2S production	Acid Slant / Acid butt without gas & H_2S production	*Staphylococcus aureus*
A/A with gas and without H_2S production	Acid Slant / Acid butt with gas & without H_2S production	*E. coli, Klebsiella*
K/A with gas and without H_2S production	Alkaline slant / Acid butt with gas & without H_2S production	*Salmonella paratyphi* A
K/K without gas and H_2S production	Alkaline slant / Acid butt without gas & H_2S production	*Pseudomonas* sp.
K/A with H_2S production	Alkaline slant / Acid butt with H_2S production	*Salmonella typhi*

Interpretation

- A/A: ferments glucose and either sucrose, lactose, or both.
- K/A: does not ferment lactose or sucrose; does ferment glucose.
- K/K: a non-fermenter.
- Black precipitate in stab: produces H_2S (and ferments glucose).

11) Catalase Test

Aim

To demonstrate the presence of catalase in an organism.

Principle

Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments to

Fig. 23. Catalase Test

neutralize toxic forms of oxygen metabolites and H_2O_2. The catalase enzyme neutralizes the bactericidal effects of hydrogen peroxide and protects them. Anaerobes generally lack the catalase enzyme.

Catalase mediates the breakdown of hydrogen peroxide H_2O_2 into oxygen and water. To find out if a particular bacterial isolate is able to produce catalase enzyme, small inoculums of bacterial isolate is mixed into hydrogen peroxide solution (3%) and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production.

Catalase-positive bacteria include strict aerobes as well as facultative anaerobes. They all have the ability to respire using oxygen as a terminal electron acceptor.

Catalase-negative bacteria may be anaerobes, or they may be facultative anaerobes that only ferment and do not respire using oxygen as a terminal electron acceptor (ie. *Streptococci*).

Uses

- The catalase test is primarily used to distinguish among Gram-positive cocci: Member of the genus *Staphylococcus* is catalase-positive, and members of the genera *Streptococcus* and *Enterococcus* are catalase-negative.
- Catalase test is used to differentiate aero tolerant strains of *Clostridium*, which are catalase negative, from *Bacillus* species, which are positive.
- Semi quantitative catalase test is used for the identification of *Mycobacterium tuberculosis*
- Catalase test can be used as an aid to the identification of *Enterobacteriaceace*. Members of Enterobacteriaceace family are Catalase positive.

Materials Required

24 hours old bacterial culture, glass slide, petridish, 3% H_2O_2, applicator sticks

Procedure

- Transfer a small amount of bacterial colony to a surface of clean, dry glass slide using a loop or sterile wooden stick
- Place a drop of 3% H_2O_2 on to the slide and mix.
- A positive result is the rapid evolution of oxygen (within 5-10 s) as evidenced by bubbling.
- A negative result is no bubbles or only a few scattered bubbles.
- Dispose of your slide in the biohazard glass disposal container.

Precautions

- Do not use a metal loop or needle with H_2O_2; it will give a false positive and degrade the metal.
- If using colonies from a blood agar plate, be very careful not to scrape up any of the blood agar as blood cells are catalase positive and any contaminating agar could give a false positive.

Observation

The release of bubbles was observed and compared with control.

Interpretation

Bubble Formation : Catalase Positive

No Bubble Formation: Catalase Negative

Examples

Catalase Positive : *Staphylococcus aureus*

Catalase Negative: *Streptococcus pyogenes*

Note

Care must be taken while performing catalase test of growth from blood agar plate because blood (RBC) contains RBC catalase.

12) Oxidase Test

Aim

To test the production of oxidase bacteria

Principle

The oxidase test is a key test to differentiate between the families of *Pseudomonadaceae* (ox +) and Enterobacteriaceace (ox-), and is useful for speciation and identification of many other bacteria those that have to use oxygen as the final electron acceptor in aerobic respiration. The enzyme cytochrome oxidase is involved with the reduction of oxygen at the end of the electron transport chain.

Fig. 24. Oxidase Test

There may be different types of oxidase enzymes produced by bacteria. The colorless redox reagent, tetra methyl-p-phenylenediamine dihydrochloride (or dimethyl) used in the test will detect the presence of the enzyme oxidase and reacting with oxygen, turn a colour. The oxidase reagent contains a chromogenic reducing agent, a compound that changes color when it becomes oxidized, so it acts as an artificial electron acceptor for the enzyme oxidase. The oxidized reagent forms the coloured compound indophenol blue.

Materials Required

Oxidase disc, 24 hours old test organism, applicator stick or glass rod.

Procedure

- The test organisms was rubbed over the reagent impregnated, filter paper disc using sterile applicator sticks or glass rod.
- Controls were also kept along with the test and the reaction was observed within 10 seconds.

Observation

The colour changes to purple were observed with the prescribed time.

Important

Acidity inhibits oxidase enzymes activity therefore the oxidase test must not be performed on colonies that produce fermentation on carbohydrates containing media like Mac Conkey Agar.

Interpretation

Formation of purple colour indicates a positive test. No colour changes show a negative test.
eg. Oxidase Positive: *Pseudomonas* sp., *Vibrio* sp.
Oxidase Negative: *E. coli, Klebsiella*

Precautions

- The test reagent is to be freshly prepared
- Nichrome wire is not used to take bacterial growth
- Cultures should not be very cold
- Culture from selective media should not be used
- The colour changes should be observed within the prescribed time

13) Coagulase Test

Aim

To distinguish coagulase producing *Staphylococcus aureus* from other species of *Staphylococcus*

Principle

Staphylococcus aureus is known to produce coagulase, which can clot plasma into gel in tube or agglutinate cocci in slide. This test is useful in differentiating *S. aureus* from other coagulase-negative staphylococci. Most strains of *S.aureus* produce two types of coagulase, free coagulase and bound coagulase. While free coagulase is an enzyme that is secreted extracellular, bound coagulase is a cell wall associated protein. Free coagulase is detected in tube coagulase test and bound coagulase is detected in slide coagulase test. Slide coagulase test may be used to screen isolates of *S. aureus* and tube coagulase may be used for confirmation. While there are seven antigenic types of free coagulase, only one antigenic type of bound coagulase exists. Free coagulase is heat labile while bound coagulase is heat stable.

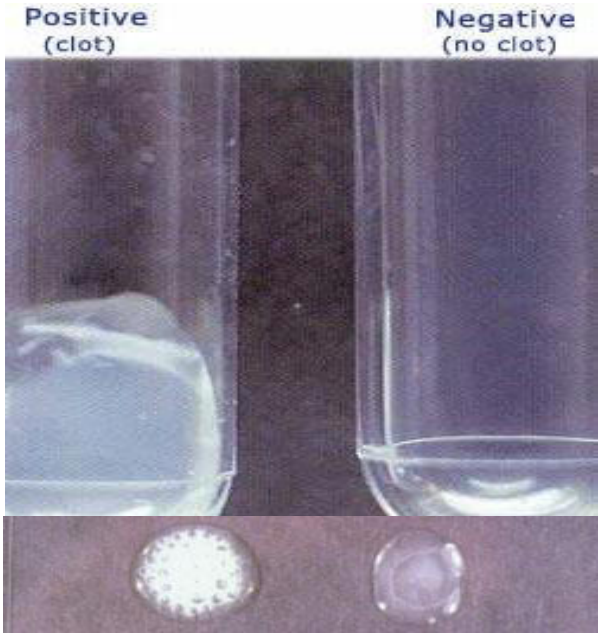


Fig. 25. Coagulase Test

Slide Coagulase Test: The bound coagulase is also known as clumping factor. It cross-links α and β chain of fibrinogen in plasma to form fibrin clot that deposits on the cell wall. As a result, individual coccus sticks to each other and clumping is observed.

Tube Coagulase Test: The free coagulases secreted by *S. aureus* act with coagulase reacting factor (CRF) in plasma to form a complex, which is thrombin. This converts fibrinogen to fibrin resulting in clotting of plasma.

Materials Required

EDTA anticoagulant human plasma, clean glass slide, test tubes, pipettes, distilled water and inoculation loop.

Procedure

*Slide Coagulase Test***:** Dense suspensions of Staphylococci from culture are made on two ends of clean glass slide. One should be labeled as "test" and the other as "control". The control suspension serves to rule out false positivity due to auto agglutination. The test suspension is treated with a drop of citrated plasma and mixed well. Agglutination or clumping of cocci within 5-10 seconds is taken as positive. Some strains of *S. aureus* may not produce bound coagulase, and such strains must be identified by tube coagulase test.

