Microbial Techniques And Bacteriology Lab

BML-393

Sterilization and Disinfection technique

Sterilization is defined as the process where all the living microorganisms, including bacterial spores are killed. Sterilization can be achieved by physical, chemical and physiochemical means. Chemicals used as sterilizing agents are called chemisterilants.
Disinfection is the process of elimination of most pathogenic microorganisms (excluding bacterial spores) on inanimate objects. Disinfection can be achieved by physical or chemical methods. Chemicals used in disinfection are called disinfectants. Different disinfectants have different target ranges, not all disinfectants can kill all microorganisms. Some methods of disinfection such as filtration do not kill bacteria, they separate them out. Sterilization is an absolute condition while disinfection is not. The two are not synonymous.

Decontamination is the process of removal of contaminating pathogenic microorganisms from the articles by a process of sterilization or disinfection. It is the use of physical or chemical means to remove, inactivate, or destroy living organisms on a surface so that the organisms are no longer infectious.
Sanitization is the process of chemical or mechanical cleansing, applicable in public health systems. Usually used by the food industry. It reduces microbes on eating utensils to safe, acceptable levels for public health.

Asepsis is the employment of techniques (such as usage of gloves, air filters, uv rays etc) to achieve microbe-free environment.
Antisepsis is the use of chemicals (antiseptics) to make skin or mucus membranes devoid of pathogenic microorganisms.

Bacteriostasis is a condition where the multiplication of the bacteria is inhibited without killing them.
Bactericidal is that chemical that can kill or inactivate bacteria. Such chemicals may be called

variously depending on the spectrum of activity, such as bactericidal, virucidal, fungicidal, microbicidal, sporicidal, tuberculocidal or germicidal. Antibiotics are substances produced by one microbe that inhibits or kills another microbe. Often the term is used more generally to include synthetic and semi-synthetic antimicrobial agents.

PHYSICAL METHODS OF STERILIZATION:

Sunlight: The microbicidal activity of sunlight is mainly due to the presence of ultra violet rays in it. It is responsible for spontaneous sterilization in natural conditions. In tropical countries, the sunlight is more effective in killing germs due to combination of ultraviolet rays and heat. By killing bacteria suspended in water, sunlight provides natural method of disinfection of water bodies such as tanks and lakes. Sunlight is not sporicidal, hence it does not sterilize. Heat: Heat is considered to be most reliable method of sterilization of articles that can withstand heat. Heat acts by oxidative effects as well as denaturation and coagulation of proteins. Those articles that cannot withstand high temperatures can still be sterilized at lower temperature by prolonging the duration of exposure.

Action of heat:

Dry heat acts by protein denaturation, oxidative damage and toxic effects of elevated levels of electrolytes. The moist heat acts by coagulation and denaturation of proteins. Moist heat is superior to dry heat in action. Temperature required to kill microbe by dry heat is more than the moist heat. Thermal death time is the minimum time required to kill a suspension of organisms at a predetermined temperature in a specified environment.

DRY HEAT:

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

Flaming: This is a method of passing the article over a Bunsen flame, but not heating it to redness. Articles such as scalpels, mouth of test tubes, flasks, glass slides and cover slips are passed through the flame a few times. Even though most vegetative cells are killed, there is no guarantee that

spores too would die on such short exposure. This method too is limited to those articles that can be exposed to flame. Cracking of the glassware may occur.

Incineration: This is a method of destroying contaminated material by burning them in incinerator. Articles such as soiled dressings; animal carcasses, pathological material and bedding etc should be subjected to incineration. This technique results in the loss of the article, hence is suitable only for those articles that have to be disposed. Burning of polystyrene materials emits dense smoke, and hence they should not be incinerated.

Hot air oven: This method was introduced by Louis Pasteur. Articles to be sterilized are exposed to high temperature (160^{O} C) for duration of one hour in an electrically heated oven. Since air is poor conductor of heat, even distribution of heat throughout the chamber is achieved by a fan. The heat is transferred to the article by radiation, conduction and convection. The oven should be fitted with a thermostat control, temperature indicator, meshed shelves and must have adequate insulation.

