Laboratory Manual
Microbiology
(BT102)UPDATED 2020



VIRTUAL UNIVERSITY OF PAKISTAN

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Practical No: 1
Biosafety levels

Biosafety

Safety from exposure to Infectious Agents

Biosafety Level:

A biosafety level is a set of biocontainment precautions required to isolate dangerous biological agents in an enclosed laboratory facility.

Importance:

- Laboratorians recognize hazards of processing infectious agents
- Guidelines developed to protect workers in microbiological and medical labs through engineering controls, management policies, work practices
- Precautions so people researching or trying to identify organisms do not become infected
- While handling or testing clinical specimens, workers could accidentally infect themselves or coworkers
- Labs must adhere to very specific safety regulations to work with organisms that pose a threat to human health

Biosafety levels (1-4):

There are four biosafety levels; protective practices increase with each

Biosafety Level 1 labs - work with least dangerous agents, require fewest precautions.

Biosafety Level 4 labs - have strictest methods because dealing with agents that are most dangerous to human health

Primary barriers: Physical barriers or personal protective equipment between lab worker and pathogen. For example: Gloves, masks, lab coat, goggle, shoes cover

Secondary barriers: Structural aspects of the laboratory that make working environment safer against infection. For example: Sinks for hand washing, special containment areas, special air ventilation patterns

BIOSAFETY LEVEL 1

- Work is generally conducted on open bench tops using standard microbiological practices.
- Special containment equipment or facility designs is not required nor generally used.

- Laboratory personnel have specific training in the procedures conducted in the laboratory and are supervised by a scientist with general training in microbiology.

Standard Microbiological Practices:

- Eating, drinking, smoking, handling contact lenses, and applying cosmetics are not permitted in the work areas where there is reasonable likelihood of exposure to potentially infectious materials.
- Food is stored outside the work area in cabinets or refrigerators designated and used for this purpose only.
- Mouth pipetting is prohibited; mechanical pipetting devices are used.
- All procedures are performed carefully to minimize the creation of splashes or aerosols.
- Work surfaces are decontaminated at least once a day and after any spill of viable material.
- All cultures, stocks, and other regulated wastes are decontaminated before disposal by an approved decontamination method.
- Materials to be decontaminated outside of the immediate laboratory are to be placed in a durable, leak-proof container and closed for transport from the laboratory.
- Materials to be decontaminated at off-site from the laboratory are packaged in accordance with applicable local, state, and federal regulations, before removal from the facility.
- Special containment devices or equipment such as a biological safety cabinet is generally not required for manipulations of agents assigned to Biosafety Level 1.
- It is recommended that laboratory coats, gowns, or uniforms be worn to prevent contamination.
- Gloves should be worn if the skin on the hands is broken or if a rash exists.

Laboratory Facilities:

- Each laboratory contains a sink for hand washing.
- The laboratory is designed so that it can be easily cleaned. Rugs in laboratories are not appropriate, and should not be used because proper decontamination following a spill extremely difficult to achieve.
- Bench tops are impervious to water and resistant to acids, alkalis, organic solvents, and moderate heat.
- Laboratory furniture is sturdy. Spaces between benches, cabinets, and equipment are accessible for cleaning.
- If the laboratory has windows that open, they are fitted with fly screens.

BIOSAFETY LEVEL 2

- Risk Group 2 infectious agents are pathogens that can cause human or animal disease but, under normal circumstances, are unlikely to be a serious hazard to laboratory workers, the community, livestock, or the environment.
- Infections by risk group 2 organisms are not considered to be a serious hazard. They are a moderate individual risk and limited community risk.
- Laboratory exposures rarely cause infection leading to serious disease; effective treatment and preventive measures are available and the risk of spread is limited.
- Examples of infectious agents in this risk level are E. coli, many influenza viruses, some fungi like ringworm; human herpes simplex viruses; Transmissible Gastro-enteritis of swine; Mouse Hepatitis Virus; and a few parasites.
- **Primary hazards:** accidental needle sticks, exposure to eyes and nose, ingestion of infectious materials
- Agents do not cause lethal infections that are not transmissible via airborne route (do not cause infection if tiny droplets become airborne and are inhaled, which might occur if the material were spattered)
- Agents are pathogens for which immunization or antibiotic treatment is available
- Extreme care should be taken with contaminated needles and sharp lab instruments

Procedures required to contain risk group 2 organisms:

- The laboratory should be separated from all other activities.
- A biohazard sign should be present and visible. (policies to restrict access to lab)
- All the surfaces in the laboratory should be readily cleanable
- An autoclave should be present.
- A certified HEPA filtered class 1 or 2 biological cabinet should be available for the manipulation of organisms.

Gloves and a laboratory coat should be worn at all times in the laboratory



Fig 1.1: Biohazard Symbol

BIOSAFETY LEVEL 3

- Risk Group 3 infectious agents are pathogens that usually cause serious human or animal disease, or which can result in serious economic consequences, but do not ordinarily spread by casual contact from one individual to another (high individual risk, low community risk), or that can be treated by antimicrobial or antiparasitic agents.
- Risk Group 3 pathogens include bacteria such as anthrax, Q Fever, tuberculosis, and viruses such as hanta viruses, Human immunodeficiency viruses (HIV - all isolates), eastern and western equine encephalitis viruses.

Procedures required to contain risk group 3 organisms:

- Laboratory staff must be fully trained in the handling of pathogenic and other hazardous material, in the use of safety equipment, disposal techniques, handling of contaminated waste, and emergency response.
- Standard operating procedures must be pasted in visible spot.
- Equipment must include an autoclave and a certified HEPA filtered class II biological safety cabinet
- All activities involving infectious materials to be conducted in biological safety cabinets or other appropriate combinations of personal protective and physical containment devices.

BIOSAFETY LEVEL 4

Designed for use with:

- Highly toxic/infectious agents
- Agents that are at a very high risk for forming infectious aerosols
- Life threatening agents

Examples: Ebola virus, Marburg virus, Lassa virus

Special Practices:

❖ Only those that work in the BSL-4 lab will be allowed entry

❖ A logbook must be signed each time one comes in or out of the lab with the date and time

❖ The doors must have a universal hazard label with….

- Description of the infectious agent (s)
- Responsible director

❖ All lab personnel are trained specially for BSL-4 by lab director

❖ Lab personnel must be vaccinated for the agents:

- Present in the lab
- That have potential to be in the lab

❖ Baseline serum samples are collected and stored for serological surveillance

- Make sure the personnel have sufficient antibodies for the pathogens they are working with

❖ Supplies/materials are brought into the lab by a double door autoclave that….

- is secured so that materials can be removed from the interior door
- is decontaminated after each use

❖ Plastic is substituted for glass when possible

- Eliminate sharps such as capillaries, scalpels, needles and syringes
- Broken glass should be handled with a dust pan and broom
- Non-disposable and disposable sharps collected in a hard container to be transported to an autoclave for decontamination; disposables are thrown out

Safety Equipment:

Primary barriers:

❖ Class III biological safety cabinets for handling infectious material or….

❖ BSC must be validated on a 12 month period

❖ The PPE required is a

- one-piece jumpsuit
- gloves
- closed toe shoes
- All will be removed in the inner room after showering

Secondary barrier:

❖ BSL-4 labs are housed in separate buildings or isolated within a building with lower BSL labs

❖ Outer and inner change rooms

❖ Walls, floors and ceilings have sealed internal shells

- keep pests out
- Liquid and heat stabile for decontaminating purposes

❖ Drains have chemical disinfectant traps attached to liquid waste decontamination system

- All waste must be decontaminated including shower waste

❖ HEPA filters in vents for sterile air

- Filter air exhaust from the class III biological safety cabinets.



Fig 1.2: One-piece suit

Biosafety Cabinet Classification

Class I biosafety cabinet	A ventilated cabinet with an inward airflow and outlet HEPA filters. It was previously referred to as the CDC Hood and served a valuable function in its time by protecting personnel and the environment. Because it offers no product protection, it has been essentially obsolete for the past several decades.
Class II (types A and B)	Laminar Flow Biological Safety Cabinets that protect personnel, product and environment. They provide inward airflow to protect personnel, downflow HEPA filtered air to the work area to protect the product and exhaust HEPA filtered air to protect the environment from particulate and aerosol hazards.
Class III cabinet	Essentially a ventilated glovebox. This is a gas-tight chamber operated through sealed gloves which provide a complete barrier between the worker and hazardous material. The glovebox is maintained under negative pressure with HEPA filtered supply air and double HEPA filtered exhaust air.

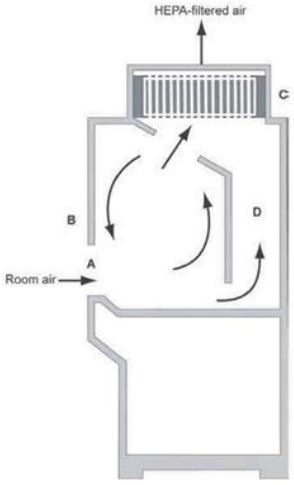


Fig 1.3: Biosafety Cabinet Class I

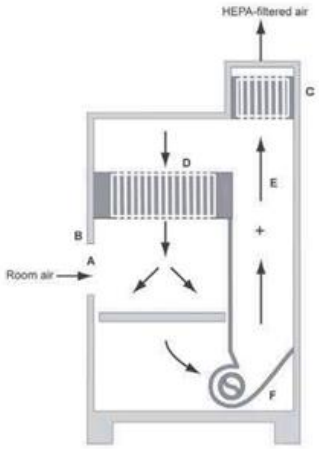


Fig 1.4: Biosafety Cabinet Class II

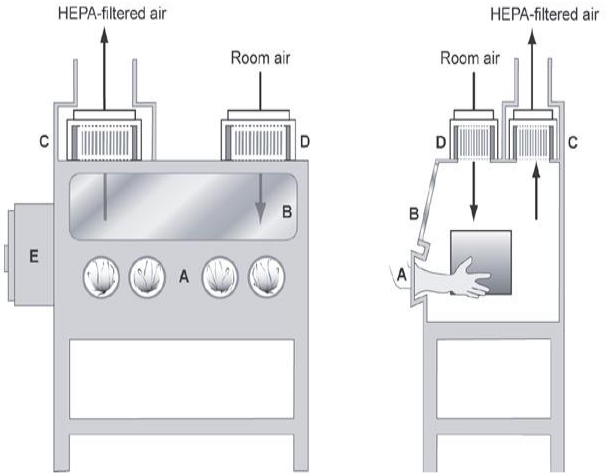


Fig 1.5: Biosafety Cabinet Class III

Table 1.1: Type of biosafety rules in details

BSL	Agents	Practices	Safety Equipment (Primary Barriers)	Facilities (Secondary Barriers)
1	Not known to consistently cause disease in healthy adults	Standard Microbiological Practices	None required	Open bench top sink required
2	Associated with human disease, hazard = percutaneous injury, ingestion, mucous membrane exposure	BSL-1 practice plus: Limited access Biohazard warning signs "Sharps" precautions Biosafety manual defining any needed waste decontamination or medical surveillance policies	Primary barriers = Class I or II BioSafety Cabinets (BSCs) or other physical containment devices used for all manipulations of agents that cause splashes or aerosols of infectious materials; Personal Protective Equipments (PPEs): laboratory coats; gloves; face protection as needed	BSL-1 plus: Autoclave available
3	Indigenous or exotic agents with potential for aerosol transmission; disease may have serious or lethal consequences	BSL-2 practice plus: Controlled access Decontamination of all waste Decontamination of lab clothing before laundering Baseline serum	Primary barriers = Class I or II BCSs or other physical containment devices used for all open manipulations of agents; PPEs: protective lab clothing; gloves; respiratory protection as needed	BSL-2 plus: Physical separation from access corridors Self-closing, double-door access Exhausted air not recircul-ated Negative airflow into laboratory
4	Dangerous/exotic agents which pose high risk of life-threatening disease, aerosol-transmitted lab infections; or related agents with unknown risk of transmission	BSL-3 practices plus: Clothing change before entering Shower on exit All material decontaminated on exit from facility	Primary barriers = All procedures conducted in Class III BSCs or Class I or II BSCs in combination with full-body, air-supplied, positive pressure personnel suit	BSL-3 plus: Separate building or isolated zone Dedicated supply and exhaust, vacuum, and decontamination systems Other requirements outlined in the text

Practical No: 2	Sterilization techniques (Physical/Heat method of sterilization)

Introduction

Sterilization is defined as total destruction of all microorganisms (whether or not pathogenic) and their spores, usually through the use of drastic methods such as concentrated toxic chemicals (chlorine, formaldehyde, glutar-aldehydes, etc.), very high temperature, or intense radiation. A sterilized item cannot support life in any form.