Observation

The slides were observed for clumping or not within prescribed time.

Interpretation

Clumping formation - Positive reaction

No clumping formation – Negative reaction

Tube Coagulase Test

Three test tubes are taken and labeled "test", "negative control" and "positive control". Each tube is filled with 0.5 ml of 1 in 10 diluted rabbit plasma. To the tube labeled test, 0.1 ml of overnight broth culture of test bacteria is added. To the tube labeled positive control, 0.1 ml of overnight broth culture of known [[*S. aureus* is added and to the tube labeled negative control, 0.1 ml of sterile broth is added. All the tubes are incubated at 37°C and observed up to four hours. Positive result is indicated by gelling of the plasma, which remains in place even after inverting the tube. If the test remains negative until four hours at 37°C, the tube is kept at room temperature for overnight incubation.

Observation

The tubes were observed for clotting in the prescribed time.

Interpretation

Clot formation - Positive reaction

No clot formation – Negative reaction

Examples

Coagulase Positive: *Staphylococcus aureus*
Coagulase Negative: *E. coli*

11

FUNGI

Fungi are unicellular or multi cellular organisms which live either as saprophytes or parasites. They are the major contaminating organisms in the tissue cultures because of their simple rapid reproductive processes through asexual and sexual spores. Elimination of contaminants is crucial for the successful tissue culture production as the fungi species during their rapid growth, utilize the culture media and destroy the explants.

Techniques

1) *Lacto Phenol Cotton Blue*

Lacto Phenol Cotton Blue (LPCB) of tear mount staining technique was employed for identification. Two methods are employed for LPCB staining, namely, Tear mount method and slide culture method. In LPCB staining, cotton blue stain gives dark blue colour to the fungal structure against light blue background. The cytoplasm will also be in light blue colour. Phenol acts as fungicide and lactic acid acts as clearing agent. To perform tear mount method, one or two drops of Lacto phenol cotton blue stain was added to a clean slide. Using a flame sterilized needle a few fungal mycelia was placed on the stain and the mycelia was gently teased and spread using a sterile needle. Cover glass was carefully placed taking extra caution to avoid air bubbles. Excess stain was removed using tissue paper and observed under 10X and 45X objective of microscope.

2) *Slide Culture Techniques*

Contaminations were also studied using slide culture method for a double confirmation. Slides were arranged over the V- shaped tube in a petriplate. 1 cm X 1 cm square block of Sabouraud's dextrose agar (SDA) was carefully placed on the center of the glass slide block. Cover slip was placed with sterile forceps and moistened cotton in petriplate was kept for promoting the fungal growth. After two to three days incubation, agar block was carefully placed on a glass slide containing Lacto phenol cotton blue staining. Block was later observed under 10X and 45X magnification of microscope.

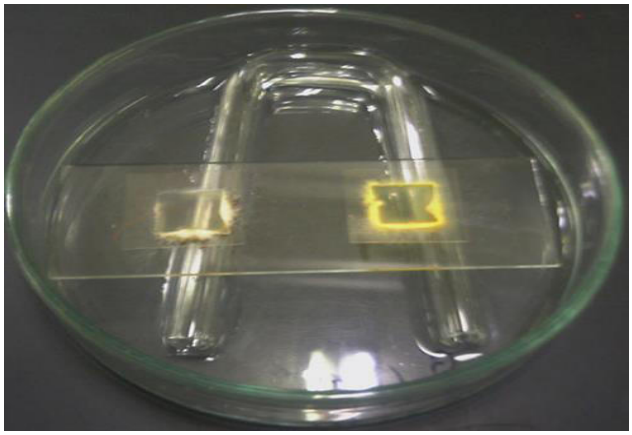


Fig. 26. Slide Culture Technique

3) *Germ Tube Test*

It is used for the observation of germ tubes in *Candida* sp. Using micropipette, dispense 3drops of fresh pooled human serum into test tubes with a sterile wooden applicator sticker/ needle touch a yeast (suspected sample) place the stick into serum. Incubated the sample 35°C for 2-3hours. By placing on clean glass slide, examine it with microscope.

Study of Characteristics of Some Common Fungi

1) *Aspergillus* species

Colony Morphology

Colonies are wooly at first, white to yellow, then turning dark brown to black. Reverse is white to yellow.

Microscopic Morphology

Conidiophores are smooth and colourless and turned dark toward vesicles. The vesicle was globes and bearing phialides mycelium septate.

2) *Penicillium* species

Colony Morphology

Colony appeared as bluish green mycelium was septate and ridged

Microscopic Morphology

Penicillium has brush like appearance formed of chains of spores extending from the ends of phialides borne on short branches of conidiophores on the hyphae.

3) *Mucor* species

Colony Morphology

Colony was rapidly growing, filling the test tube or petridish in 5-7 days with a fluffy asexual mycelia ie, at first white but later become gray to brown.

Microscopic Morphology

Mycelium was broad, non-septate, and colourless without rhizoids shows few irregular cross wall, sporangiophores arose singly from them the mycelium forming a thick fluff. They were either unbranched with terminal sporangia or branched with spherical multispored sporangia on cell at the end of the hyphae.

4) *Rhizopus* species

Colony Morphology

Colonies are columnar, fast growing and cover an agar surface with a dense cottony growth that is at first white becoming grey or yellowish brown with sporulation.

Microscopic Morphology

Sporangiophores up to 1500 µm in length and 18 µm in width, smooth walled, non - septate, simple or branched, arising from stolons opposite rhizoids usually in groups of 3 or more. Sporangia are globose, often with a flattened base, grayish black, powdery in appearance, up to 175 µm in diameter and many spored.

5) *Fusarium* species

Colony Morphology

Colony was fluffy to cottony, owing to extensive mycelium some diffusible pigment produced on reverse side (orange).

Microscopic Morphology

Conidiophores singly or grouped, septate, micro conidia are one walled and often numerous in chain or balls. Macroconidia were elongate and cylindrical.

6) *Candida* species

Colony Morphology

Colonies were creamy white, pasty, smooth, dull with foul odour and yeast-like in appearance.

Microscopic Morphology

All Candida species produce blastoconidia singly or in small clusters. Blastoconidia may be round or elongate. Most species produce pseudohyphae which may be long, branched or curved. True hyphae and chlamydospores are produced by strains of some *Candida* spp.