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

Sterilization process: Articles to be sterilized must be perfectly dry before placing them inside to avoid breakage. Articles must be placed at sufficient distance so as to allow free circulation of air in between. Mouths of flasks, test tubes and both ends of pipettes must be plugged with cotton wool. Articles such as petri dishes and pipettes may be arranged inside metal canisters and then placed. Individual glass articles must be wrapped in kraft paper or aluminum foils.

Sterilization cycle: This takes into consideration the time taken for the articles to reach the sterilizing temperature, maintenance of the sterilizing temperature for a defined period (holding time) and the time taken for the articles to cool down. Different temperature-time relations for holding time are 60 minutes at 160^{O}C, 40 minutes at 170^{O}C and 20 minutes at 180^{O}C. Increasing temperature by 10 degrees shortens the sterilizing time by 50 percent. The hot air oven must not be opened until the temperature inside has fallen below 60^{O}C to prevent breakage of glasswares.

Sterilization control: Three methods exist to check the efficacy of sterilization process, namely physical, chemical and biological.

- Physical: Temperature chart recorder and thermocouple.
- Chemical: Browne's tube No.3 (green spot, color changes from red to green)
- Biological: 10^6 spores of Bacillus subtilis var niger or Clostridium tetani on paper strips are placed inside

envelopes and then placed inside the hot air oven. Upon completion of sterilization cycle, the strips are removed and inoculated into thioglycollate broth or cooked meat medium and incubated at 37^{O}C for 3-5 days. Proper sterilization should kill the spores and there should not be any growth.

Advantages: It is an effective method of sterilization of heat stable articles. The articles remain dry after sterilization. This is the only method of sterilizing oils and powders. Disadvantages:

Since air is poor conductor of heat, hot air has poor penetration. Cotton wool and paper may get slightly charred.
Glasses may become smoky.
Takes longer time compared to autoclave.

Infra red rays: Infrared rays bring about sterilization by generation of heat. Articles to be sterilized are placed in a moving conveyer belt and passed through a tunnel that is heated by infrared radiators to a temperature of 180^{O}C. The articles are exposed to that temperature for a period of 7.5 minutes. Articles sterilized included metallic instruments and glassware. It is mainly used in central sterile supply department. It requires special equipments, hence is not applicable in diagnostic laboratory. Efficiency can be checked using Browne's tube No.4 (blue spot).

MOIST HEAT:

Moist heat acts by coagulation and denaturation of proteins.

At temperature below 100^{O}C:

- **Pasteurization:** This process was originally employed by Louis Pasteur. Currently this procedure is employed in food and dairy industry. There are two methods of pasteurization, the holder method (heated at 63^{O}C for 30 minutes) and flash method (heated at 72^{O}C for 15 seconds) followed by quickly cooling to 13^{O}C. Other pasteurization methods include Ultra-High Temperature (UHT), 140^{O}C for 15 sec and 149^{O}C for 0.5 sec. This method is suitable to destroy most milk borne pathogens like Salmonella, Mycobacteria, Streptococci, Staphylococci and Brucella, however Coxiella may survive pasteurization. Efficacy is tested by phosphatase test and methylene blue test.
- **Vaccine bath:** The contaminating bacteria in a vaccine preparation can be inactivated by heating in a water bath at 60^{O}C for one hour. Only vegetative bacteria are killed and spores survive.
- **Serum bath:** The contaminating bacteria in a serum preparation can be inactivated by heating in a water bath at 56^{O}C for one hour on several successive days. Proteins in the serum will coagulate at higher temperature. Only vegetative bacteria are killed and spores survive.
- **Inspissation:** This is a technique to solidify as well as disinfect egg and serum containing media. The medium containing serum or egg are placed in the slopes of an inspissator and heated at 80-85^{O}C for 30 minutes on three successive days. On the first day, the vegetative bacteria would die and those spores that germinate by next day are then killed the following day. The process depends on germination of spores in between inspissation. If the spores fail to germinate then this technique cannot be considered sterilization.