Methods involved in sterilization

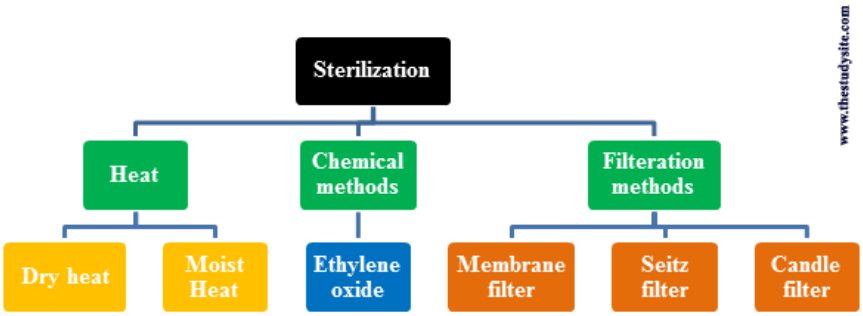


Fig 2.1: List of methods

This is the most common method of sterilization. The heat used kills the microbes in the substance. The temperature of the heat and duration of heating are the factors that affect the extent of sterilization. In heat sterilization process, the longer the exposure to heat the better is the sterilization at a given temperature. As the temperature of heat raises the time span required for sterilization decreases. Further, the sterilization time increases with a decrease in temperature and vice-versa. But one needs to maintain minimum sterilization time or minimum contact time for the heat to be in touch with microbes or bacteria and thereby kill them. The heat method of sterilization is again of two types based on the type of heat used.

A) Moist heat methods. B) Dry heat methods.

A. Moist heat method of sterilization:

Here heat is applied in the form of steam or just boiling. This method includes techniques like

1. Boiling. 2. Pasteurization. 3. By use of steam (Autoclave).

1. Boiling is preferred for metallic devices like surgical scissors, scalpels, needles, etc. Here substances are boiled to sterilize them.

2. Pasteurization is the process of heating the milk at a temperature of 6o degrees or 72 degrees 3 to four times. Here alternative heating and cooling kills all the microbes and molds without boiling the milk.

3. Using Steam (autoclaving): It is the most common method used for drugs as it is powerful enough even to kill bacterial spores. Bacterial spores are the forms of bacteria which are inert. They form a rigid cover over the cell wall during harsh climate. This cover prevents any damage to cell and drying of the cell. By steam sterilization, these forms of bacteria are also killed as steam destroys the cell wall.

Principle:

In this method sterilization is done by steam under pressure. Steaming at temperature higher than 100°C is used in autoclaving. The temperature of boiling depends on the surrounding atmospheric pressure. A higher temperature of steaming is obtained by employing a higher pressure. When the autoclave is closed and made

air-tight, and water starts boiling, the inside pressures increases and now the water boils above 100°C. At 15 ib per sq. inch pressure, 121°C temperatures is obtained. This is kept for 15 minutes for sterilization to kill spores. It works like a pressure cooker.

Construction of Autoclave

Autoclave is a metallic cylindrical vessel. On the lid, there are:

(1) A gauge for indicating the pressure,

(2) A safety valve, which can be set to blow off at any desired pressure, and

(3) A stopcock to release the pressure. It is provided with a perforated diaphragm. Water is placed below the diaphragm and heated from below by electricity, gas or stove.

Working of Autoclave

(a) Place materials inside,

(b) Close the lid. Leave stopcock open,

(c) Set the safety valve at the desired pressure,

(d) Heat the autoclave. Air is forced out and eventually steam ensures out through the tap,

(e) Close the tap. The inside pressure now rises until it reaches the set level (i.e. 15 Win), when the safety valve opens and the excess steam escapes,

(f) Keep it for 15 minutes (holding time),

(g) Stop heating,

(h) Cool the autoclave below 100°C,

(i) Open the stopcock slowly to allow air to enter the autoclave.

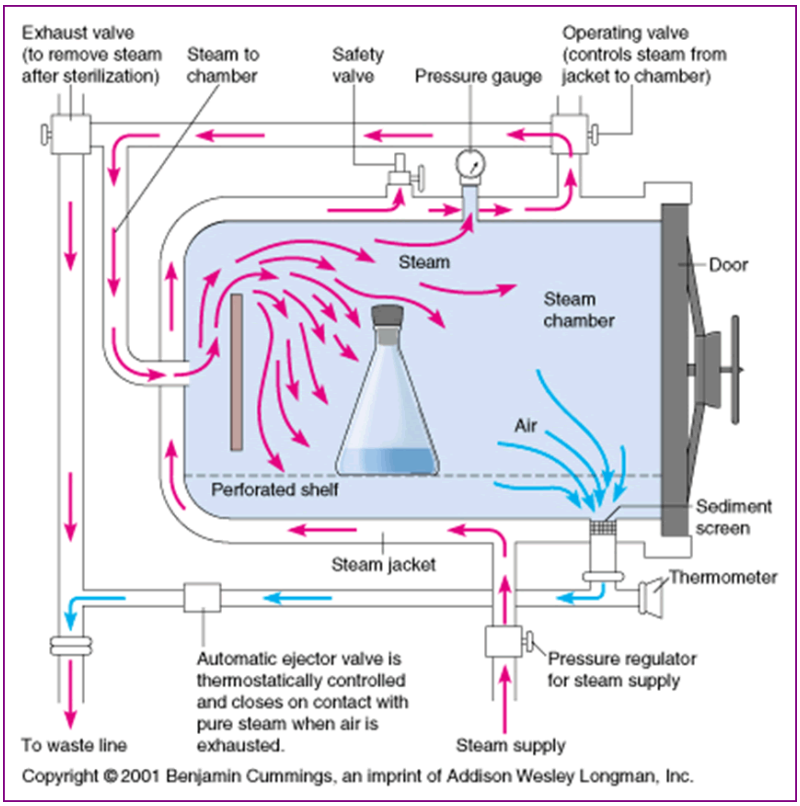


Fig 2.2: Procedure of autoclave

Advantages – The penetrating nature of steam makes it a great solution to destroy proteins in any microorganism after a certain amount of time. It is environmentally safe having no toxic byproducts.

B. Dry heat methods

Here the substances are subjected to dry heat like

1. Flaming 2. Incineration 3. Hot air oven. 4. Radiation sterilization

1. Flaming

It is the process of exposing metallic device like the needle, scalpels, and scissors to flame for few minutes. The fire burns the microbes and other dust on the instrument directly.

2. Incineration

It is done especially for inoculating loops used in microbe cultures. The metallic end of the loop is heated to red hot on the flame. This exposure kills all the germs.

3. Hot Air Oven

It is one of the most common methods used for sterilization. Glass wares, swab sticks, all glass ware, syringes, powder and oily substances are sterilized in hot air oven. For sterilization, a temperature of 160°C is maintained (holding) for one hour. Spores are killed at this temperature.

It leads to sterilization. Hot air oven is suitable for dry material like powders, metal devices, glassware, etc.

Working and Principle

It is an apparatus with double metallic walls and a door. There is an air space between these walls. The apparatus is heated by electricity or gas at the bottom. On heating, the air at the bottom becomes hot and passes between the two walls from below upwards, and then passes in the inner chamber through the holes of the apparatus. A thermostat is fitted to maintain a constant temperature of 160°C.

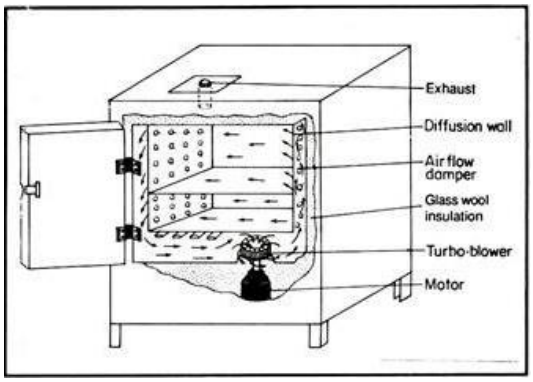


Fig 2.3: Hot air oven

Advantages– Dry heat sterilization is preferred for heat stable products that are sensitive to moisture.

4. Radiation

This method involves exposing the packed materials to radiation for sterilization. There are two types of radiations available for sterilization i.e. non-ionic and ionic radiation.

 Non-ionic radiations are safe to the operator of sterilization, and they are like Ultra Violet radiations, they can be used even at the door entrances to prevent entry of live microbes through the air.

 Ionizing radiation sterilization. They are powerful radiation and very useful for sterilization. The operator needs to protect himself from exposure from these radiations by use of special clothing. Ex: X-rays, γ-rays, etc.

Practical No: 3 **Sterilization techniques (Chemical method of sterilization)**

Here the articles are subjected to sterilization by using toxic gasses. The gas penetrates quickly into the material like steam so, the sterilization is effective. But the chances of explosion and cost factors are to be considered. The gasses used for sterilization are very poisonous. The commonly used gas is ethylene oxide with a combination of carbon-dioxide. Carbon dioxide is added to minimize the chances of an explosion.

Modes of action of chemicals:

- Protein coagulation
- Disruption of cell membrane resulting in exposure, damage or loss of the contents.
- Removal of free sulfhydryl groups essential for the functioning of the enzymes.
- Substrate competition – a compound resembling the essential substrate of the enzyme diverts or misleads the enzymes necessary for the metabolism of the cell and causes cell death.

Alcohols

The types of alcohol used in disinfection are ethanol (80%), propanol (60%), and isopropanol (70%). Alcohols are quite effective against bacteria and fungi, less so against viruses. They do not kill bacterial spores. Due to their rapid action and good skin penetration, the main areas of application of alcohols are surgical and hygienic disinfection of the skin and hands.

One disadvantage is that their effect is not long-lasting (no depot effect). Alcohols denature proteins.

Aldehydes

Formaldehyde (HCHO) is the most important aldehyde. It can be used in a special apparatus for gas sterilization. Its main use, however, is in disinfection. Formaldehyde is a water-soluble gas. Formalin is a 35% solution of this gas in water. Formaldehyde irritates mucosa; skin contact may result in inflammations or allergic eczemas. Formaldehyde is a broad-spectrum germicide for bacteria, fungi, and viruses. At higher concentrations, spores are killed as well. The mechanism of action of formaldehyde is based on protein denaturation.

Ethylene oxide

This highly reactive gas (C2H4O) is flammable, toxic, and a strong mucosal irritant. Ethylene oxide can be used for sterilization at low temperatures (20–60 8C). The gas has a high penetration capacity and can even get through some plastic foils. One drawback is that this gas cannot kill dried microorganisms and requires a relative humidity level of 40–90% in the sterilizing chamber. Ethylene oxide goes into solution in plastics, rubber, and similar materials, therefore sterilized items must be allowed to stand for a longer period to ensure complete desorption.

Chlorine compounds

Chlorine denatures proteins by binding to free amino groups. Generally, chlorine is used in the form of sodium hypochlorite which is commonly known as household bleach. It is very useful for sterilization purpose as it is very inexpensive and also effective against bacteria and viruses.

But it has some disadvantages as well; First of all high concentration of this agent corrodes metals and also damages cloths. Secondly the strength of this solution decreases with time so whenever you want to use it always prepare its fresh solution.

Oxidants

This group includes ozone, hydrogen peroxide, and peracetic acid. Their relevant chemical activity is based on the splitting off of oxygen. Most are used as mild antiseptics to disinfect mucosa, skin, or wounds.

Practical No: 4 Sterilization techniques (Mechanical/Filtration methods)

This method is used for sterilizing thermolabile solutions, which will otherwise be degraded by other conventional heating methods. The drug solutions are passed through the sterile bacteria proof filter unit and subsequently transferring the product aseptically into the sterile containers which are then sealed

Procedure:

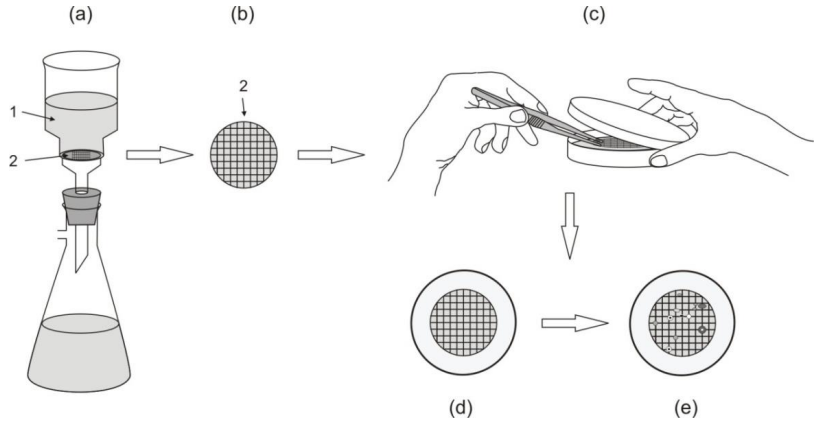
The solutions to be sterilized is passed through the filter and collected in the sterile receiver by the application of positive pressure to the non-sterile compartment or negative pressure to the sterile side.

Membrane filters

- Membrane filters are made of cellulose-derivative (acetate or nitrate). They are very fine. They are fixed in some suitable holders.
- Nominal pore size is 0. 22 ± 0. 02 mm or less is required.
- The membranes are brittle when dry. In this condition they can be stored for years together. They become very tough when dipped in water.
- They are sterilized by autoclaving or by ethylene oxide gas. They cannot be sterilized by dry heat as they decompose above 120^0C.
- They are suitable for sterilizing aqueous and oily solutions but not for organic solvents such as alcohol, chloroform etc.
- Membrane filters are generally blocked by dirt particles and organisms. Pre-filtration (through glass-fibre paper prefilter) reduces the risks of blockage of the final filter.