7) *Phytophthora* species

Colony Morphology

White cottony colonies, media colour changed into red

Microscopic Morphology

Highly branched, septate and nucleated

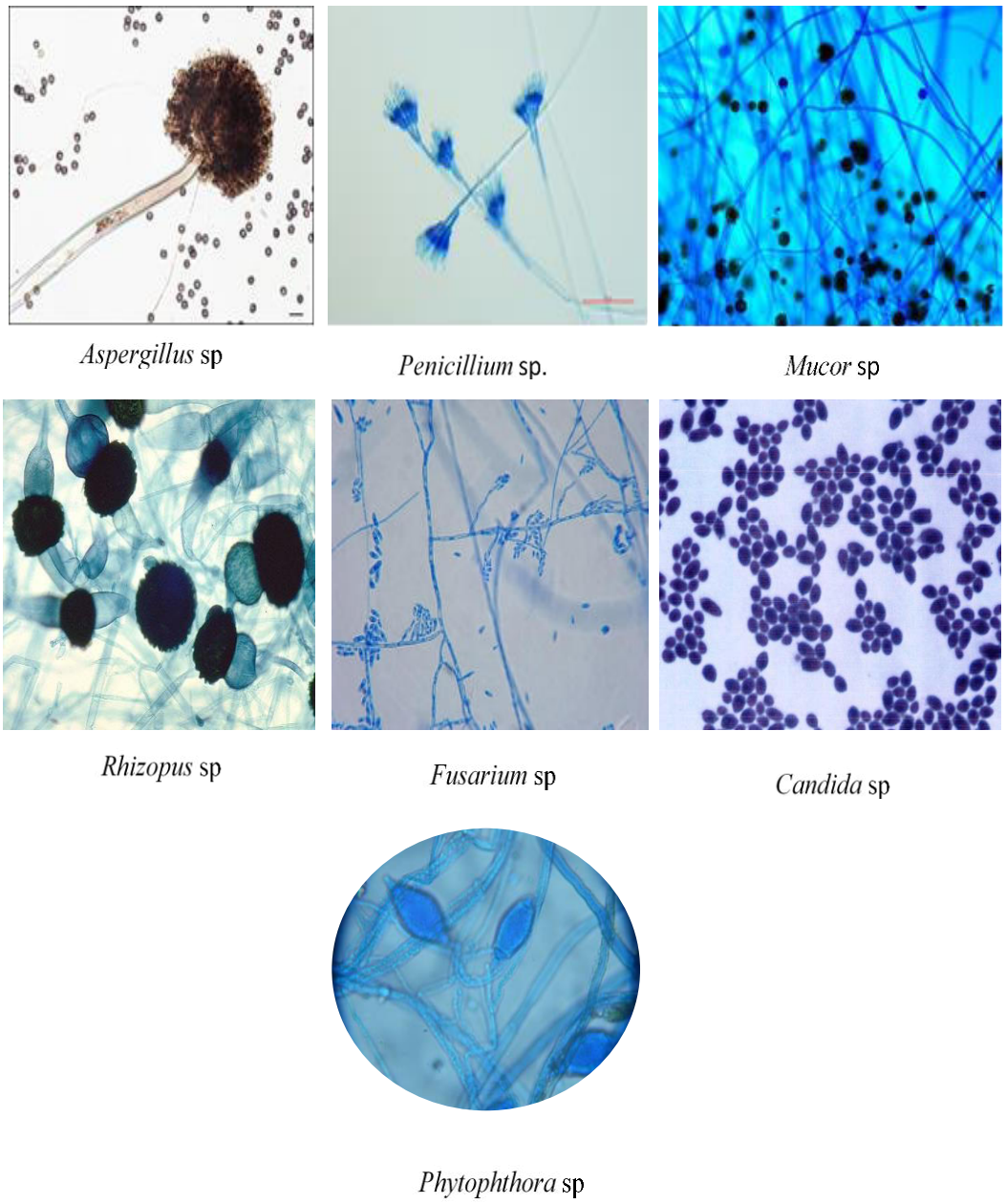


Fig. 27. Microscopic View of Some Fungi

12
IDENTIFICATION OF FUNGAL CONTAMINANTS IN PLANT TISSUE CULTURE LAB

Objective

To identify various fungal contaminants in plant tissue culture lab

Technical Programme

Lacto Phenol Cotton Blue (LPCB) of tear mount staining technique was employed for identification. Two methods are employed for LPCB staining, namely, tear mount method and slide culture method. In LPCB staining blue colour gives to cytoplasm against light blue background walls of hyphae can be visualized easily. Phenol act as fungicide and lactic acid act as clearing agent. To perform tear mount method, one or two drops of Lacto phenol cotton blue stain was added to a clean slide. Using a flame sterilized needle a few fungal mycelia was placed on the stain and the mycelia was gently teased and spread using a sterile needle. Cover glass was carefully placed taking extra caution to avoid air bubbles. Excess stain was removed using tissue paper and observed under 10X and 45X objectives of microscope. Various fungal smears were identified based on their morphological characteristics from the banana, pineapple and passion fruit tissue culture bottles.

Contaminations were also studied using slide culture method for a double confirmation. Slides were arranged over the V- shaped tube in a petriplate. 1 cm X 1 cm square block of Sabouraud's dextrose agar (SDA) was carefully placed on the center of the glass slide block. Cover slip was placed with sterile forceps and moistened cotton in petriplate was kept for promoting the fungal growth. After two to three days incubation, agar block was carefully placed on a glass slide containing Lacto phenol cotton blue staining. Block was later observed under 10X and 45X magnification of microscope.

Observation

Table 7. Tissue Culture Contaminants

Macroscopic Observation	Microscopic Observation	Organism
White color, creamy growth on the media surface	Pink color large cells obtained by Gram's Staining large, oval, budding cells obtained by LPCB staining	*Yeast sp.*
Blackish- brown, rough colonies spread all over the media	Mycelium is septate and branched. Conidia developed as a stalk and heads from foot cells	*Aspergillus sp.*
Greyish - green colour colonies, smooth colonies	Brush like conidiophores and branched mycelium spores arranged on conidiophores	*Penicillium sp.*

Result

A wide range of microorganisms cause contamination in tissue culture laboratory, fungi, yeast, molds and bacteria were the predominant microbes. Among them fungi were the major contaminants, 73.135% of consisting of fungal contamination and of bacteria were 26.87%.

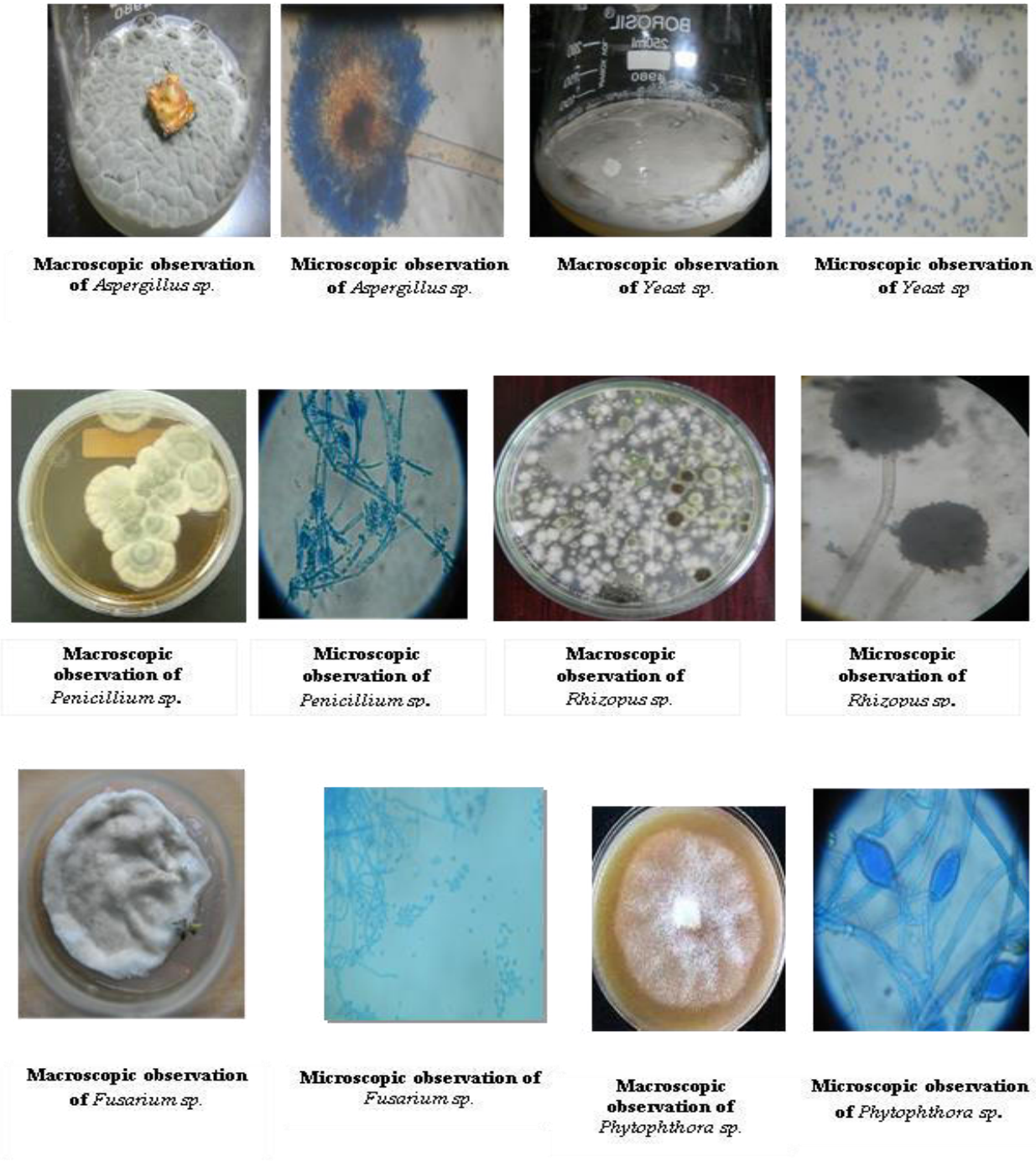


Fig. 28. Macroscopic and Microscopic observations of some fungal contaminants

13

IDENTIFICATION OF DISEASE CAUSING FUNGAL PATHOGEN OF PASSION FRUIT NURSERY PLANTS

Objective

To identify the infective agent on passion fruit seedling from roof top nursery

Technical Programme

The soil and plant samples were collected from the roof top nursery and weighed 1g of sample and suspended in 9 ml sterile distilled water in tubes (10^{-1}). Arranged 5 sets of tubes, each set contained 9 ml of sterile distilled water. Shaked and homogenized the first and transferred 1 ml from it to the second. Similarly, 1ml sample was serially transferred 10^{-2} dilutions into third tube containing 9ml of sterile water to get a final dilution of 10^{-3}. Repeated the procedure for 10^{-4}, 10^{-5}, 10^{-6} dilutions. The same procedure was followed in plant samples. Aseptically poured 1 ml soil suspension from 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} into sterile petriplate mixed with 15 ml of SDA at 45-50°C and mixed well. Incubate the plates at room temperature for 4-5 days. After proper incubation stained the colonies using LPCB stain method.