At temperature 100^{O}C:

- **Boiling: Boiling water (100^{O}C)** kills most vegetative bacteria and viruses immediately. Certain bacterial toxins such as Staphylococcal enterotoxin are also heat resistant. Some bacterial spores are resistant to boiling and survive; hence this is not a substitute for sterilization. The killing activity can be enhanced by addition of 2% sodium bicarbonate.

When absolute sterility is not required, certain metal articles and glasswares can be disinfected by placing them in boiling water for 10-20 minutes. The lid of the boiler must not be opened during the period.

- **Steam at 100^{O}C**: Instead of keeping the articles in boiling water, they are subjected to free steam at 100^{O}C. Traditionally Arnold's and Koch's steamers were used. An autoclave (with discharge tap open) can also serve the same purpose. A steamer is a metal cabinet with perforated trays to hold the articles and a conical lid. The bottom of steamer is filled with water and heated. The steam that is generated sterilizes the articles when exposed for a period of 90 minutes. Media such as TCBS, DCA and selenite broth are sterilized by steaming. Sugar and gelatin in medium may get decomposed on autoclaving, hence they are exposed to free steaming for 20 minutes for three successive days. This process is known as tyndallisation (after John Tyndall) or fractional sterilization or intermittent sterilization. The vegetative bacteria are killed in the first exposure and the spores that germinate by next day are killed in subsequent days. The success of process depends on the germination of spores.

At temperature above 100^{O}C:

Autoclave: Sterilization can be effectively achieved at a temperature above 100^{O}C using an autoclave. Water boils at 100^{O}C at atmospheric pressure, but if pressure is raised, the temperature at which the water boils also increases. In an autoclave the water is boiled in a closed chamber. As the pressure rises, the boiling point of water also raises. At a pressure of 15 lbs inside the autoclave, the temperature is said to be 121^{O}C. Exposure of articles to this temperature for 15 minutes sterilizes them. To destroy the infective agents associated with spongiform encephalopathies (prions), higher temperatures or longer times are used; 135^{O}C or 121^{O}C for at least one hour are recommended.

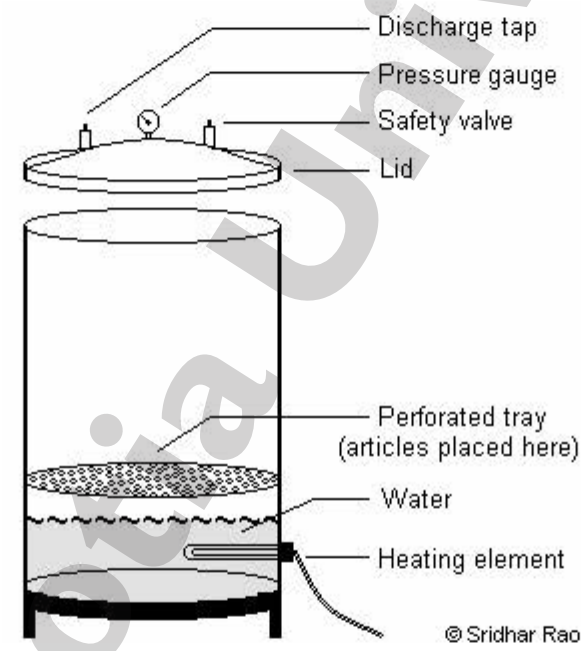
Advantages of steam: It has more penetrative power than dry air, it moistens the spores (moisture is essential for coagulation of proteins), condensation of steam on cooler surface releases latent

heat, condensation of steam draws in fresh steam.