Examples of membrane filters:

MF-Millipore – it is a mixture of cellulose esters



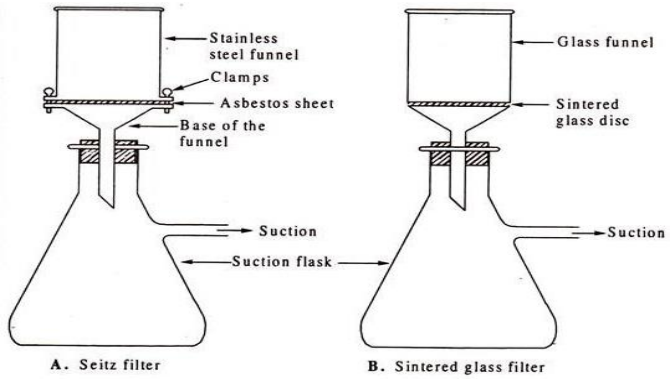
Sintered (or fritted) glass filters

Borosilicate glass is finely powdered in a ball-mill and the particles of required size are separated. This is packed into disc mounted and heated till the particles get fused. The discs thus made have pore size of 2 mm and are used for filtration. As these are made of glass and hence do not absorb liquids during filtration. The disadvantage is that they are very brittle and break easily

They are cleaned with the help of sulfuric acid.

Seitz filter:

These are made of asbestos or other material. They are pad like and thicker than membrane filters. They do not rupture during filtration. But the solution might get absorbed by the filter pad itself



Advantages of sterilization by filtration:

1. Thermolabile solutions can be sterilized.
2. It removes all the living microorganisms.

Disadvantages of sterilization by filtration:

1. Filters may break down suddenly or gradually on use.
2. Sterility testing is obligatory on the filtered solution.
3. Filter media may be absorbed on the filter surface.
4. Viruses are not removed by filtration.
5. Suspensions and oils cannot be sterilized by this method due to their heavy load of particulate matters and viscosity.

Practical No: 5 Culturing of bacteria in liquid medium

Luria broth (LB) is a nutrient-rich media commonly used to culture bacteria in the lab. However, a liquid culture is capable of supporting a higher density of bacteria and is used to grow up sufficient numbers of bacteria necessary for an experimental use. LB is the standard medium used to grow bacteria, principally Escherichia Coli. Also known as Luria broth, or Luria-Bertani medium or Lennox broth. Bertani was the inventor of LB and was a student with Max Delbruck at Caltech. They were investigating phage lysis of *E. coli*. The following protocol is for inoculating an overnight culture of liquid LB with bacteria.

Protocol:

1. Prepare liquid LB. For example, to make 1 liter of LB, weigh out the following into a 500mL glass bottle:

- 10g NaCl
- 10g Tryptone
- 5g Yeast Extract
- and dH_2O to 1 liter

Note: If your lab has pre-mixed LB agar powder, use the suggested amount, instead of the other dry ingredients above.

1. Loosely close the cap on the bottle (do NOT close all the way or the bottle may explode!) and then loosely cover the entire top of the bottle with aluminum foil. Autoclave and allow to cool to room temperature. Now screw on the top of the bottle and store the LB at room temperature.

2. When ready to grow your culture, add liquid LB to a tube or flask and add the appropriate antibiotic to the correct concentration.

Note: If you intend to do a mini-prep you will usually want to start 2mL in a falcon tube, but for larger preps you might want to use as much as a liter of LB in a 2L Erlenmeyer flask.

3. Using a sterile pipette tip or toothpick, select a single colony from your LB agar plate.
4. Drop the tip or toothpick into the liquid LB + antibiotic.
5. Loosely cover the culture with sterile aluminum foil or a cap that is not air tight.
6. Incubate bacterial culture at 37°C for 12-18hr in a shaking incubator.
7. After incubation, check for growth, which is characterized by a cloudy haze in the media.

Note: Some protocols require bacteria to be in the log phase of growth. Check the instructions for your specific protocol and conduct an OD_{600} to measure the density of your culture if needed.

Note: A good negative control is LB media + antibiotic without any bacteria inoculated. You should see no growth in this culture after overnight incubation.

8. *(Optional)* For long term storage of the bacteria, you can proceed with Creating a Glycerol Stock.



Antibiotic Concentrations

Commonly Used Antibiotics	**Recommended Concentration**
Ampicillin	100 µg/mL
Bleocin	5 µg/mL
Carbenicillin	100 µg/mL
Chloramphenicol	25 µg/mL
Coumermycin	25 µg/mL
Gentamycin	10 µg/mL
Kanamycin	50 µg/mL
Spectinomycin	50 µg/mL
Tetracycline	10 µg/mL

Practical No: 6 **Culturing of bacteria on solid medium**

Organisms can be grown in liquid media (broth) or on solid media. For example, Nutrient media is referred to as Nutrient Broth when in the liquid form, and Nutrient Agar when in the solid form. Agar, a galactan obtained from marine algae is used as the hardening agent in solid media. Broth, with added agar powder, is heated to 121oC in an autoclave, dissolving the agar and sterilizing the medium. The molten medium may be poured into plates or tubes. The agar-media will remain liquid at temperatures above 45oC. Below 45oC the agar will harden, and supply a solid surface for the growth of bacteria. Agar plates and slants may be inoculated after they have solidified. To avoid any condensation-droplets from falling onto agar surfaces and smearing the inoculum, agar plates are inverted during incubation.

Batch makes about X plates

The below mentioned values is for 1L media preparation and at least 40 plates, you can minimize it into what would you need.

Making the LB Agar

1. Add 250 mL of dH2O to a graduated cylinder.
2. Weigh out 8.50g of pre-mix LB Agar powder or:

5.0 g tryptone

2.5 g yeast extract

5.0 g NaCl

3.5 g agar

3. Mix powder well to bring into solution
4. Add dH2O to total volume of 500 mL and transfer to 1 L flask
5. Put on stirring hot plate and heat to boil for 1 min while stirring.
6. Transfer to 1 L pyrex jar and label with autoclave tape.
7. Autoclave at liquid setting for 20 minutes in a basin making sure to loosen top
8. Let agar cool to ~55C (you should be able to pick up the jar without a glove)

Pouring the Plates

1. Make sure bench top has wiped down with bleach/EtOH.
2. Remove sterile Petri dishes from plastic bag (save the bag for storage).
3. Pour a thin layer (5mm) of LB Agar (~10mL) into each plate being careful to not lift the cover off excessively (you should be able to just open up enough to pour).
4. Swirl plate in a circular motion to distribute agar on bottom completely.
5. Let each plate cool until its solid (~20 minutes) then flip so as to avoid condensation on the agar.
6. Store plates in plastic bags in fridge with: name, date and contents.

Streak Plate Method

For economy of materials and time, this method is best. It requires a certain amount of skill, however, which is forthcoming with experience. A properly executed streak plate will give as good isolation as is desired for most work. The important thing is to produce good spacing between colonies.

Materials:

Electric hot plate (or tripod and wire gauze), Bunsen burner and beaker of water

Wire loop, thermometer, and china marking Pencil, LB agar pour and sterile Petri plate,

One mixed culture of *Serratia marcescens, Escherichia coli, and Micrococcus luteus* (or *Chromobacterium violaceum*) etc etc.

1. Prepare your tabletop by disinfecting its surface with the disinfectant that is available in the laboratory. Use a sponge to scrub it clean.

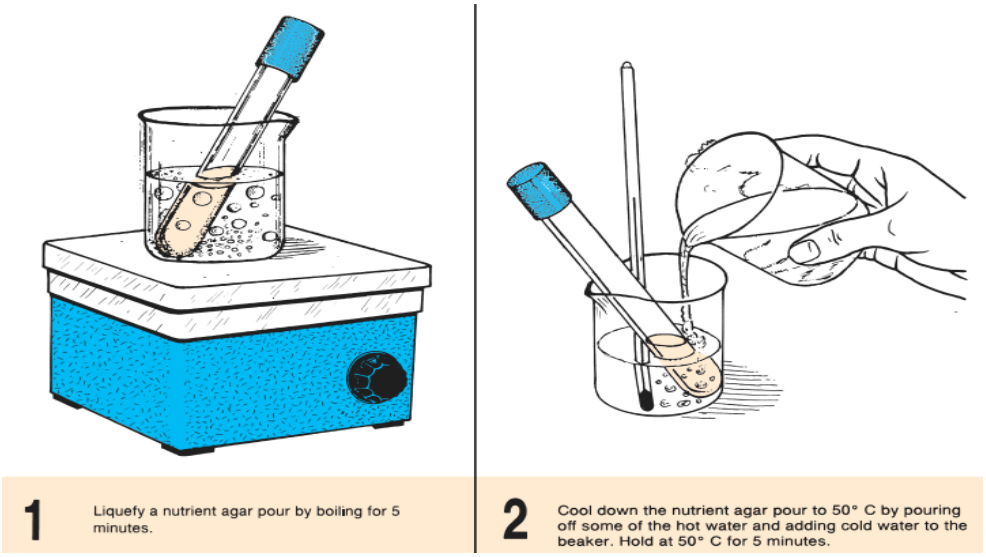
2. Label the bottom surface of a sterile Petri plate with your name and date. Use china marking pencil.

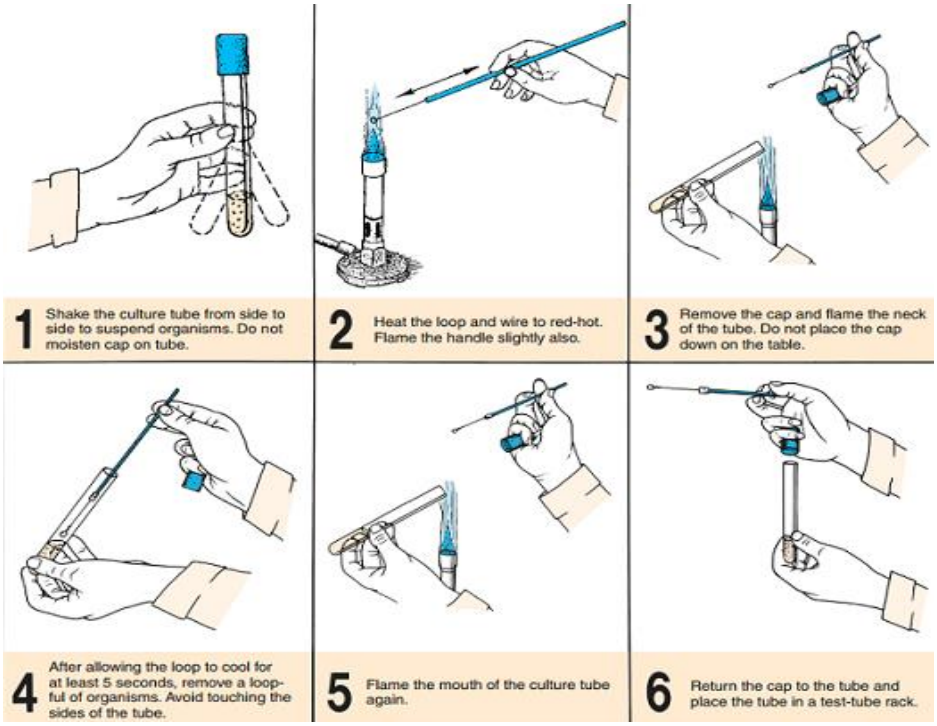
3. Liquefy a tube of nutrient agar, cool to 50° C, and pour the medium into the bottom of the plate. Be sure to flame the neck of the tube prior to pouring to destroy any bacteria around the end of the tube. After pouring the medium into the plate, gently rotate the plate so that it becomes evenly distributed, but do not splash any medium up over the sides.

Agar-agar, the solidifying agent in this medium becomes liquid when boiled and resolidifies at around 42° C. Failure to cool it prior to pouring into the plate will result in condensation of moisture on the cover. Any moisture on the cover is undesirable because if it drops down on the colonies, the organisms of one colony can spread to other colonies, defeating the entire isolation technique.

4. Streak the plate by one of the methods, see below figure.

5. Incubate the plate in an inverted position at 25° C for 24–48 hours. By incubating plates upside down, the problem of moisture on the cover is minimized.