Results

Table 8. Soil Sample observations in different dilutions

Dilution	Macroscopic	Microscopic
10^{-1}	White cottony appearance	*Fusarium sp.*
10^{-2}	White fluffy large colonies	*Fusarium sp.*
10^{-3}	White fluffy large colonies Green colored colonies	*Fusarium sp.* *Penicillium sp.*
10^{-4}	White fluffy large colonies Black powdery colonies	*Fusarium sp.* *Aspergillus sp.*
10^{-5}	White fluffy large colonies	*Fusarium sp.*

Table 9. Plant samples in different dilutions

Dilutions	Macroscopic	Microscopic
10^{-1}	White cottony appearance Green colored colonies	*Fusarium sp.* *Penicillium sp.*
10^{-2}	White fluffy large colonies Green colored colonies	*Fusarium sp.* *Penicillium sp.*
10^{-3}	White fluffy large colonies Green colored colonies	*Fusarium sp.* *Penicillium sp.*
10^{-4}	White fluffy large colonies Green colored colonies	*Fusarium sp.* *Penicillium sp.*
10^{-5}	White fluffy large colonies Black powdery colonies Green colored colonies	*Fusarium sp.* *Aspergillus s.* *Penicillium sp.*

Result

Fusarium sp. was found to be in large number in both plant and soil samples studied. This is the reason for the damping off disease of seedlings in passion fruit planted in roof top nursery.

Fig. 29. *Fusarium* sp. (45X)

14
IDENTIFICATION OF DISEASE CAUSING PATHOGENS IN PASSION FRUIT PLANTS OF PRS FIELD

Aim

To identify the disease causing pathogens for infected plants 55-8, 55-9,Vazhakulam purple variety plants 12, 10, 9, 8, 7 and 6, 86-7, 134-4, 55-5 and 125

Materials Required

SDA, Nutrient Agar (NA) plates, sterile distilled water, test tubes and routine lab equipments

Technical Programme

As per the information from the field, that the passion fruit plants started to wilt, plant pathologist visited the field and collected the soil samples, stem samples etc. Aseptically collected the samples and packed well. Checked the symptoms of the disease.

Symptoms

Sample varieties showed wilting, root rot, stem rot, and stem discoloration. During that time leaves were falling from the plant. When removed the soil nearby the root system had shown rotting except tap root.

Techniques Include

1. Surface sterilization of the sample
2. Serial dilution of the soil sample
    - Serial dilution
    - Plating (Spread plate)
    - Colony Counting
    - Sub culturing (Streaking)
    - Gram's Staining
    - Biochemical tests (if applicable)
3. Inoculation of leaf, stem, root samples on SDA plates
    - Preparation of SDA
    - Inoculation of samples
    - LPCB for fungal identification
4. Gram Staining
5. Hanging Drop Motility

Surface Sterilization of the Samples

- Washed the samples (root & stem samples collected from the diseased plants) in running tap water for few minutes.
- Washed again using sterile distilled water.
- Rinsed the samples using 70% alcohol.
- Washed again using distilled water for removing the alcohol.
- Rinsed the samples using 0.1% Mercuric chloride ($HgCl_2$) for 1 minute
- Washed the samples with distilled water for removing the excess mercuric chloride

Serial Dilution

Weigh 1 g of soil sample and add it to 10 ml sterile distilled water and mix well. The sample was serially diluted up to 10^{-4} by transferring 1ml. first three dilutions spread on 3 Nutrient Agar (NA) and 3 SDA plates by transferring 0.1ml of each dilution. Incubate NA plates in incubator at 37°C for 24 hrs and also incubate SDA plates at room temperature for 3-4 days.

Table 10. Serial dilution of 55-8, 55-9 on NA plates

Sample	Dilutions	No. of Colonies	Cultural Characters
55-8	10^{-1}	TNTC	Off White, Mucoid, Round, Small, Opaque, Flat Colonies
	10^{-2}	110 Colonies	
	10^{-3}	No Growth	
55-9	10^{-1}	TNTC	
	10^{-2}	TFTC	
	10^{-3}	TFTC	

N B: TNTC – Too Numerous to Count

TFTC - Too Few to Count

Sub cultured the colonies for getting pure culture by streak plate method and performed Gram staining.

Gram Staining Result

Purple-rods were obtained by gram staining, Gram positive bacteria. Both 55-8 and 55-9 have the same bacteria. It's a common soil microbe, which is not responsible for this infection.

Table 11. Serial Dilution of 55-8, 55-9 on SDA plates

Sample	Dilutions	No: of colonies	Cultural characters
55-8	10^{-1}	TNTC	white puffy, yellow puffy, blackish-brown, off- white, large, fibrous colonies
	10^{-2}	TNTC	
	10^{-3}	TNTC	
55-9	10^{-1}	TNTC	
	10^{-2}	TNTC	

NB:– TNTC-Too Numerous to Count
TFTC - Too few to Count

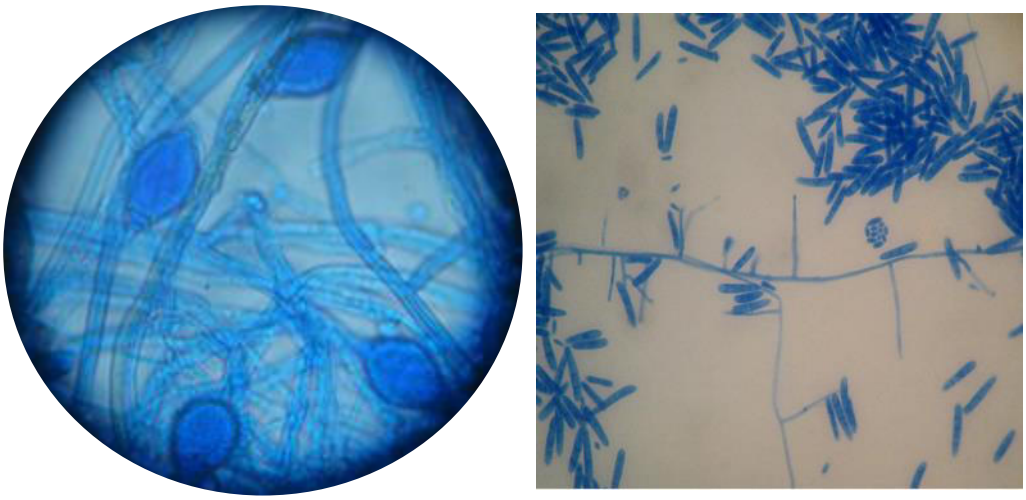
Performed LPCB for the fungal growths obtained from leaf and root samples on SDA plates. Macroscopic observations of the fungal growth obtained from leaf and root samples on SDA plates are discussed below:

Table 12. Macroscopic and Microscopic Observations of Different Passion Fruit Samples on SDA plates

Macroscopic observations	Microscopic observations
Off white colored, fibrous, spreading colonies all over the surface with yellowish base	Mycelium with highly branched hyphae, single or many branched sporangiospores are present. Lemon or egg shaped sporangia.

LPCB Results

The fungal pathogen was *Phytophthora sp.*



Phytophthora sp. (45X) *Fusarium* sp. (45X)

Fig. 30. Microscopic view of *Phytophthora* & *Fusarium* sp.