Different types of autoclave:

Simple "pressure-cooker type" laboratory autoclave, Steam jacketed downward displacement laboratory autoclave and high pressure pre-vacuum autoclave

Construction And Operation Of Autoclave:



A simple autoclave has vertical or horizontal cylindrical body with a heating element, a perforated try to keep the articles, a lid that can be fastened by screw clamps, a pressure gauge, a safety valve and a discharge tap. The articles to be sterilized must not be tightly packed. The screw caps and cotton plugs must be loosely fitted. The lid is closed but the discharge tap is kept open and the water is heated. As the water starts boiling, the steam drives air out of the discharge tap. When all the air is displaced and steam start appearing through the discharge tap, the tap is closed. The pressure inside is allowed to rise upto 15 lbs per square inch. At this pressure the articles are held for 15 minutes, after which the heating is stopped and the autoclave is allowed to cool. Once the pressure gauge shows the pressure equal to atmospheric pressure, the discharge tap is opened to

let the air in. The lid is then opened and articles removed. Articles sterilized: Culture media, dressings, certain equipment, linen etc.

Precautions: Articles should not be tightly packed, the autoclave must not be overloaded, air discharge must be complete and there should not be any residual air trapped inside, caps of bottles and flasks should not be tight, autoclave must not be

opened until the pressure has fallen or else the contents will boil over, articles must be wrapped in paper to prevent drenching, bottles must not be overfilled.
Advantage: Very effective way of sterilization, quicker than hot air oven.
Disadvantages: Drenching and wetting or articles may occur, trapped air may reduce the efficacy, takes long time to cool

Sterilization control: Physical method includes automatic process control, thermocouple and temperature chart recorder. Chemical method includes Browne's tube No.1 (black spot) and succinic acid (whose melting point is $121^{o}C$) and Bowie Dick tape. Bowie Dick tape is applied to articles being autoclaved. If the process has been satisfactory, dark brown stripes will appear across the tape. Biological method includes a paper strip containing 10^{6} spores of Geobacillus stearothermophilus.

RADIATION:

Two types of radiation are used, ionizing and non-ionizing. Non-ionizing rays are low energy rays with poor penetrative power while ionizing rays are high-energy rays with good penetrative power. Since radiation does not generate heat, it is termed "cold sterilization". In some parts of Europe, fruits and vegetables are irradiated to increase their shelf life up to 500 percent.

Non-ionizing rays: Rays of wavelength longer than the visible light are non-ionizing. Microbicidal wavelength of UV rays lie in the range of 200-280 nm, with 260 nm being most effective. UV rays are generated using a high-pressure mercury vapor lamp. It is at this wavelength that the absorption by the microorganisms is at its maximum, which results in the germicidal effect. UV rays induce formation of thymine-thymine dimers, which ultimately inhibits DNA replication. UV readily induces mutations in cells

irradiated with a non-lethal dose. Microorganisms such as bacteria, viruses, yeast, etc. that are exposed to the effective UV radiation are inactivated within seconds. Since UV rays don't kill spores, they are considered to be of use in surface disinfection. UV rays are employed to disinfect hospital wards, operation theatres, virus laboratories, corridors, etc. Disadvantages of using uv rays include low penetrative power, limited life of the uv bulb, some bacteria have DNA repair enzymes that can overcome damage caused by uv rays, organic matter and dust prevents its reach, rays are harmful to skin and eyes. It doesn't penetrate glass, paper or plastic.

Ionizing rays: Ionizing rays are of two types, particulate and electromagnetic rays. o Electron beams are particulate in nature while gamma rays are electromagnetic in nature. High-speed electrons are produced by a linear accelerator from a heated cathode. Electron beams are employed to sterilize articles like syringes, gloves, dressing packs, foods and pharmaceuticals. Sterilization is accomplished in few seconds. Unlike electromagnetic rays, the instruments can be switched off. Disadvantage includes poor penetrative power and requirement of sophisticated equipment.

FILTRATION:

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

Different types of filters are:
1. Earthenware filters: These filters are made up of diatomaceous earth or porcelain. They are usually baked

into the shape of candle. Different types of earthenware filters are:

a. **Pasteur-Chamberland filter:** These candle filters are from France and are made up of porcelain (sand and kaolin). Similar filter from Britain is Doulton. Chamberland filters are made with various porosities, which are graded as L1, L1a, L2, L3, L5, L7, L9 and L11. Doulton filters are P2, P5 and P11.

b. **Berkefeld filter:** These are made of Kieselguhr, a fossilized diatomaceous earth found in Germany. They are available in three grades depending on their porosity (pore size); they are V (veil), N (normal) and W (wenig). Quality of V grade filter is checked using culture suspension of Serrtia marcescens (0.75 μm).

c. **Mandler filter:** This filter from America is made of kieselguhr, asbestos and plaster of Paris.