1 Shake the culture tube from side to side to suspend organisms. Do not moisten cap on tube.

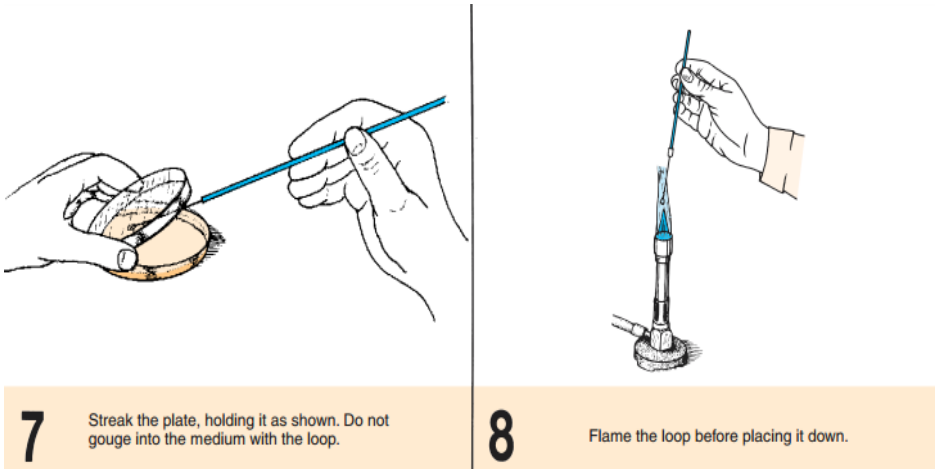
2 Heat the loop and wire to red-hot. Flame the handle slightly also.

3 Remove the cap and flame the neck of the tube. Do not place the cap down on the table.

4 After allowing the loop to cool for at least 5 seconds, remove a loop-ful of organisms. Avoid touching the sides of the tube.

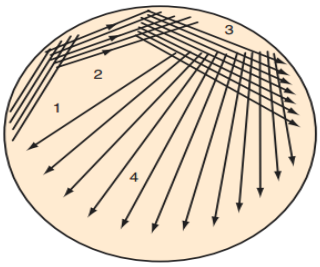
5 Flame the mouth of the culture tube again.

6 Return the cap to the tube and place the tube in a test-tube rack.



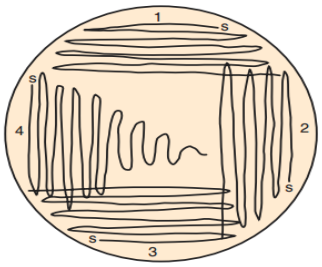
7 Streak the plate, holding it as shown. Do not gouge into the medium with the loop.

8 Flame the loop before placing it down.



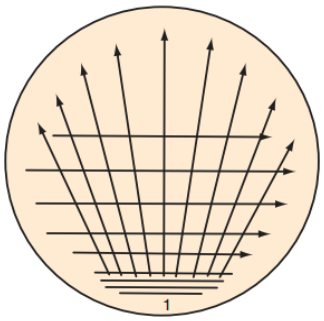
QUADRANT STREAK
(Method A)

1. Streak one loopful of organisms over Area 1 near edge of the plate. Apply the loop lightly. Don't gouge into the medium.
2. Flame the loop, cool 5 seconds, and make 5 of 6 streaks from Area 1 through Area 2. Momentarily touching the loop to a sterile area of the medium before streaking insures a cool loop.
3. Flame the loop again, cool it, and make 6 or 7 streaks from Area 2 through Area 3.
4. Flame the loop again and make as many streaks as possible from Area 3 into Area 4, using up the remainder of the plate surface.
5. Flame the loop before putting it aside.



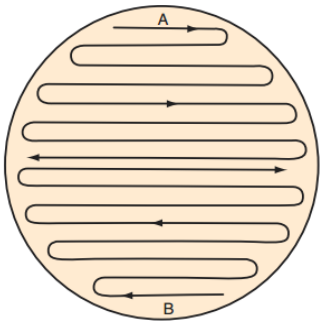
QUADRANT STREAK
(Method B)

1. Streak one loopful of organisms back and forth over Area 1, starting at point designated by "s". Apply loop lightly. Don't gouge into the medium.
2. Flame the loop, cool 5 seconds and touch the medium in sterile area momentarily to insure coolness.
3. Rotate the dish 90 degrees while keeping the dish closed. Streak Area 2 with several back and forth strokes, hitting the original streak a few times.
4. Flame the loop again. Rotate the dish and streak Area 3 several times, hitting last area several times.
5. Flame the loop, cool it, and rotate the dish 90 degrees again. Streak Area 4, contacting Area 3 several times and drag out the culture as illustrated.
6. Flame the loop before putting it aside.



RADIANT STREAK

1. Spread a loopful of organisms in small area near the edge of the plate in Area 1. Don't gouge medium.
2. Flame the loop and allow it to cool for 5 seconds. Touching a sterile area of the medium will insure coolness.
3. **From the edge** of Area 1 make 7 or 8 straight streaks to the opposite side of the plate.
4. Flame the loop again, cool it sufficiently, and cross streak over the last streaks, **starting near Area 1.**
5. Flame the loop again before putting it aside.



CONTINUOUS STREAK

1. Starting at the edge of the plate (Area A) with a loopful of organisms, spread the organisms in a single continuous movement to the center of the plate. Use light pressure and avoid gouging the medium.
2. Rotate the plate 180 degrees so that the uninoculated portion of the plate is away from you.
3. Without flaming loop, and using the same face of the loop, continue streaking the other half of the plate by starting at Area B and working toward the center.
4. Flame your loop before putting it aside.

Practical No: 7 **Types of media and culturing**

Introduction

All micro-organisms require water, sources of energy, carbon, nitrogen, mineral element and vitamin plus oxygen in their growth medium. On a small scale, it is simple to device a medium containing pure compounds, but the resulting medium although satisfy the growth, may be unsuitable for use in a large scale process.

The essential part of media designing is to be considering the stoichiometry of growth and formation of desired product which is given below:

Carbon and energy source + Nitrogen source + O2 + Other requirements ----> Biomass + Products + CO2 + H2O+ Heat Essential characteristics of formulated media

1. It should produce the maximum yield, maximal concentration with maximal rate of production of desired biomass, of consistent quality which is always readily available.

2. It should also have minimal problems during media designing.

3. It should have minimal problems during recovery of desired product/ biomass especially during aeration and agitation, extraction, purification and waste treatment.

Basic media constituents

1. Water

It is major component of all fermentation media. Following factors need to be considered about water for media preparation

- pH
- Dissolve salt
- Effluent contamination

Mineral water content of water is very important in brewing, and most critical in the mashing process, and historically influenced the siting of breweries and the type of beer produced.

2. Energy Sources

Energy for growth comes from either the oxidation of medium components or from light. Most industrial micro-organisms are chemo-organotrophs, therefore the commonest sources of energy will be the carbon source such as carbohydrates, lipids and proteins.

3. Carbon sources

Carbon requirement for the medium is normally provided by:

- **Sucrose**: Sugarcane, sugar beet molasses
- **Glucose**: Corn sugar, starch, cellulose
- **Lactose**: Milk whey
- **Fats**: Vegetable oil
- **Starch**: Maize grains, cereals, potatoes and cassava
- **Hydrocarbons**: Petroleum fractions

4. Nitrogen Sources

Microorganisms generally can use inorganic or organic N.

- Inorganic sources: ammonia, ammonium salts –
- Organic sources: amino acid, proteins and urea, Corn steep liquor, Yeast extract, Peptones, Soya bean meal.

5. Oxygen sources

Oxygen is always provided in water.

Some organisms require molecular oxygen as terminal oxidizing agents to fulfill their energetic needs through aerobic respiration. These organisms are obligatorily aerobic.

For obligate anaerobes molecular 02, is a toxic substance.

Some organisms are facultative anaerobes and can grow with or without molecular 02.

Types of media and culturing

Culture media contains nutrients and physical growth parameters necessary for microbial growth. All microorganisms cannot grow in a single culture medium and in fact many can't grow in any known culture medium.

Organisms that cannot grow in artificial culture medium are known as obligate parasites. *Mycobacterium leprae, rickettsias, Chlamydias,* and *Treponema pallidum* are obligate parasites. Bacterial culture media can be distinguished on the basis of composition, consistency and purpose**.**

Classification of culture media used in Microbiology laboratory on the basis of consistency

1. **Solid medium**

   solid medium contains agar at a concentration of 1.5-2.0% or some other, mostly inert solidifying agent. Solid medium has physical structure and allows bacteria to grow in physically informative or useful ways (e.g. as colonies or in streaks). Solid medium is useful for **isolating bacteria** or for determining the colony characteristics of the isolate.

2. **Semisolid media**

   They are prepared with agar at concentrations of 0.5% or less. They have soft custard like consistency and are useful for the cultivation of **microaerophilic bacteria** or for **determination of bacterial motility.**

3. **Liquid (Broth) medium**

   These media contains specific amounts of nutrients but don't have trace of gelling agents such as gelatin or agar. Broth medium serves various purposes such as propagation of large number of organisms, fermentation studies, and various other tests. e.g. sugar fermentation tests, MR-VR broth

Classification of culture media based on the basis of composition

1. Synthetic or chemically defined medium

   A chemically defined medium is one prepared from purified ingredients and therefore whose exact composition is known.

2. Non synthetic or chemically undefined medium

Non-synthetic medium contains at least one component that is neither purified nor completely characterized nor even completely consistent from batch to batch. Often these are partially digested proteins from various organism sources. Nutrient broth, for example, is derived from cultures of yeasts.

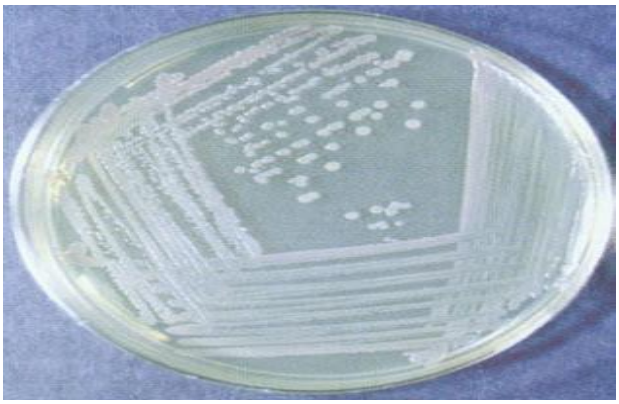
Synthetic medium may be simple or complex depending up on the supplement incorporated in it. A simple non-synthetic medium is capable of meeting the nutrient requirements of organisms requiring relatively few growth factors where as complex non-synthetic medium support the growth of more fastidious microorganisms.

Classification of Bacterial Culture Media based on the basis of purpose/ functional use/ application

Many special purpose media are needed to facilitate recognition, enumeration, and isolation of certain types of bacteria. To meet these needs, numerous media are available.

1. General purpose media/ Basic media

Basal media are basically simple media that supports most non-fastidious bacteria. Peptone water, nutrient broth and nutrient agar are considered as basal medium. These media are generally used for the primary isolation of microorganisms.



Nutrient Agar

2. Enriched medium (Added growth factors):

Addition of extra nutrients in the form of blood, serum, egg yolk etc, to basal medium makes them enriched media. Enriched media are used to grow nutritionally exacting (fastidious) bacteria. B**lood agar**, chocolate agar, Loeffler's serum slope etc are few of the enriched media. Blood agar is prepared by adding 5-10% (by volume) blood to a blood agar base. **Chocolate agar** is also known as heated blood agar or lysed **blood agar.**



Blood Agar

3. Selective and enrichment media are designed to inhibit unwanted commensal or contaminating bacteria and help to recover pathogen from a mixture of bacteria. While selective media are agar based, enrichment media are liquid in consistency. Both these media serve the same purpose. Any agar media can be made selective by addition of certain inhibitory agents that don't affect the pathogen of interest. Various approaches to make a medium selective include **addition of antibiotics, dyes, chemicals, alteration of pH or a combination of these.**

a. Selective medium

Principle: Differential growth suppression

Selective medium is designed to suppress the growth of some microorganisms while allowing the growth of others. Selective medium are agar based (solid) medium so that individual colonies may be isolated.

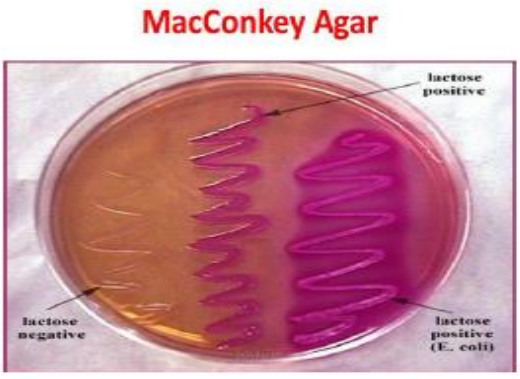
Examples of selective media include:

1. **Mannitol Salt Agar** and Salt Milk Agar used to recover *S.aureus* contains 10% NaCl.



Mannitol Salt Agar grows halophilic (salt-loving) bacteria. Pathogenic *Staph* is growing on left (yellow side) and normal flora *Staph* growing on right (pink).

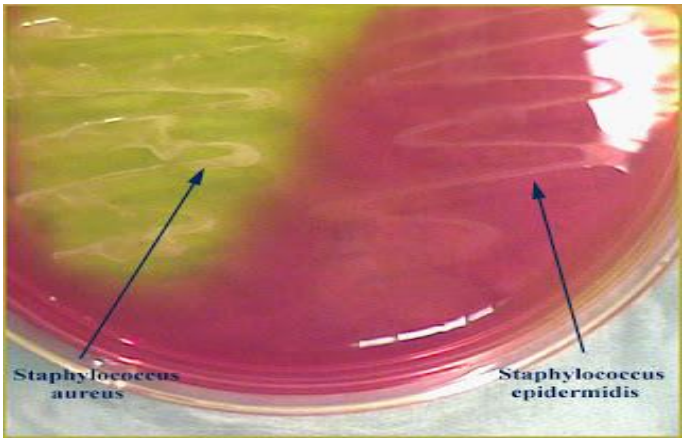
2. **MacConkey's Agar** used for **Enterobacteriaceae** members contains bile salt that inhibits most gram positive bacteria.