Table 13. Results of 86, 134 and 55 varieties of passion Fruits

Sample	LPCB Result	Gram's Staining	Hanging drop
86Y-R2 Root	*Fusarium sp.*	Gram +ve cocci	Non Motile
86Y-R2 Leaf	*Fusarium sp.*	Gram +ve rods	Non Motile
134P-R1 Root	*Mucor sp.*+ *Fusarium sp.*	Gram +ve cocci	Motile
134P-R1 Leaf	*Fusarium sp.*	Gram +ve cocci	Non Motile
134P-R1 Root	*Fusarium sp.*	Gram +ve rods	Motile
134P-R1 Leaf	*Fusarium sp.*	Gram +ve cocci	Motile
125Y-R1 Leaf	*Fusarium* + *Mucor sp.*	Gram +ve cocci	Non Motile
55Y-R2 Root	*Fusarium sp.*	Gram +ve cocci	Non Motile
66Y-R1 Root	*Fusarium sp.*	Gram +ve rods	Motile

Table 14. Macroscopic and Microscopic observations of 86, 134 and 55 varieties of passion Fruits

Macroscopic Observation	Microscopic Observation
White, cottony, rhizoid, irregular growth all over the media	Short and branched conidiophores are present. Microconidia, Microconidia and kidney shaped spores are present. Chlamydospore are produced at terminal position with dark, thick wall

15
IDENTIFICATION OF PATHOGENS FROM MD-2 PINEAPPLE FRUIT ROT

Objective

To identify the causative agent of pineapple fruit rot.

Materials Required

Slide, Staining Kit, Cover slip, Needle, Loop, Petriplates

Technical Programme

The sample was inoculated on SDA plates and NA plates and incubated at 25°C for 3-4 days and at 37°C for 24 hours, respectively. The growth was observed on the plates and further subjected to the following tests for identification of the organism.

Techniques Include

- Gram staining
- LPCB staining
- Hanging drop method
- Biochemical tests
- Germ tube test
- Urea hydrolysis
- Starch Hydrolysis
- Phenylalanine Agar

Observation

Table 15. Observations of samples

1	On SDA plates	White coloured large colonies
2	Gram staining	Gram Positive Rods
3	LPCB staining	Yeast like cells, round/ovoid in nature
4	Hanging drop method	Motile rods
5	Catalase test	-
6	Oxidase test	-
7	Urea Hydrolysis	+
8	Starch Hydrolysis	+
9	Phenylalanine Test	-
10	Germ Tube Test	+
Biochemical tests		
1	Sugar Fermentation Tests	
a	Glucose	Fermented with acid & gas production
b	Lactose	Fermented with acid & gas production
c	Sucrose	Non-fermented
4	Indole Production	-
5	Methyl Red	-
6	Voges – Proskauer	-
7	Citrate Utilization	-
8	Urease test	No change
9	Triple Sugar Iron Agar	Pink – yellow, No H_2S production

Result

The sample may be *Candida* sp.

16
MICROBIOLOGICAL EXAMINATION OF MILK

Methylene Blue Reductase Test

Principle

This reductase test is based on the oxidation-reduction activities of the bacteria present in the milk sample. The indicator used in the reaction is methylene blue which is color sensitive to oxygen concentration. The indicator is blue in the oxidized state and leuco or white in the reduced conditions. The speed of color disappearance of methylene blue is proportional to the microbial load in the milk sample. The more the bacteria present the faster will be the reduction.

The classification of milk as per methylene blue reductase test is as follows.

1. Class I- Excellent, not decolorized in 8 hours (<500 bacteria/ml)
2. Class II-Good, decolorized in less than 8 hours but not less than 6 hours (>500 bacteria/ml)
3. Class III-Fair, decolorized in less than 6 hours but not less than 2 hours (>40,00,000 bacteria/ml)
4. Class IV- Poor, decolorized in less than 2 hours. (> 2,00,00,000 bacteria/ml)

Materials Required

Milk sample, methylene blue solution, Mc Cartney bottles, pipettes, water bath set at 37^{o}C, distilled water, Bunsen burner.

Note

All the glass wares were sterilized before use.

Procedure

- Methylene blue solution was prepared by dissolving 1 mg methylene blue powder aseptically in 25 ml of distilled water.
- Transferred 10 ml of milk sample into sterile Mc Cartney bottle using sterile pipettes.
- Added 1 ml of methylene blue solution to the milk sample using a separate sterile pipette.
- The bottle was closed with the stopper.
- The contents of the tube were mixed by gently inverting it 2-3 times.
- Incubate the Mc Cartney bottle in a water bath at 37^{o}C for 6 hours.
- Controlled tubes containing 10 ml boiled milk and 1 ml of methylene blue was also incubated.
- Recorded the time for discoloration.

17
MICROBIAL ANALYSIS OF FOOD ITEMS

Microbial analysis of ice cream and soft drink

Principle

The bottled beverages including non-pasteurized non-carbonated soft drink should confirm as a minimum requirement for microbiological criteria of the WHO standard. Only pasteurized milk was used in the manufacture of ice cream. Samples were received in the frozen state molted one were rejected. The frozen samples were melted immediately before examination. The spread plates ensure an aerobic environment for microorganisms present in the food sample.

Materials Required

Ice cream sample, soft drink sample, nutrient agar, pipettes, petriplates, test tubes, L rod, alcohol.

Procedure

a) *Ice cream sample*:

- 1 g of ice cream sample was weighed and added to 10 ml sterilized distilled water blanks and was labeled as 10^{-1} dilution.
- Mixed gently by inverting the test tubes for several times.
- Prepared serial dilutions of ice cream sample by transferring 1 ml from first dilution to sterile distilled water and mixed well.
- This test tube was labeled as 10^{-2}.
- The procedure was repeated up to 7th test tube with respective dilution 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} using different sterile pipettes.
- Discarded 1 ml of the sample from 10^{-7} dilution.
- 0.1 ml of the diluted sample from particular dilution were pipetted out into nutrient agar plates and was spread uniformly using a sterilized L-rod.
- Kept the plates in an upright position for few minutes.
- Incubated the plates in an inverted position at 37°C for 24 hours.
- Examined the plates for bacterial colonies.

b) Soft drink sample:

- 1 ml of soft drink was added to 9 ml sterilized distilled water blank and was labeled as 10^{-1} dilution.
- Mix gently by inverting the test tubes for several times.
- Prepared serial dilution of the soft drink sample by transferring 1 ml from first dilution to sterile distilled water blank and mixed well.
- This test tube was labeled as 10^{-2} dilution.
- The procedure was repeated up to 7th test tube with respective dilution 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} using different sterile pipettes.
- Discarded 1 ml of the sample from 10^{-7} dilution.
- 0.1 ml of the diluted sample from particular dilution was pipetted out into nutrient agar plates and was spread uniformly using a sterilized L-rod.
- Kept the plates in an upright position for few minutes.
- Incubated the plates in an inverted position at 37°C for 24 hours.
- Examined the plates for bacterial colonies.

Observation

The number of colonies formed in each plate was counted for both ice cream and soft drink samples. Plates with fewer than 30 colonies were designated as “too few to count” (TFTC) and plates with more than 300 colonies as “too numerous to count” (TNTC).

Microbiological analysis of fruits and vegetables

Principle

Fruits and vegetables are readily susceptible to microbial decomposition, and hence considered as perishables. The total number of bacteria present in the sample of fruits and vegetables and other frozen food can be enumerated by traditional pour plate or spread plate technique. A sterile food blender is essential to obtain the microorganism in suspension.