2. **Asbestos filters:** These filters are made from chrysotile type of asbestos, chemically composed of magnesium silicate. They are pressed to form disc, which are to be used only once. The disc is held inside a metal mount, which is sterilized by autoclaving. They are available in following grades; HP/PYR (for removal of pyrogens), HP/EKS (for absolute sterility) and HP/EK (for claryfying).

3. **Sintered glass filters:** These are made from finely ground glass that are fused sufficiently to make small particles adhere to each other. They are usually available in the form of disc fused into a glass funnel. Filters of Grade 5 have average pore diameter of 1-1.5 μm. They are washed in running water in reverse direction and cleaned with warm concentrated H_2SO_4 and sterilized by autoclaving.

Membrane filters: These filters are made from a variety of polymeric materials such as cellulose nitrate, cellulose diacetate, polycarbonate and polyester. The older type of membrane, called gradocol (graded colloidion) membrane was composed of cellulose nitrate. Gradocol membranes have average pore diameter of 3-10 μm. The newer ones are composed of cellulose diacetate. These membranes have a pore diameter ranging from 0.015 μm to 12 μm. These filters are sterilized by autoclaving. Membrane filters are made in two ways, the capillary pore membranes have pores produced by radiation while the labyrinthine pore membranes are produced by forced evaporation of solvents from cellulose esters.

The disadvantages of depth filters are migration of filter material into the filtrate, absorption or retention of certain volume of liquid by the filters, pore sizes are not definite and viruses and mycoplasma could pass through. The advantages of membrane filters are known porosity, no retention of fluids, reusable after autoclaving and compatible with many chemicals. However, membrane filters have little loading capacity and are fragile.

Air Filters: Air can be filtered using HEPA (High Efficiency Particle Air) filters. They are usually used in biological safety cabinets. HEPA filters are at least 99.97% efficient for removing particles >0.3 μm in diameter. Examples of areas where HEPA filters are used include rooms housing severely neutropenic patients and those operating rooms designated for orthopedic implant procedures. HEPA filter efficiency is monitored with the dioctylphthalate (DOP) particle test using particles that are 0.3 μm in diameter.

SONIC AND ULTRASONIC VIBRATIONS: Sound waves of frequency >20,000 cycle/second kills bacteria and some viruses on exposing for one hour. Microwaves are not particularly antimicrobial in themselves, rather the killing effect of microwaves are largely due to the heat that they generate. High frequency sound waves disrupt cells. They are used to clean and disinfect instruments as well as to reduce microbial load. This method is not reliable since many viruses and phages are not affected by these waves.

CHEMICAL METHODS OF DISINFECTION:

Disinfectants are those chemicals that destroy pathogenic bacteria from inanimate surfaces. Some chemical have very narrow spectrum of activity and some have very wide. Those chemicals that can sterilize are called chemisterilants. Those chemicals that can be safely applied over skin and mucus membranes are called antiseptics. An ideal antiseptic or disinfectant should have following properties:

Such an ideal disinfectant is not yet available. The level of disinfection achieved depends on contact time, temperature, type and concentration of the active ingredient, the presence of organic

matter, the type and quantum of microbial load. The chemical disinfectants at working concentrations rapidly lose their strength on standing.