4. Differential/ indicator medium: differential appearance:

Certain media are designed in such a way that different bacteria can be recognized on the basis of their colony colour. Various approaches include incorporation of dyes, metabolic substrates etc, so that those bacteria that utilize them appear as differently coloured colonies. Such media are called differential media or indicator media. Differential media allow the growth of more than one microorganism of interest but with morphologically distinguishable colonies.

Examples of differential media include:



Mannitol salts agar (mannitol fermentation = yellow)

Mac Conkey agar (lactose fermenters, pink colonies **(*E. coli*)** whereas non- lactose fermenter produces pale or colorless colonies.

Practical No: 8	**Gram staining of bacteria**

A technique that is used to define and examine different types of microbes

Types of staining techniques

1. **Simple stains techniques**

Simple staining is performed with basic dyes such as crystal violet or methylene blue.

2. **Differential stains techniques**

Differential stain technique distinguishes two kinds of organisms. There are two types of this technique; one is Gram stain technique while other is acid fast technique.

Gram stain technique

A most commonly used technique for study of microbes like bacteria.

Principle

This technique separates bacteria into two groups, Gram-positive bacteria and Gram-negative bacteria.

Materials:

- Clean glass slides
- Inoculating loop
- Bunsen burner
- Bibulous paper
- Microscope
- Lens paper and lens cleaner
- Immersion oil
- Distilled water
- 18 to 24 hour cultures of organisms

Reagents:

1. Primary Stain - Crystal Violet

2. Mordant - Grams Iodine
3. Decolourizer - Ethyl Alcohol
4. Secondary Stain - Safranin

Procedure:

1. **Smear preparation**

A small sample of microorganisms is placed on a slide and permitted to air dry. The smear is heat fixed by quickly passing it over a flame. Heat fixing kills the organisms, makes them adhere to the slide, and permits them to accept.

2. **Primary stain**

Apply crystal violet as primary stain

3. **Mordant**

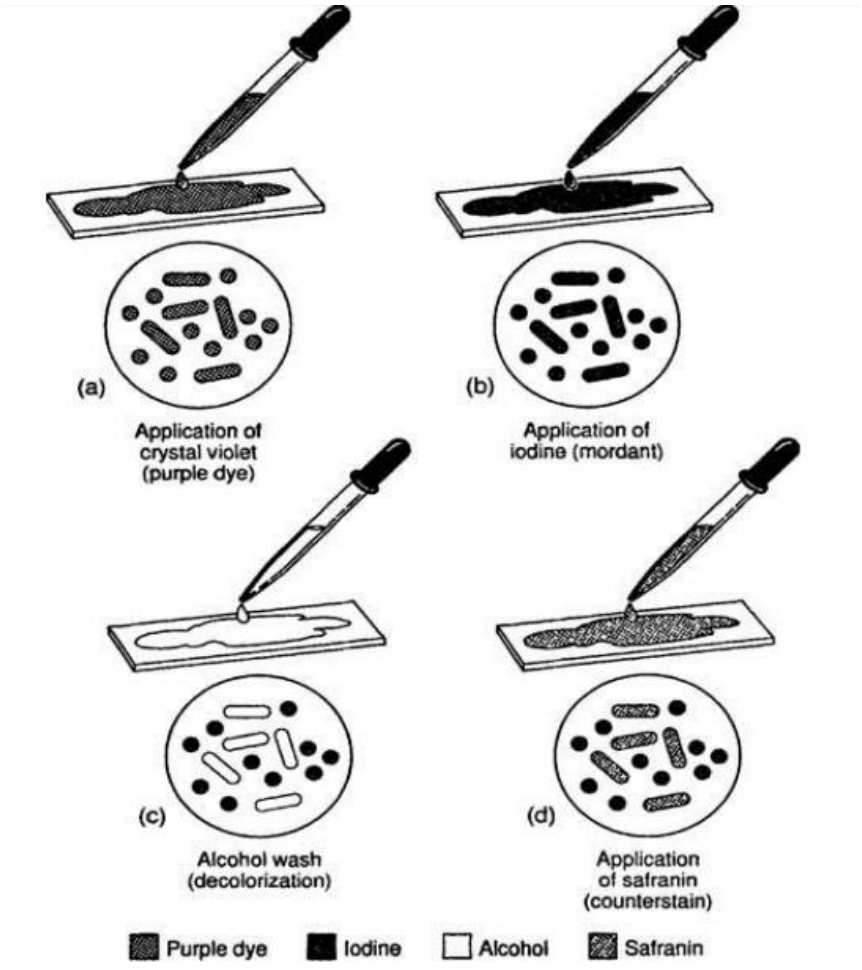
Use iodine as a mordant

4. **Decolourizer**

Wash with ethyl alcohol that acts as a decolourizing agent. Gram-positive bacteria retain the crystal-violet iodine stain; however, the Gram-negative bacteria lose the stain.

5. **Secondary stain**

The Gram-negative bacteria subsequently stain with the Safranin dye, the counterstain. These bacteria appear red under the oil-immersion lens, while Gram-positive bacteria appear blue or purple, reflecting the crystal violet retained during the washing step.



The Gram staining procedure used for differentiating bacteria into two groups

Practical No: 9	Bacterial cell count

A viable cell count allows one to identify the number of actively growing/dividing cells in a sample. The plate count method or spread plate relies on bacteria growing a colony on a nutrient medium. The colony becomes visible to the naked eye and the number of colonies on a plate can be counted. Colony-forming unit (CFU or cfu) is a measure of viable bacterial. CFU measures only viable cells. For convenience the results are given as CFU/mL (colony-forming units per milliliter) for liquids and CFU/g (colony-forming units per gram) for solids.

To quantify the number of cells in a culture, the cells can be simply plated on a petri dish with growth medium. If the cells are efficiently distributed on the plate, it can be generally assumed that each cell will give rise to a single colony or Colony Forming Unit (CFU). The colonies can then be counted, and based on the known volume of culture that was spread on the plate; the cell concentration can be calculated. As is with counting chambers, cultures usually need to be heavily diluted prior to plating; otherwise, instead of obtaining single colonies that can be counted, a so-called "lawn" will form: thousands of colonies lying over each other. Additionally, plating is the slowest method of all: most microorganisms need at least 12 hours to form visible colonies.

Although this method can be time consuming, it gives an accurate estimate of the number of viable cells (because only they will be able to grow and form visible colonies). It is therefore extensively used in experiments aiming to quantify the number of cells resisting drugs or other external conditions. In addition, the enumeration of colonies on agar plates can be greatly facilitated by using colony counters.

The materials needed to perform a plate count are:

- Sterile 0.9% NaCl (sterile saline)
- Sterile tubes, tips and spreaders
- Agar plates (three per sample)

Procedure:

- Make a ten-fold dilution serial dilution of your bacterial culture (broth). Dilute the suspension to a dilution factor of 10-6 (a million-fold dilution).
- Spread out aliquots using a sterile bacterial spreader (0.1 ml) of each dilution onto 3 agar plates.
- Incubate the plates for 1-2 at 37C.
- Count the number of bacterial colonies that appear on each of the plates that has between 30 and 200 colonies.
- Any plate which has more than 200 colonies is designated as "too numerous to count". Plates with fewer than 30 colonies do not have enough individuals to be statistically acceptable.
- To compute the estimated number of bacteria on the surface that you tested, use the following formula:

$B = N/d$

where: B = number of bacteria; N = average number of colonies counted on three plates; d = dilution factor. Example: Plate 1: 56 CFU; Plate 2: 75 CFU; Plate 3: 63 CFU; Average (N) = 64.7; Dilution (d) = 1/1,000; B = (64.7x1,000) = 64,700 bacteria in 0.1 ml, 647,000 bacteria per ml.

Practical No: 10	Biochemical tests

Introduction

Biochemical tests are among the most important methods for microbial identification. Routine biochemical tests include tests for carbohydrate fermentation, IMViC test, urease, oxidase and catalase test.

1. Carbohydrate fermentation tests:

The carbohydrate fermentation test is used to determine whether or not bacteria can ferment a specific carbohydrate. Carbohydrate fermentation patterns are useful in differentiating among bacterial groups or species. The term fermentation is often used to describe the breaking down or catabolism of a carbohydrate under anaerobic conditions. Therefore, bacteria capable of fermenting a carbohydrate are usually facultative anaerobes. It tests for the presence of acid and/or gas produced from carbohydrate fermentation. Basal medium containing a single carbohydrate source such as Glucose, Lactose, Sucrose or any other carbohydrate is used for this purpose. A pH indicator (such as Andrade's solution, Bromcresol purple (BCP), Bromothymol blue (BTB) or Phenol red) is also present in the medium; which will detect the lowering of the pH of the medium due to acid production. Small inverted tubes called Durham tube is also immersed in the medium to test for the production of the gas (hydrogen or carbondioxide).

Media

Carbohydrate broth medium

Peptone 10

Sodium chloride 5 gram

Phenol red/bromocresol purple 0.016 gram

Carbohydrate 10 gram

Distilled water 1000 ml

Procedure

1. Dissolve the media ingredients to distilled water and adjust the pH to 6.8 in case of bromocresol purple or 7.4 if using phenol red.
2. Distribute the media in small test tubes and insert Durham tubes in inverted position to detect gas production.
3. Place cotton plugs to tubes and autoclave them.
4. Alternatively, 90 ml of the basal medium (medium without the carbohydrate) is sterilized in the autoclave and to this, after cooling, 10 ml of the carbohydrate solution (10%) sterilized by membrane filtration is added. This is particularly essential for heat-sensitive carbohydrates, which undergo degradation during heat sterilization.
5. After autoclaving, inoculate each tube with 18-24 h old culture of test microorganism. Perform the test in duplicate or triplicate and incubate at 37ºC for 24 h. Unioculated media is used as negative control.
6. Next day, check tubes for color change or gas production**.**

Acid production:

a. **Positive:** After incubation the liquid in the tube turns yellow *(indicated by the change in the color of the phenol red indicator)*. It indicates that there is drop in the pH because of the production of the acid by the fermentation of the carbohydrate (sugar) present in the media. NOTE:*If you are using other pH indicators please check corresponding colors in particular pH.

b. **Negative:** The tube containing medium will remain red, indicating the bacteria cannot ferment that particular carbohydrate source present in the media.

Gas Production

c. **Positive:** A bubble (small or big depending up the amount of gas produced) will be seen in the inverted Durham tube.

d. **Negative:** There won't be any bubble in the inverted Durham tube i.e. bacteria does not produce gas from the fermentation of that particular carbohydrate present in the media i.e. anaerogenic organism.

Interpretation:

- Colour of broth changes to yellow and gas accumulates in Durham tube: Fermentative for the carbohydrate and acrogenic.
- Colour of broth changes to yellow, but no gas accumulates in Durham tube: Fermentative for the carbohydrate and anaerogenic.
- Colour of broth does not change: Non-fermentative for the carbohydrate.

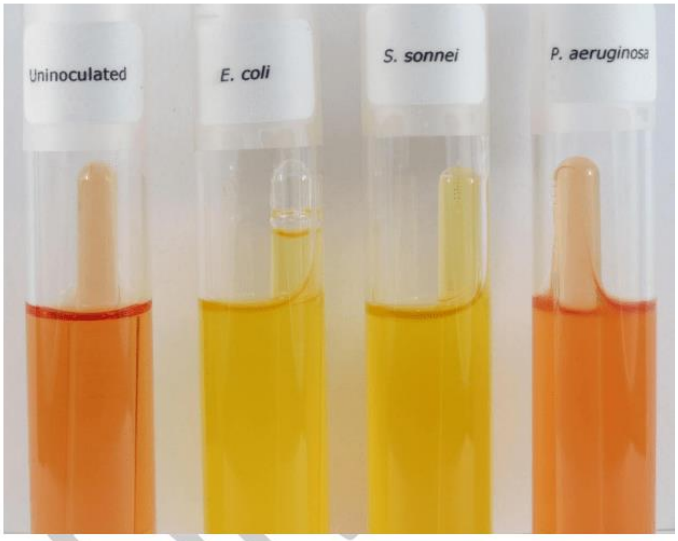


Figure 1. Peptone media with phenol red indicator. From left to right: Uninoculated tube; glucose fermenter with gas production (visible air bubble in the inverted Durham tube); glucose fermenter without gas production (no visible air bubble in the inverted Durham tube); non-fermenter.