Materials Required

Nutrient agar, fruit sample, vegetable sample, petriplate, pipettes, L-rod, Homogenizer (food blender), alcohol

Procedure

a) *Fruit sample*

- Using aseptic technique, 1 g of fruit was weighed.
- Weighed food sample was transferred into 10 ml sterile distilled water blank and was labeled as 10^{-1} dilution.
- Mix gently by inverting the test tubes for several times.
- Using a sterile pipette, 1 ml of sample was transferred from 10^{-1} dilution to sterile distilled water blank and was labeled as 10^{-2} dilution.
- Mixed gently.
- The procedure was repeated up to 7th test tube with respective dilution 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} using different sterile pipettes.
- Discarded 1 ml of the sample from 10^{-7} dilution.
- 0.1 ml of the diluted sample from particular dilution was pipetted out into nutrient agar plates and was spread uniformly using a sterilized L-rod.
- Kept the plates in an upright position for few minutes.
- Incubated the plates in an inverted position at 37°C for 24 hours.
- Examined the plates for bacterial colonies.

b) *Vegetable sample*

- Using aseptic technique, 1 g of vegetable was weighed.
- Weighed sample was transferred into 10 ml sterile distilled water blank and was labeled as 10^{-1} dilution.
- Mix gently by inverting the test tubes for several times.
- Using a sterile pipette, 1 ml of sample was transferred from 10^{-1} dilution to sterile distilled water blank and was labeled as 10^{-2} dilution.
- Mixed gently.
- The procedure was repeated up to 7th test tube with respective dilution 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} using different sterile pipettes.
- Discarded 1 ml of the sample from 10^{-7} dilution.
- 0.1 ml of the diluted sample from particular dilution were pipetted out into nutrient agar plates and was spread uniformly using a sterilized L-rod.
- Kept the plates in an upright position for few minutes.
- Incubated the plates in an inverted position at 37°C for 24 hours.
- Examined the plates for bacterial colonies.

Observation

The number of colonies formed in each plate were counted for both fruit and vegetable samples. Plates with fewer than 30 colonies were designated as “too few to count” (TFTC) and plates with more than 300 colonies as “too numerous to count” (TNTC).

18

BACTERIOLOGICAL EXAMINATION OF WATER BY MULTIPLE TUBE FERMENTATION TEST

Aim

To determine the presence of coliform bacteria in the given water sample and to estimate the number of coliforms present in the same

Principle

With increasing industrialization, water sources available for consumption and recreation have been polluted with industrial as well as animal and human wastes. As a result, water has become a formidable factor in disease transmission. Polluted water contains vast amount of organic matter that serves as excellent nutritional sources for the growth and multiplication of microorganisms. The presence of non-pathogenic organisms is not of major concern, but intestinal contaminants of fecal origin are important. These pathogens are responsible for intestinal infections such as bacillary dysentery, typhoid fever, cholera and paratyphoid fever. Since *Escherichia coli* are always present in the human intestine, its presence in water indicates the presence of other human or animal intestinal pathogens. Both qualitative and quantitative methods are used to determine the sanitary conditions of water.

Multiple tube fermentation tests are used to detect coliform bacteria which are used as indicator of fecal contamination. This test is performed as sequentially in 3 stages: Presumptive, Confirmed and Completed tests. Coliform bacteria are aerobic or facultative anaerobic, gram negative, rod shaped, and non-endospore forming, capable of fermenting lactose with the production of acid and gas within 24 hours of incubation at 37°C.

a) Presumptive Test

The presumptive test is specific for detection for coliform bacteria. It is used to detect and estimate the coliform population of a water sample. Measured the water sample to be added to a lactose fermentation broth containing an inverted glass vial. Because these bacteria are capable of using lactose as the carbon source, this detection is facilitated by use of this medium. In addition to lactose, the medium also contains a surface tension depressant, bile salt, used to suppress the growth of organisms other than coliform bacteria. The presumptive test also enables to obtain the number of coliform organisms present by means of most portable number test (MPN). The MPN is estimated by determining the number of tubes in each group that show gas following the incubation period.

Procedure

- Arranged three double strength broth (each tube with 10 ml medium) and six single strength broth (each tube containing 5 ml medium) with inverted Durham's tube.
- Added 10 ml, 1 ml and 0.1 ml water sample to three test tubes with 10 ml double strength broth, 3 tubes with 5 ml single strength broth and three tubes with 5 ml single strength broth respectively using different sterile pipettes.
- Mixed it gently
- Incubated the tubes at 37°C for 24-48 hours.
- After incubation, the tubes were observed for more gas production.

Observation

After incubation, the tubes were observed for 10% or more gas production

- Positive: 10% or more gas production in the Durham's tube after 24 hours of incubation.

- Doubtful : gas developed after 48 hours of incubation
- Negative: No gas production after 48 hours.

b) Confirmed Test

This test is used to confirm the presence of coliforms in water samples showing positive or doubtful presumptive tests. Confirmation of these results is necessary, since positive presumptive tests may be the result of organisms of non-coliform bacteria that are not recognized as indicators of fecal pollution. The confirmed test requires selective and differential media such as Eosin Methylene Blue. EMB contains the dye methylene blue, which inhibits the growth of gram positive organisms. In the presence of an acid environment, EMB forms a complex that precipitates out in to the coliform colonies, producing a dark centre and green metallic sheen. This reaction is characteristic for *Escherichia coli*, the major indicator of fecal pollution.

Procedure

- Sterilized, dried EMB agar plates were inoculated with positive 24 hours broth culture selected from presumptive tests by using sterile loop
- The inoculated plates were incubated at 37°C for 24 hours
- Examined the inoculated plates for *E. coli* colonies

Observation

Appearance of green metallic sheen with dark centers indicates the presence of *Escherichia coli* in the water sample.

c) Complete Test

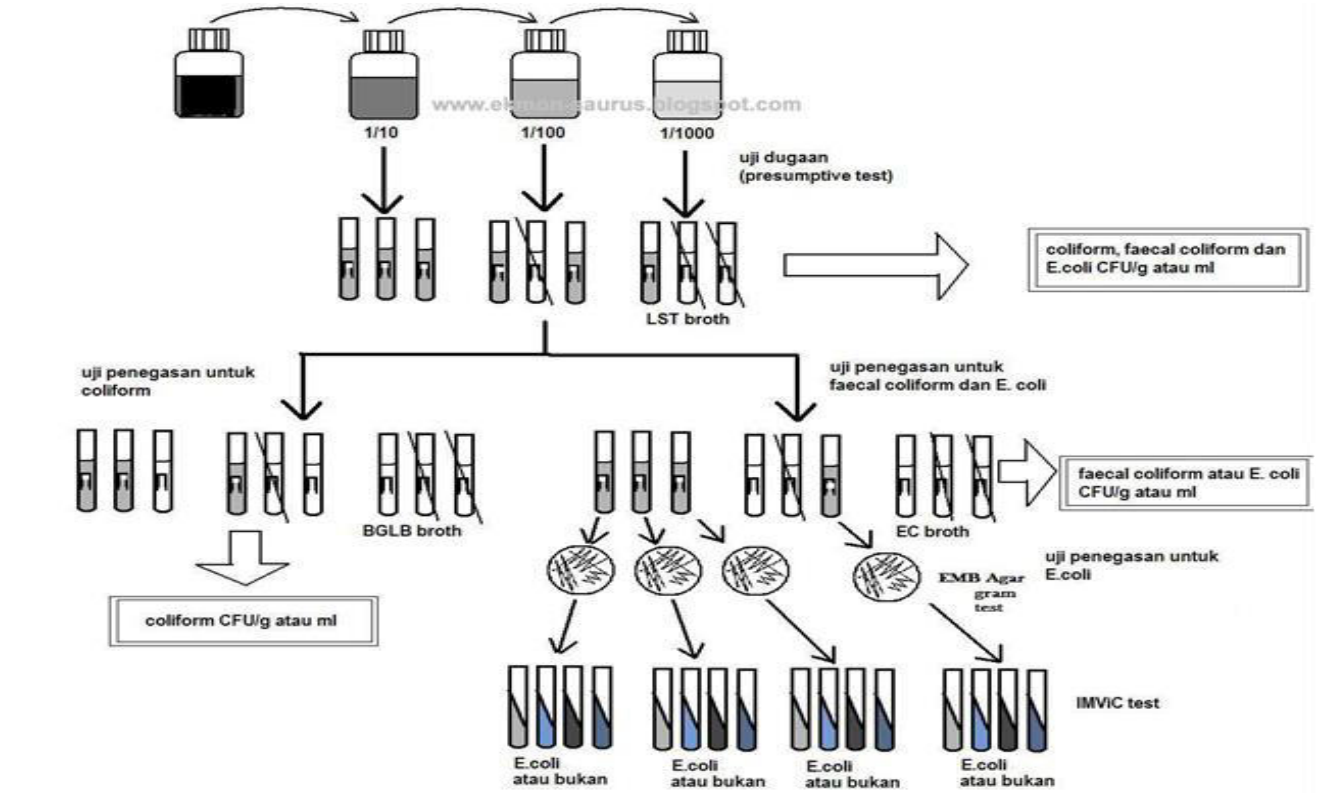
The completed test is the final analysis of water sample. It is used as a confirmatory test for the presence of *E. coli* in a water sample. It's used to examine the coliform colonies that appeared on the EMB plates used in the confirmed test. An isolated colony is picked from the confirmatory test plate and inoculated onto a tube of lactose broth and the presence of gram negative bacilli on microscopic examination are further confirmation of the presence of *E. coli* and they are indicative of positive completed test.