Classification of disinfectants:

1. Based on consistency
    1. Liquid (E.g., Alcohols, Phenols)
    2. Gaseous (Formaldehyde vapor, Ethylene oxide)
2. Based on spectrum of activity
    1. High level
    2. Intermediate level
    3. Low level
3. Based on mechanism of action
    1. Action on membrane (E.g., Alcohol, detergent)
    2. Denaturation of cellular proteins (E.g., Alcohol, Phenol)

c. Oxidation of essential sulphydryl groups of enzymes (E.g., H2O2, Halogens)

4. Alkylation of amino-, carboxyl- and hydroxyl group (E.g., Ethylene Oxide, Formaldehyde)
5. Damage to nucleic acids (Ethylene Oxide, Formaldehyde)
6. **ALCOHOLS:**

   Mode of action: Alcohols dehydrate cells, disrupt membranes and cause coagulation of protein.

   Examples: Ethyl alcohol, isopropyl alcohol and methyl alcohol

   Application: A 70% aqueous solution is more effective at killing microbes than absolute alcohols. 70% ethyl alcohol (spirit) is used as antiseptic on skin. Isopropyl alcohol is preferred to ethanol. It can also be used to disinfect surfaces. It is used to disinfect clinical thermometers. Methyl alcohol kills fungal spores, hence is useful in disinfecting inoculation hoods.

   Disadvantages: Skin irritant, volatile (evaporates rapidly), inflammable
7. **ALDEHYDES:**

   Mode of action: Acts through alkylation of amino-, carboxyl- or hydroxyl group, and

probably damages nucleic acids. It kills all microorganisms, including spores. Examples: Formaldehyde, Gluteraldehyde

Application: 40% Formaldehyde (formalin) is used for surface disinfection and fumigation of rooms, chambers, operation theatres, biological safety cabinets, wards, sick rooms etc. Fumigation is achieved by boiling formalin, heating paraformaldehyde or treating formalin with potassium permanganate. It also sterilizes bedding, furniture and books. 10% formalin with 0.5% tetraborate sterilizes clean metal instruments. 2% gluteraldehyde is used to sterilize thermometers, cystoscopes, bronchoscopes, centrifuges, anasethetic equipments etc. An exposure of at least 3 hours at alkaline pH is required for action by gluteraldehyde.

2% formaldehyde at 40^{O}C for 20 minutes is used to disinfect wool and 0.25% at 60^{O}C for six hours to disinfect animal hair and bristles.

Disadvantages: Vapors are irritating (must be neutralized by ammonia), has poor penetration, leaves non-volatile residue, activity is reduced in the presence of protein. Gluteraldehyde requires alkaline pH and only those articles that are wettable can be sterilized.

8. **PHENOL:**

Mode of action: Act by disruption of membranes, precipitation of proteins and inactivation of enzymes.

Examples: 5% phenol, 1-5% Cresol, 5% Lysol (a saponified cresol), hexachlorophene, chlorhexidine, chloroxylenol (Dettol)

Applications: Joseph Lister used it to prevent infection of surgical wounds. Phenols are coal-tar derivatives. They act as disinfectants at high concentration and as antiseptics at low concentrations. They are bactericidal, fungicidal, mycobactericidal but are inactive against spores and most viruses. They are not readily inactivated by organic matter. The corrosive phenolics are used for disinfection of ward floors, in discarding jars in laboratories and disinfection of bedpans. Chlorhexidine can be used in an isopropanol solution for skin disinfection, or as an aqueous solution for wound irrigation. It is often used as an antiseptic hand wash. 20% Chlorhexidine gluconate solution is used for pre-operative hand and skin preparation and for general skin disinfection. Chlorhexidine gluconate is also mixed with quaternary ammonium compounds such as cetrimide to get stronger and broader antimicrobial effects (eg. Savlon). Chloroxylenols are less irritant and

can be used for topical purposes and are more effective against gram positive bacteria than gram negative bacteria. Hexachlorophene is chlorinated diphenyl and is much less irritant. It has marked effect over gram positive bacteria but poor effect over gram negative bacteria, mycobacteria, fungi and viruses. Triclosan is an organic phenyl ether with good activity against gram positive bacteria and effective to some extent against many gram negative bacteria including Pseudomonas. It also has fair activity on fungi and viruses.