2. IMViC test

The IMViC tests are useful for differentiating the Enterobacteriaceae, especially when used alongside the urease test. When used alone, the IMViC tests are particularly useful for differentiating Escherichia coli, Enterobacter aerogenes, Enterobacter cloacae, and Klebsiella pneumoniae. Each of the letters in "IMViC" stands for one of these tests. "I" is for indole; "M" is for methyl red; "V" is for Voges-Proskauer, and "C" is for citrate, lowercase "i" is added for the ease of pronunciation. IMViC is an acronym that stands for **four different tests**

- Indole test
- Methyl red test
- Voges-Proskauer test
- Citrate utilization test

a. Indole test

Indole test is used to determine the ability of an organism to split amino acid tryptophan to form the compound indole. Tryptophan is hydrolysed by tryptophanase to produce three possible end products – one of which is indole. Indole production is detected by Kovac's or Ehrlich's reagent which contains 4 (p)-dimethylamino benzaldehyde, this reacts with indole to produce a red coloured compound. Indole test is a commonly used biochemical test (e.g. in IMVIC test, SIM test etc). Indole test helps to differentiate *Enterobacteriaceae* and other genera.

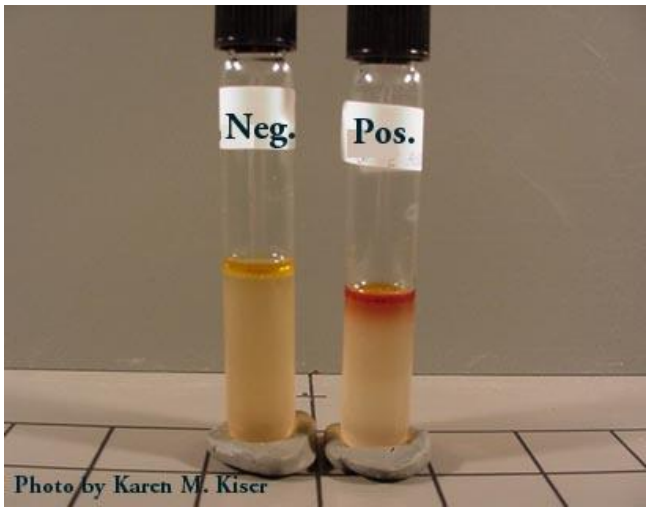
Tryptophan medium Composition

Ingredients	Gms / Litre
Meat peptone/ Casein enzymic hydrolysate	10.000
Sodium chloride	5.000
DL-Tryptophan	1.000

Final pH (at 25°C) 7.2±0.2

Procedure

1. Inoculate the tryptophan broth with broth culture or emulsify isolated colony of the test organism in tryptophan broth.
2. Incubate at 37°C for 24-28 hours in ambient air.
3. After incubation, add 0.5 ml of Kovac's reagent to the broth culture.



Expected results:

- **Positive**: Pink colored rink after addition of appropriate reagent
- **Negative**: No color change even after the addition of appropriate reagent. e.g. *Klebsiella pneumoniae*

b. Methyl Red / Voges-Proskauer (MR/VP)

This test is used to determine which fermentation pathway is used to utilize glucose. In the mixed acid fermentation pathway, glucose is fermented and produces several organic acids (lactic, acetic, succinic, and formic acids). The stable production of enough acid to overcome the phosphate buffer will result in a pH of below 4.4. If the pH indicator (methyl red) is added to an aliquot of the culture broth and the pH is below 4.4, a red color will appear. If the MR turns yellow, the pH is above 6.0 and the mixed acid fermentation pathway has not been utilized. The 2,3 butanediol fermentation pathway will ferment glucose and produce a 2,3 butanediol end product instead of organic acids. In order to test this pathway, an aliquot of the MR/VP culture is removed and a-naphthol and KOH are added. They are shaken together vigorously and set aside for about one hour until the results can be read. The Voges-Proskauer test detects the presence of acetoin, a precursor of 2,3 butanediol. If the culture is positive for acetoin, it will turn "brownish-red to pink". If the culture is negative for acetoin, it will turn "brownish-green to yellow". Note: A culture will usually only be positive for one pathway: either MR+ or VP+. *Escherichia coli* is MR+ and VP-. In contrast, *Enterobacter aerogenes* and *Klebsiella pneumoniae* are MR- and VP+. *Pseudomonas aeruginosa* is a glucose nonfermenter and is thus MR- and VP-.

Media and Reagents

Methyl red-Voges-Proskauer (MR/VP) broth (formulated by Clark and Lubs) is used in this test. The composition of MR/VP broth is as follows:

Ingredient	MR/VP broth (g/L)
Polypeptone	7 g
Glucose	5 g
Dipotassium phosphate	5 g
Distilled water	1 L
Final pH	6.9

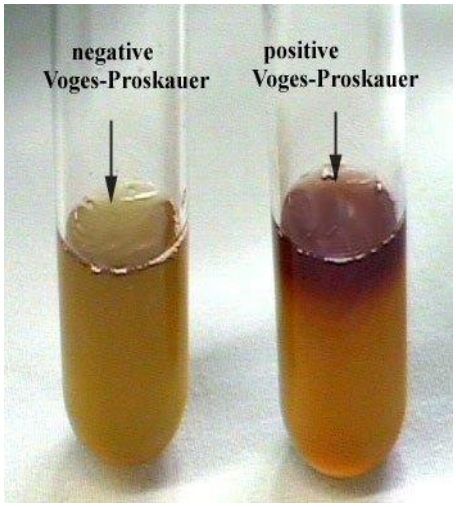
Methyl Red pH Indicator

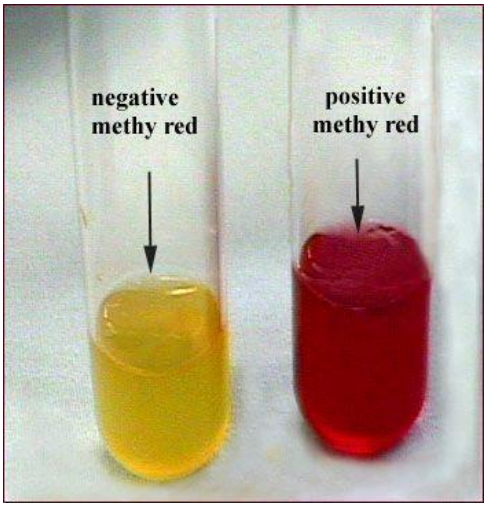
- Methyl red: 0.1 g in 300 mL of 95% ethyl alcohol.
- Distilled water: 200 mL

Procedure for Methyl Red (MR) Test

MR-VP broth is used for both MR Test and VP test. Only the addition of reagent differs, and both tests are carried out consecutively.

1. Inoculate two tubes containing MR-VP Broth with a pure culture of the microorganisms under investigation.
2. Incubate at 35 °C for up to 4 days.
3. Add about 5 drops of the methyl red indicator solution to the first tube (for Voges-Proskauer test, Barrit's reagent is added to another tube).
4. A positive reaction is indicated, if the colour of the medium changes to red within a few minutes.





c. Citrate test

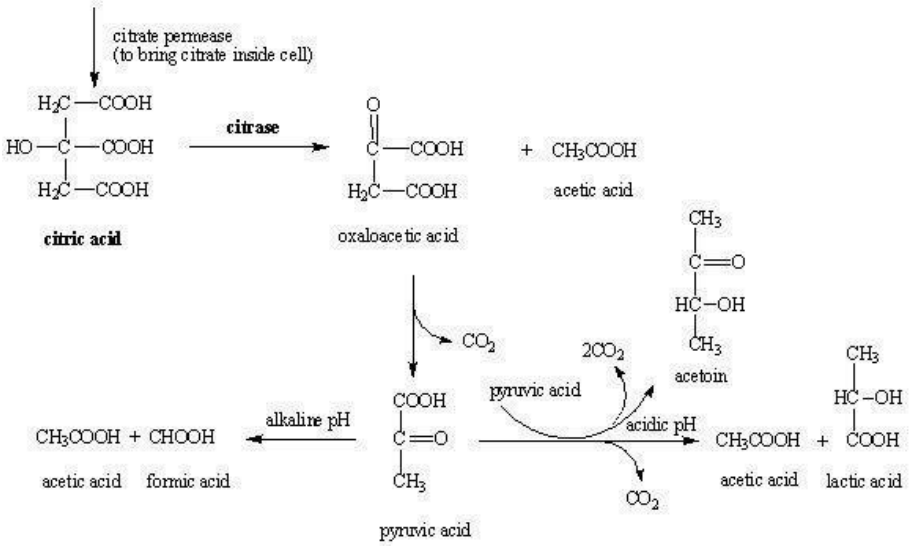
Citrate utilization test is commonly employed as part of a group of tests, the IMViC (Indole, Methyl Red, VP and Citrate) tests, that distinguish between members of the Enterobacteriaceae family based on their metabolic by-products. Citrate utilization test is used to determine the ability of bacteria to utilize sodium citrate as its only carbon source and inorganic ammonium dihydrogen phosphate (NH4H2PO4) is the sole fixed nitrogen source.

Principle of citrate utilization test

When an organic acid such as citrate (*remember Krebs cycle)* is used as a carbon and energy source, alkaline carbonates and bicarbonates are produced ultimately. In addition, ammonium hydroxide is produced when the ammonium salts in the medium are used as the sole nitrogen source. Utilization of exogenous citrate requires the presence of citrate transport proteins (permeases). Upon uptake by the cell, citrate is cleaved by citrate lyase to oxaloacetate and acetate. The oxaloacetate is then metabolized to pyruvate and CO_2.

Citrate = oxaloacetate + acetate

oxalacetate = pyruvate + CO_2



Further metabolic breakdown is **dependent upon the pH of the medium**.

A. Under alkaline conditions, pyruvate is metabolized to acetate and formate.

pyruvate = acetate + formate

B. At pH 7.0 and below, lactate and acetoin are also produced.

pyruvate = acetate + lactate + CO_2

pyruvate = acetoin + CO_2

The carbon dioxide that is released will subsequently react with water and the sodium ion in the medium to produce sodium carbonate, an alkaline compound that will raise the pH. In addition, ammonium hydroxide is produced when the ammonium salts in the medium are used as the sole nitrogen source.

Growth usually results in the bromothymol blue indicator, turning from green to blue. The bromothymol blue pH indicator is a deep forest green at neutral pH. With an increase in medium pH to above 7.6, bromothymol blue changes to blue

Citric acid media composition

Ingredient	Simmons Citrate Agar (g/L)
Ammonium dihydrogen phosphate	1 g
Dipotassium phosphate	1 g
Sodium chloride	5 g
Sodium citrate	2 g
Magnesium sulfate	0.20 g
Agar	15 g
Bromothymol blue	0.08 g
Distilled water	1 L
Final pH	6.9

Procedure of citrate utilization test:

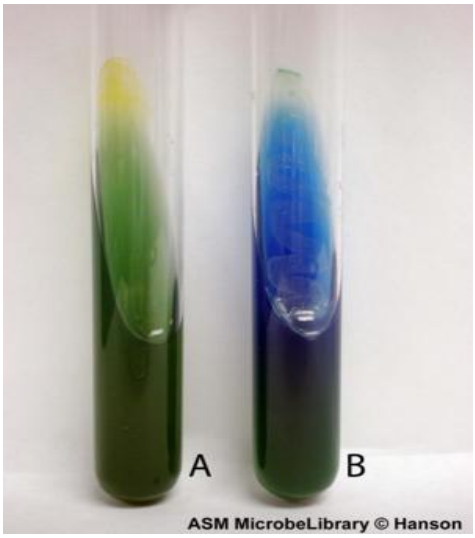
1. Inoculate Simmons citrate agar lightly on the slant by touching the tip of a needle to a colony that is 18 to 24 hours old.

2. Incubate at 35°C to 37°C for 18 to 24 hours. Some organisms may require up to 7 days of incubation due to their limited rate of growth on citrate medium.
3. Observe the development of blue color; denoting alkalinization.

Expected results in citrate utilization test:

Citrate positive: growth will be visible on the slant surface and the medium will be an intense Prussian blue. The alkaline carbonates and bicarbonates produced as by-products of citrate catabolism raise the pH of the medium to above 7.6, causing the bromothymol blue to change from the original green color to blue .

Citrate negative: trace or no growth will be visible. No color change will occur; the medium will remain the deep forest green color of the uninoculated agar. Only bacteria that can utilize citrate as the sole carbon and energy source will be able to grow on the Simmons citrate medium, thus a citrate-negative test culture will be virtually indistinguishable from an uninoculated slant



3. Catalase test

Introduction

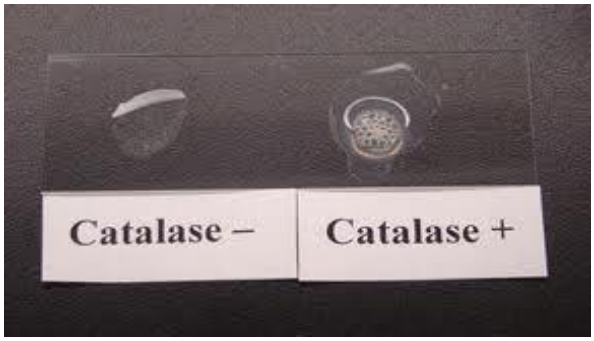
Catalase is the enzyme that breaks hydrogen peroxide (H_2O_2) into H_2O and O_2. Hydrogen peroxide is often used as a topical disinfectant in wounds, and the bubbling that is seen is due to the evolution of O_2 gas. H_2O_2 is a potent oxidizing agent that can wreak havoc in a cell; because of this, any cell that uses O2 or can live in the presence of O_2 must have a way to get rid of the peroxide. One of those ways is to make catalase. The catalase reaction is used in the identification of gram positive cocci (differentiate between Streptococcus and Staphylococcus species) and some gram positive bacilli.