Procedure

- The broth and nutrient agar slants were inoculated with the organism obtained from the EMB agar plates of confirmed test using sterile loop.
- Incubated the inoculated media at 37°C for 24 hours
- Examined the Brilliant green lactose broth for gas production
- Preformed gram stain from nutrient agar slant

Observation

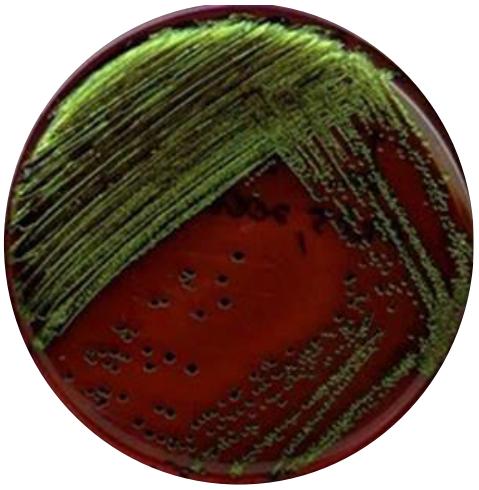
- Gas production was seen in broth
- Gram negative bacilli were observed on Gram staining.



MPN Procedure

nomor tabung yang positif			indeks MPN	95% batas kepercayaan	
10 ml	1 ml	0,1 ml	per 100 ml	terendah	tertinggi
0	0	1	3	<0.5	9
0	1	0	3	<0.5	13
1	0	0	4	<0.5	20
1	0	1	7	1	21
1	1	0	7	1	23
1	1	1	11	3	36
1	2	0	11	3	36
2	0	0	9	1	36
2	0	1	14	3	37
2	1	0	15	3	44
2	1	1	20	7	89
2	2	0	21	4	47
2	2	1	28	10	150
3	0	0	23	4	120
3	0	1	39	7	130
3	0	2	64	15	380
3	1	0	43	7	210
3	1	1	75	14	230
3	1	2	120	30	380
3	2	0	93	15	380
3	2	1	150	30	440
3	2	2	210	35	470
3	3	0	240	36	1300
3	3	1	460	71	2400
3	3	2	1100	150	4800

MPN Index Chart



***E.coli* on EMB Agar Plate**

Fig. 31. Most Probable Number

19

ANTIBIOTIC SENSITIVITY TEST (KIRBY – BAUER METHOD OR DISC DIFFUSION METHOD)

Aim

To determine the susceptibility of a pathogen to a range of antibiotics

Principle

Antibiotics are natural antimicrobial agents produced by microorganisms. One type of penicillin, for example, is produced by the mold *Penicillium notatum*. The Kirby-Bauer test, also called the disc diffusion test, is a valuable standard tool for measuring the effectiveness of antimicrobics against pathogenic microorganisms. In the test, antimicrobics-impregnated paper disks are placed on a plate that is inoculated to form a bacterial lawn. The plates are incubated to allow growth of the bacteria and time for the agent to diffuse into the agar. As the drug moves through the agar, it establishes a concentration gradient. If the organism is susceptible to it, a clear zone will appear around the disk where growth has been inhibited.

The size of this zone of inhibition depends upon the sensitivity of the bacteria to the specific antimicrobial agent and the point at which the chemical's minimum inhibitory concentration (MIC) is reached. Some drugs kill the organism and are said to be bactericidal. Other drugs are bacteriostatic; they stop growth but don't kill the microbe.

Materials and Methods

Broth culture, Muller- Hinter agar Plates, antibiotic disc, cotton swab and sterile forceps

Procedure

- The test organism was inoculated on to sterile peptone water and was incubated at 37°C for 2-4 hours.
- After proper incubation, the suspension was then swabbed on to sterile Muller – Hinter Agar using sterile cotton swab and kept in position for some time.
- The antibiotic discs of known potency were placed on to agar surface using sterile forceps and gently pressed it
- Incubated the plates at 37°C for 24 hours and observed it carefully.

Observation

Observed the zone of growth inhibition around the disc and measured it and compared the value with standard antibiogram. Based on the comparison the organism can be differentiated in to sensitive, intermediate sensitive and resistant.

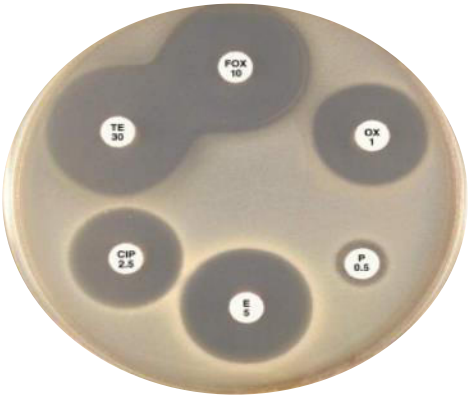


Fig. 32. Antibiotic Sensitivity Test

UNITS, MEASUREMENTS, CONVERSIONS AND USEFUL DATA

SI prefixes and multiplication factors

Multiplication factor		Prefix	Symbol
1 000 000 000 000 000 000 000 000	$=10^{24}$	yotta	Y
1 000 000 000 000 000 000 000	$=10^{21}$	zetta	Z
1 000 000 000 000 000 000	$=10^{18}$	exa	E
1 000 000 000 000 000	$=10^{15}$	peta	P
1 000 000 000 000	$=10^{12}$	tera	T
1 000 000 000	$=10^{9}$	giga	G
1 000 000	$=10^{6}$	mega	M
1 000	$=10^{3}$	kilo	k
100	$=10^{2}$	hecto	h
10	$=10^{1}$	deca	da
0.1	$=10^{-1}$	deci	d
0.01	$=10^{-2}$	centi	c
0.001	$=10^{-3}$	milli	m
0.000 001	$=10^{-6}$	micro	μ
0.000 000 001	$=10^{-9}$	nano	n
0.000 000 000 001	$=10^{-12}$	pico	p
0.000 000 000 000 001	$=10^{-15}$	femto	f
0.000 000 000 000 000 001	$=10^{-18}$	atto	a
0.000 000 000 000 000 000 001	$=10^{-21}$	zepto	z
0.000 000 000 000 000 000 000 001	$=10^{-24}$	yocto	y

SI base, derived and other units

Phisical quantity	Unit	Symbol
length	metre	m
mass	kilogram	kg
time	second	s
electric current	ampere	A
thermodynamic temperature	kelvin	K
amount of substance	mole	mol
luminous intensity	candela	cd
time	minute	min
time	hour	h
time	day	d
plane angle	degree	º
plane angle	minute	’
plane angle	second	’’
length	foot	’
length	inch	’’
length	angstrom	A
area	barn	b
volume	litre	l
mass	tonne	t
pressure	bar	bar

SI Units

Length and area

Micron (μm, μ)	$=10^{-6}$ m
Angstrom (A)	$=10^{-10}$ m
Fermi (fm)	$=10^{-15}$ m
Are (a)	$=100$ m^2
Barn (b)	$=10^{-28}$ m^2

Mass

Tonne (t)	$=10^6$g=1000kg

fps Units

Length

12 inches	=1 foot (ft)
3 feet	=1 yard (yd)
22 yards	=1 chain
10 chains	=1 furlong
8 furlongs	=1 mile (mi)
6 feet	=1 fanthom
6080 feet	=1UK nautical mile

Area

4840 yard^2	=1 acre
640 acres	=1 mile^2

Mass

16 ounces (oz)	=1 pound (lb)
14 pounds	=1 stone
28 pounds	=1 quarter
4 quarters	=1 hundredweight
20 hundredweight	=1 ton

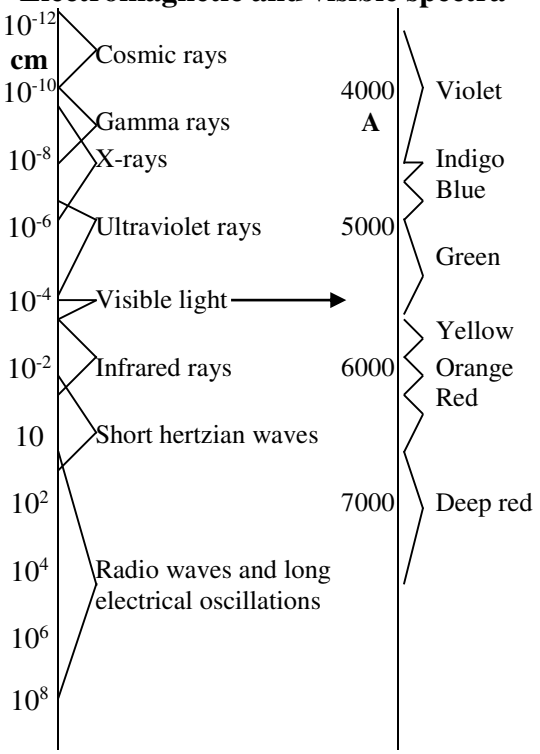
Volume

20 fluid ounces	=1 pint (pt)
2 pints	=1 quart (qt)
4 quarts	=1 gallon

Conversion Table

fps Units	SI Units	Reciprocal
Length		
1 inch (in)	$=2.54 \times 10^{-2}$m	39.370079
1 foot (ft)	=0.3048 m	3.280839
1 yard (yd)	=0.9144 m	1.093613
1 fanthom	=1.8288 m	0.546806
1 chain	=20.1168 m	4.97097×10^{-2}
1 furlong	$=2.01168 \times 10^2$ m	4.97097×10^{-3}
1 mile (mi)	1.609344×10^3 m	6.213712×10^{-4}
Area		
1 in^2	$=6.4516 \times 10^{-4}$m^2	1.550003×10^3
1 ft^2	$=9.290304 \times 10^{-2}$m^2	10.763910
1 yd^2	=0.836127 m^2	1.195990
1 mi^2	$=2.589988 \times 10^6$ m^2	3.861022×10^{-7}
1 acre	$=4.046856 \times 10^3$ m^2	2.471054×10^{-4}
Volume		
1 in^3	$=1.638706 \times 10^{-5}$ m^3	6.102374×10^4
1 ft^3	$=2.831685 \times 10^{-2}$m^3	35.31467
1yd^3	=0.764555 m^3	1.307950
1 fluid ounce (fl oz)	$=2.841306 \times 10^{-5}$m^3	3.519508×10^4
1 pint (pt)	5.682613×10^{-4}m^3	1.759754×10^3
1 quart (qt)	$=1.136523 \times 10^{-3}$m^3	8.798770×10^2
1 gallon (gal)	$=4.54609 \times 10^{-3}$m^3	2.199692×10^2
1 bushel (bu)	=0.036369 m^3	27.495944
1 US gallon (=231in^3)	$=3.785412 \times 10^{-3}$ m^3	2.641721×10^2
Mass		
1 ounce (oz)	$=2.834952 \times 10^{-2}$ kg	35.273962
1 pound (lb)	=0.45359237 kg	2.204623
1 stone	=6.350293 kg	0.158473
1 quarter	=12.700586 kg	7.873652×10^{-2}
1 hundredweight	=50.802345 kg	1.968413×10^{-2}
1 ton	$=1.016047 \times 10^3$ kg	9.842065×10^{-4}

Electromagnetic and visible spectra



Designation of large numbers

	USA	UK
10^6	million	million
10^9	billion	milliard
10^{12}	trillion	billion
10^{15}	quadrillion	billiard
10^{18}	quintillion	trillion

Conversion Factors

Parameter	Conversion	Reciprocal
P_2O_5	=P x 2.29	0.43668
K_2O	=K x 1.2	0.83333
Urea	=N x 2.174	0.46
SSP	=P_2O_5 x 6.25	0.16
MOP	=K_2O x 1.67	0.275438
Organic matter %	=C x 1.74	0.5747

Soil test rating

Parameter	Low	Medium	High
O.C. %	< 0.5	0.5-0.75	> 0.75
N kg/ha	< 280	280-560	> 560
P kg/ha	< 10	10-25	> 25
K kg/ha	< 110	110-280	> 280

Useful data

1 atmo = 764 mm Hg = 1036 cm water = 1013 mbar = pF 3 = 1.03329 kg/cm^2 = 1013231 dynes/cm^2
1 ha = 0.01 km^2 = 10000 m^2 = 2.471 ac = 0.003861 mile^2
1 ac = 100 cents = 0.4 ha = 4047m^2 = 43560ft^2
1 cent = 40.47m^2 = 436ft^2
1 mile = 8 furlong = 80chains = 1760yards = 5280ft
1 ton = 20 hundredweight (cwt) = 80 quarter = 2240 pounds = 35840 ounces
1 HP = 76.0404 kg.m/s = 745.7 watts = 550 ft.lb/s
Erosion: v velocity, v^2 erosive power, v^5 amount eroded, v^6 size of materials carried away.
When slope is increased 4 times: increase in velocity 2 times, erosivity 4 times, quantity 32 times, size 64 times.
Construct bunds every 3 ft vertical drop or 300 ft length whichever is less
Solar constant: 2cal/cm^2/minute. Average energy received on earth from sun.
Plants use only 0.4-0.5% energy. Algae use the maximum, 2.5%
Beer's law $I=I_oe^{-KL}$
Hopkins bioclimatic law: Crop phenological events are delayed by 1 day for every 1° latitude, 5° longitude and 400 ft altitude.
15 cm 1 ha furrow slice = 2.2 million kg
1 ha 1 mm water = 10 m^3
1 cusec (cubic foot/s) = ft^3/s = 28.3 l/s = 1 acre inch/hour
1 cumes (cubic metre/s) = m^3/s = 35.3 cusecs
°C = (°F – 32) 5 ÷ 9 ; °F = 1.8°C + 32
Balanced fertilizer application based on balanced ratio of NPK 4:16:1 in the economic part.
Protein contains 16% N, Protein % =N% x 6.25
Organic matter contains 58% carbon, Organic matter % =C % x 1.724
Soil water potential, $\psi = \psi_{m/p} + \psi_{o/s} + \psi_g + \psi_a$
Pan evaporation, E_o=4-6 mm/day, $Et_o=E_o$ x 0.6-0.8
Irrigation requirement: depth=3-8cm, interval=8-10days, IW/CPE=0.9 for sensitive crops, 0.6 for hardy crops
N x Eq. wt=g/l, N=Eq. wt/l, ppm=µg/ml=mg/l=me/l x Eq. wt.
mmhos/cm, EC x 640=TSS, ppm; EC x 0.064=TSS%; EC x 10=TSS, me/l; EC x –0.36=$\psi_{o/s}$ (+ osmotic pressure)
NPK recovery by crop: <40, <20, 80-90%
Cation adsorption to clay: Al>Fe>Si>H>Ca>Mg>K>Na
Anion adsorption to clay: SiO_4>PO_4>MO_4>SO_4>NO_3>Cl
(Cation and anion leaching in reverse order)
Anion toxicity to crops: HCO_3>CO_3>Cl>SO_4>NO_3
Anaerobic reduction during flooding: O>NO_3>Mn>Fe>S>C
Lyophilic series/Displacement capacity of anions: F>OH>HCO_3>PO_4>SiO_4
Plant mobile nutrients: N, P, K, Mg, Cl, S
Plant immobile elements: Ca, Fe, Mn, Zn, Cu, Mo, B
Particle size: solution < 10A colloid 1000A > suspension
Neutralising value (Ca equivalent): $CaCO_3$ 100, $MgCO_3$ 119, $Ca(OH)_2$ 136, CaO 179
Residual (equivalent) acidity: $CaNH_4NO_3$ zero, Urea 80, NH_4NO_3 60, $(NH_4)_2SO_4$ 110, $(NH_4)_3PO_4$ 86, NH_4Cl 128, anhydrous NH_3 148
Essential elements: C, H, O, N, P, K, Ca, Mg, S, Fe, Mn, Zn, Cu, Mo, B, Cl, Co
Soils having >20% o.m. = organic; >70% sand = sandy; >40% clay = clayey; 27-52% silt = silty soil

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