Reagent

Catalase Reagent (Hydrogen peroxide 3%)

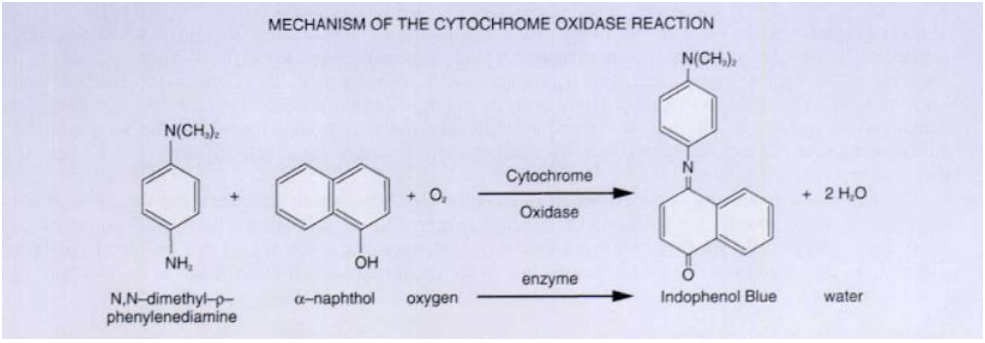
Procedure

1. Place a small amount of growth from your culture on to a clean microscope slide. If using colonies from a blood agar plate, be very careful not to scrape up any of the blood agar—blood cells are catalase positive and any contaminating agar could give a false positive.
2. Add a drop of Catalase Reagent onto the smear.
3. A positive result is the rapid evolution of O_2 as evidenced by bubbling.
4. A negative result is no bubbles or only a few scattered bubbles.
5. Dispose of your slide in the biohazard glass disposal container.



4. Oxidase test

The oxidase test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. When present, the cytochrome c oxidase oxidizes the reagent (tetramethyl-p-phenylenediamine) to (indophenols) purple color end product. When the enzyme is not present, the reagent remains reduced and is colorless.



All bacteria that are oxidase positive are aerobic, and can use oxygen as a terminal electron acceptor in respiration. This does NOT mean that they are strict aerobes. Bacteria that are oxidase-negative may be anaerobic, aerobic, or facultative; the oxidase negative result just means that these organisms do not have the cytochrome c oxidase that oxidizes the test reagent. They may respire using other oxidases in electron transport.

Oxidase test is most helpful in screening colonies suspected of being one of the *Enterobacteriaceae* (all negative) and in identifying colonies suspected of belonging to other genera such as *Aeromonas, Pseudomonas, Neisseria*, *Campylobacter*, and *Pasteurella* (positive).

Test requirements for Oxidase test: Moist filter paper with the substrate (1% tetramethyl-p-phenylenediamine dihydrochloride), or commercially prepared paper disk, wooden wire or platinum wire.

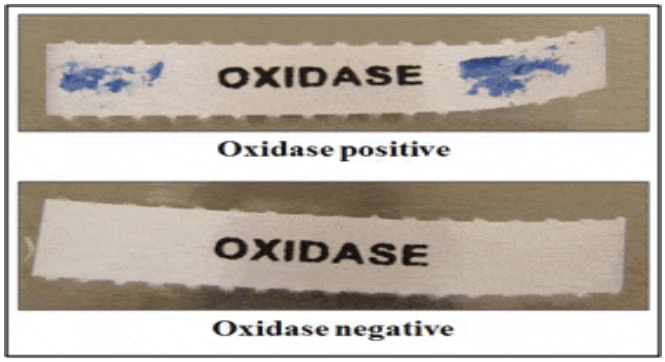
Procedure of Oxidase test:

1. Take a filter paper soaked with the substrate tetramethyl-p-phenylenediamine dihydrochloride
2. Moisten the paper with a sterile distilled water
3. Pick the colony to be tested with wooden or platinum loop and smear in the filter paper
4. Observe inoculated area of paper for a color change to deep blue or purple within 10-30 seconds

Expected results of Oxidase test

Positive: Development of dark purple color (indophenols) within 10 seconds

Negative: Absence of color



5. Urease test

Urea is a diamide of carbonic acid. It is hydrolyzed with the release of ammonia and carbon dioxide. Many organisms especially those that infect the urinary tract, have an urease enzyme which is able to split urea in the presence of water to release ammonia and carbon dioxide.

The ammonia combines with carbon dioxide and water to form ammonium carbonate which turns the medium alkaline, turning the indicator phenol red from its original orange yellow color to bright pink.

$$(NH_2)_2CO + 2\,H_2O \xrightarrow{\text{Urease}} CO_2 + H_2O + 2\,NH_3$$

Urea — Carbon dioxide — Water — Ammonia

Composition of Christensen's Urea Agar

Ingredients	**Grams/liter**
Peptone	1 g
Dextrose	1 g
Sodium chloride	5 g
Potassium phosphate, monobasic	2 g
Urea	20 g
Phenol red	0.012 g
Agar	15 to 20 g

Preparation of Christensen's Urea Agar

1. Dissolve the first six ingredients in 100 ml of distilled water and filter sterilize (0.45 mm pore size).
2. Suspend the agar in 900 ml of distilled water, boil to dissolve completely, and autoclave at 121°C and 15 psi for 15 minutes
3. Cool the agar to 50-55 °C
4. Aseptically add 100 ml of filter-sterilized urea based to the cooled agar solution and mix thoroughly
5. Distribute 4 to 5 ml per sterile tube (13 x 100 mm) and slant the tubes during cooling until solidified (It is desirable to have a long slant and short butt).

Procedure for urease test

1. Streak the entire slant surface with a heavy inoculum from an 18-24 hour pure culture (Do not stab the butt as it will serve as a color control).
2. Incubate tubes with loosened caps at 35°C.

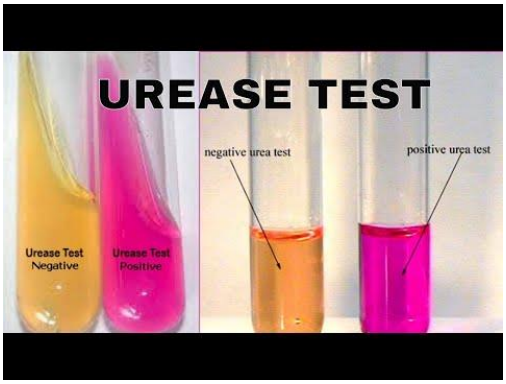
3. Observe the slant for a color change at 6 hours and 24 hours unless specified for longer incubation.

Result and Interpretation

Organisms that hydrolyze urea rapidly (*Proteus* spp., *Morganella morganii*, and some *Providencia stuartii* strains) will produce strong positive reactions within 1 or 6 hours of incubation; delayed positive organisms (e.g. *Klebsiella* spp and *Enterobacter* species) will produce weak positive reactions in the slant in 6 hours of incubation which will be intense during further incubation. The culture medium will remain a yellowish color if the organism is urease negative e.g. *Escherichia coli.*

In routine diagnostic laboratories the urease test result is read within 24 hours.

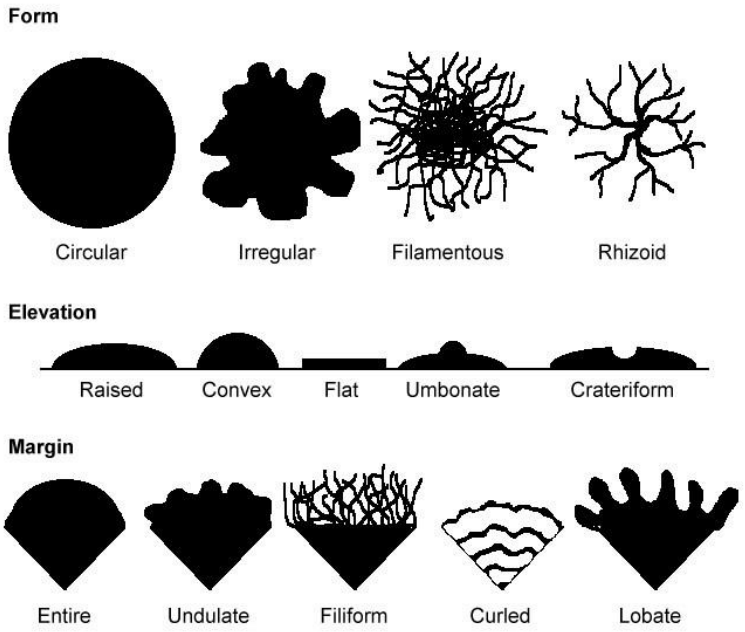
- If organism produces urease enzyme, the color of the slant changes from light orange to magenta.
- If organism do not produce urease the agar slant and butt remain light orange (medium retains original color).



Practical No: 11	**Colony and cell morphology**

Colony Morphology of Bacteria

Bacteria grow on solid media as colonies. A colony is defined as a visible mass of microorganisms all originating from a single mother cell. Key features of these bacterial colonies serve as important criteria for their identification.

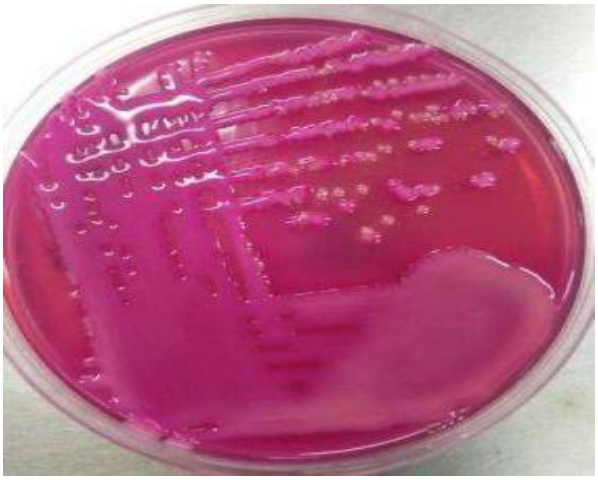


Colony morphology can sometimes be useful in bacterial identification. Colonies are described as to such properties as size, shape, texture, elevation, pigmentation, effect on growth medium. In this blog post you will find common criteria that are used to characterize the bacterial growth.

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

1. **Form of the bacterial colony**: – The form refers to the shape of the colony. These forms represent the most common colony shapes you are likely to encounter. e.g. Circular, Irregular, Filamentous, Rhizoid etc.
2. **Elevation of bacterial colony:** This describes the "side view" of a colony. These are the most common. e.g. Flat, raised, umbonate (having a knobby protuberance), Crateriform, Convex, Pulvinate (Cushion-shaped)
3. **Margin of bacterial colony:** The margin or edge of a colony may be an important characteristic in identifying an organisms. Common examples are Entire (smooth), irregular, Undulate (wavy), Lobate, Curled, Filiform etc.
   Colonies that are irregular in shape and/or have irregular margins are likely to be motile organisms. Highly motile organism swarmed over the culture media. Such as Proteus spp.
4. Size of the bacterial colony: The size of the colony can be a useful characteristic for identification. The diameter of a representative colony may be measured in millimeters or described in relative terms such as pin point, small, medium, large. Colonies larger than about 5 mm are likely to be motile organisms.

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



Mixed growth of mucoid Lactose fermenting colonies and NLF colonies in MacConkey Agar

Consistency/Texture: Several terms that may be appropriate for describing the texture or consistency of bacterial growth are: dry, moist, viscid (sticks to loop, hard to get off), brittle/friable (dry, breaks apart), mucoid (sticky, mucus-like)

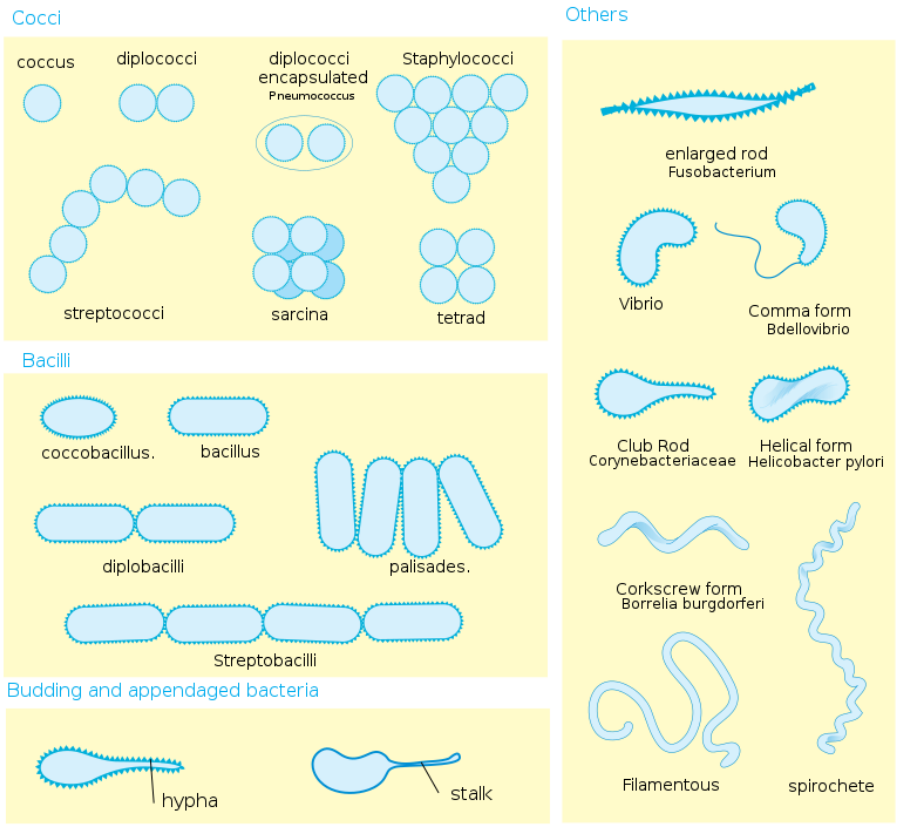
Color of the colonies (pigmentation): Some bacteria produce pigment when they grow in the medium e.g., green pigment produces by Pseudomonas aeruginosa, buff colored colonies of *Mycobacterium tuberculosis* in L.J medium, red colored colonies of *Serratia marcescens.*

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

Cell morphology of bacteria

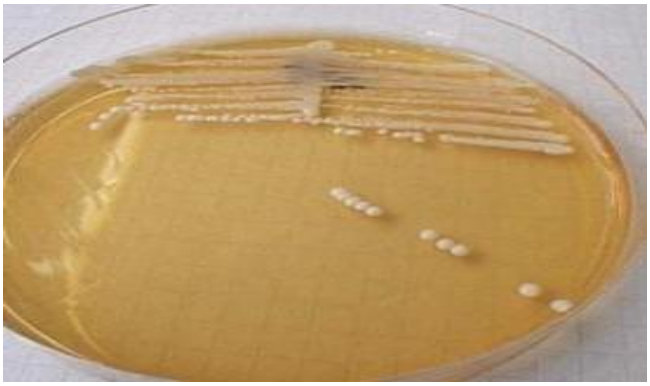
Bacteria and Archaea are classified by direct examination with the light microscope according to their morphology and arrangement.

The basic morphologies are spheres (coccus) and round-ended cylinders (bacillus). But there may be others such as helically twisted cylinders (spirochetes), cylinders curved in one plane (selenomonads) and unusual morphologies (such as the square, flat box-shaped cells of the archaean genus Haloquadratum). Arrangements include pairs, tetrads, clusters, chains and palisades.



Colony morphology of Yeasts

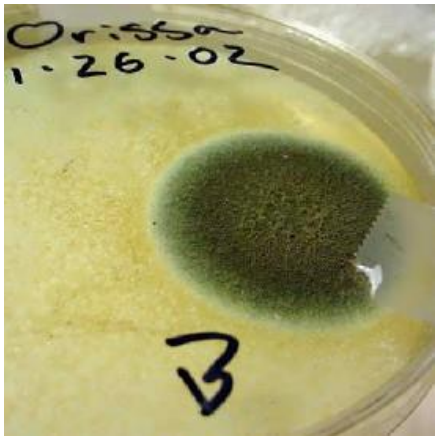
Yeast, a type of fungi (plural for fungus), is found in many places from nature, to research labs and even everyday kitchens for baking. Yeast colonies generally look similar to bacterial colonies. Some species, such as *Candida*, can grow as white patches with a glossy surface.

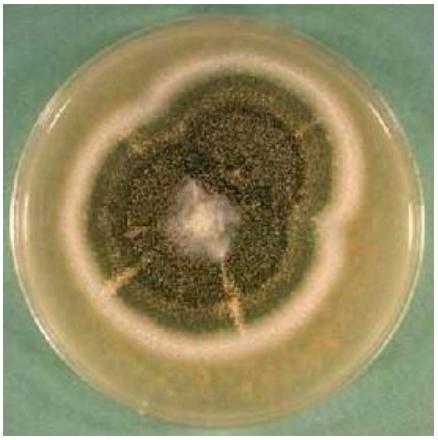


Candida Albicans

Colony morphology of Molds

Molds are actually fungi, and they often appear whitish grey, with fuzzy edges. They usually turn into a different color, from the center outwards. Two examples of molds are shown below:





Green Mold (*Trichoderma harzianum*) Black Mold (*Aspergillus nidulaus*)

Practical No: 12	**Growth Kinetics**

Objectives: To establish growth curves for an unknown bacterial species and observe the different phases of growth.

Introduction:

Bacteria normally reproduce by binary fission, forming two equal-sized progeny cells and thus doubling their number with each division. This type of cell division is called exponential; i.e., one cell divides to form two, each of these cells divides so that four cells are obtained, and so forth in a geometric progression. The unit of microbial growth is "generation time", which is the time required to achieve a doubling of the population size and is designated as tgen. Generation time can be estimated by determining cell numbers during the period of active cell division and is expressed mathematically as:

$tgen = t \ln 2 / \ln Xt - \ln X0 = t \log 2 / \log Xt - \log X0$

In the above equations, t is the elapsed time during which growth is measured, and Xo and Xt are the number of bacteria at times zero and t, respectively. The presence of 2 in the numerator of the equations is due to the fact that when doubling of the population number occurs, the quantity Xt/Xo (called the doubling constant) will be equal to 2. The generation time of many bacteria is usually several hours. However, under optimum conditions, E. coli has a generation time of about 20 minutes. Thus it can be calculated that a single E. coli cell will produce about a thousand progeny in 3.3 hours and over a million in 6.6 hours. If the generation time and the elapsed time are known, then the number of generations can be estimated as follows:

Number of generations = t/ tgen

A bacterial population follows a characteristic growth curve which has four phases: the lag phase, the log or exponential growth phase, the stationary phase, and the death phase.

1. **Lag phase:** During this phase, there is no increase in cell number; rather, bacteria are preparing for reproduction and synthesizing DNA and various inducible enzymes needed for cell division.

2. **Log phase:** This phase, also called the exponential phase, follows the lag phase and starts with a rapid increase in bacterial number. At this stage, the logarithm of bacterial biomass increases linearly with time so that numbers of bacterial cells in a given interval of time is proportional to the biomass of bacteria present.
3. **Stationary phase:** The number of bacteria reaches a maximum in this phase and does not increase further (the growth rate is exactly equal to the death rate). This phase is sometimes called the plateau stage. A bacterial population may reach stationary growth when a required nutrient is exhausted, when inhibitory end products accumulate, or when physical conditions are inappropriate for growth. The duration of the stationary phase varies, with some bacteria exhibiting very long stationary phases.
4. **Death phase:** Eventually, the number of viable bacterial cells begins to decline, signaling the onset of the death phase. No further divisions occur in this phase. Death rate, in many cases, follows the same kinetics as the exponential growth.

Principle:

Turbidity, which is a measure of the growth of microorganisms, is determined via an instrument called a spectrophotometer. The basis for the use of this instrument is that less light passes through a turbid solution than through a clear solution; i.e., the more turbid a solution (more growth), the less the amount of light transmitted through the solution. Since the optical density of the culture is proportional to the cell density, measuring the turbidity of the culture solution can be used in estimating numbers of bacterial cells, if a growth curve for the conditions used has already been established. This is the most common method used to rapidly estimate bacterial numbers.

Laboratory Supplies: 10 ml culture of an unknown microorganism, 50 ml LB broth in 125-ml flasks, Spectrophotometer, Un-inoculated LB broth in small test tube for blank.

Procedure:

1. Obtain a flask of LB and transfer 5 ml of broth to a small test tube. This is your blank. Adjust the wavelength to 550 nm on the spectrophotometer and calibrate the machine to 0.0 O.D. or 100% transmittance with your blank. Take the blank out but do not discard it.

2. Inoculate your flask with 10 ml of the culture provided and swirl the flask well.

3. Work quickly to withdraw 5 ml of the sample into a clean small test tube, using a pipette.

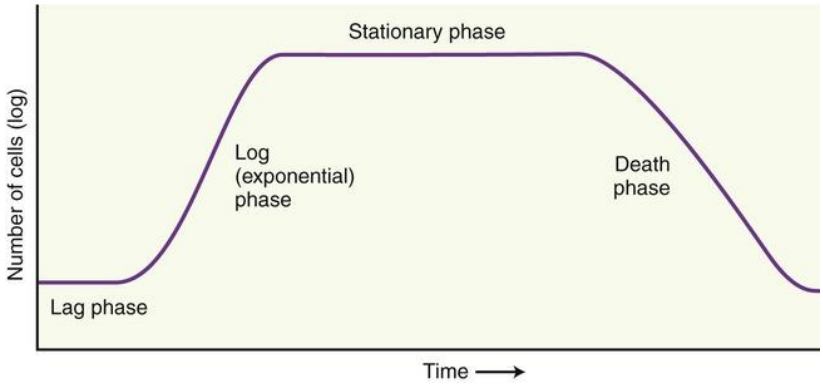
4. Place the culture flask immediately at the assigned incubating temperature and aeration condition and record the time.

5. Measure the O.D. of your sample next. Record the 0 time absorbance. Discard the contents of your sample test tube. This test tube can be re-used for your next sample without washing.

6. At 15-minute intervals for up to 90 minutes, shake your flask vigorously and transfer 5 ml of the culture to your sample test tube.

7. Record the optical density at each time period. Use your blank each time to make sure your readings are correct and other students have not altered the machine's calibration. Your instructor will ask all groups to write their results in a table on the blackboard. Copy these results to your Results Sheet. If more than one group is doing a growth condition, get their average data.

8. Plot the optical density (y-axis) versus time (x-axis) for each growth condition on the same regular graph paper.



Practical No: 13 **Testing sensitivity to antimicrobial substances**

The agar diffusion method is widely used in industry for testing the sensitivity of micro-organisms to antibiotics, antiseptics, toothpaste, mouthwashes, disinfectants, etc. The method involves preparing a pour or spread plate of a test micro-organism, adding small amount of test substance to either a well cut in the agar medium or (preferably) a paper disc which is then placed on the agar surface. After incubation, an inhibitory effect on the test organism is indicated by a clear zone (no growth) around the test substance; microbial growth is visible to the naked eye in areas of the plate that are unaffected. This is a straightforward activity that tests several practical skills and is relevant to other aspects of biology and to everyday life. In addition to using laboratory reagents, e.g. stains, and antibiotic discs, many preparations with antimicrobial activity are readily available in pharmacists and supermarkets. There is also the opportunity to think of less obvious materials, e.g. plants and their products.

Materials

- Take a nutrient agar pour or spread/lawn plate of e.g. Bacillus subtilis, Micrococcus luteus, Escherichia coli or Saccharomyces cerevisiae on malt agar.
- Sterile filter paper discs
- Sterile distilled/demineralised water (control)
- Samples to be tested, 3 (e.g. mouthwashes, selected for a range of active ingredients)
- Bunsen burner
- Forceps
- Alcohol (70 % IDA) in a small beaker covered in foil (Caution: flammable, should be kept covered away from flames)
- Incubator at 25–30 °C (if available)

Procedure

Aseptic technique should be used throughout.

1. Mark and label four sections on the base of the Petri dish, for the three different samples and control (sterile water).
2. Using sterile forceps (flamed with alcohol and cooled) remove one filter paper disc. Dip into the first test sample, drain on the side of the container and place firmly onto the appropriate section of the seeded agar plate.
3. Wash the forceps free of the sample.
4. Repeat for the remaining samples and the control (sterile water). Remember to rinse and sterilise the forceps between each sample and to open the plate for the minimum possible time.
5. Seal the lid to the base with tape.

   Incubation of the plate
6. Invert the plate and incubate at 25–30 °C or at room temperature for 48 hours.
7. Examine the plate (without opening). Measure and record the size of any zones of inhibition around the filter paper discs. Consider what factors might be affecting the size of the zones of inhibition.

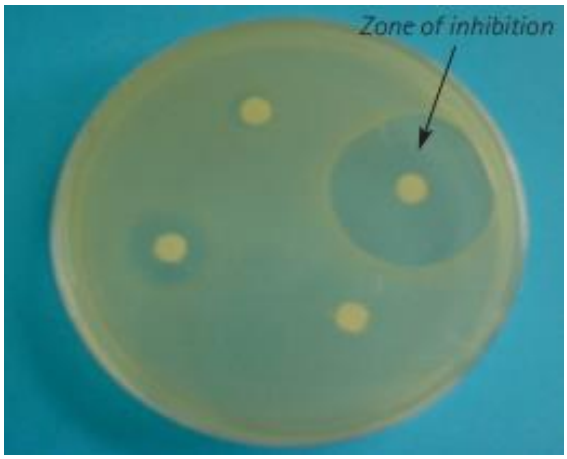


Fig 13.1: Antibiotic test