9. **Disadvantages:** It is toxic, corrosive and skin irritant. Chlorhexidine is inactivated by anionic soaps. Chloroxylenol is inactivated by hard water.
10. **HALOGENS:**

    Mode of action: They are oxidizing agents and cause damage by oxidation of essential sulfydryl groups of enzymes. Chlorine reacts with water to form hypochlorous acid, which is microbicidal.

    Examples: Chlorine compounds (chlorine, bleach, hypochlorite) and iodine compounds (tincture iodine, iodophores)

    Applications: Tincture of iodine (2% iodine in 70% alcohol) is an antiseptic. Iodine can be combined with neutral carrier polymers such as polyvinylpyrrolidone to prepare iodophores such as povidone-iodine. Iodophores permit slow release and reduce the irritation of the antiseptic. For hand washing iodophores are diluted in 50% alcohol. 10% Povidone Iodine is used undiluted in pre and postoperative skin disinfection. Chlorine gas is used to bleach water. Household bleach can be used to disinfect floors. Household bleach used in a stock dilution of 1:10. In higher concentrations chlorine is used to disinfect swimming pools. 0.5% sodium hypochlorite is used in serology and virology. Used at a dilution of 1:10 in decontamination of spillage of infectious material. Mercuric chloride is used as a disinfectant.

    Disadvantages: They are rapidly inactivated in the presence of organic matter. Iodine is corrosive and staining. Bleach solution is corrosive and will corrode stainless steel surfaces.
11. **HEAVY METALS:**

    Mode of action: Act by precipitation of proteins and oxidation of sulfydryl groups. They are bacteriostatic. Examples: Mercuric chloride, silver nitrate, copper sulfate, organic mercury salts (e.g., mercurochrome, merthiolate)

Applications: 1% silver nitrate solution can be applied on eyes as treatment for opthalmia neonatorum (Crede's method). This procedure is no longer followed. Silver sulphadiazine is used topically to help to prevent colonization and infection of burn tissues. Mercurials are active against viruses at dilution of 1:500 to 1:1000. Merthiolate at a concentration of 1:10000 is used in preservation of serum. Copper salts are used as a fungicide. Disadvantages: Mercuric chloride is highly toxic, are readily inactivated by organic matter.

12. **SURFACE ACTIVE AGENTS:**

Mode of actions: They have the property of concentrating at interfaces between lipid containing membrane of bacterial cell and surrounding aqueous medium. These compounds have long chain hydrocarbons that are fat soluble and charged ions that are water-soluble. Since they contain both of these, they concentrate on the surface of membranes. They disrupt membrane resulting in leakage of cell constituents. Examples: These are soaps or detergents. Detergents can be anionic or cationic. Detergents containing negatively charged long chain hydrocarbon are called anionic detergents. These include soaps and bile salts. If the fat-soluble part is made to have a positive charge by combining with a quaternary nitrogen atom, it is called cationic detergents. Cationic detergents are known as quaternary ammonium compounds (or quat). Cetrimide and benzalkonium chloride act as cationic detergents. Application: They are active against vegetative cells, Mycobacteria and enveloped viruses. They are widely used as disinfectants at dilution of 1-2% for domestic use and in hospitals. Disadvantages: Their activity is reduced by hard water, anionic detergents and organic matter. Pseudomonas can metabolise cetrimide, using them as a carbon, nitrogen and energy source.

13. **DYES:**

Mode of action: Acridine dyes are bactericidal because of their interaction with bacterial nucleic acids.

Examples: Aniline dyes such as crystal violet, malachite green and brilliant green. Acridine dyes such as acriflavin and aminacrine. Acriflavine is a mixture of proflavine and euflavine. Only euflavine has effective antimicrobial properties. A related dye, ethidium bromide, is also germicidal. It intercalates between base pairs in DNA. They are more effective against gram positive bacteria than gram negative bacteria and are more

bacteriostatic in action. Applications: They may be used topically as antiseptics to treat mild burns. They are used as paint on the skin to treat bacterial skin infections. The dyes are used as selective agents in certain selective media.

14. **HYDROGEN PEROXIDE:** Mode of action: It acts on the microorganisms through its release of nascent oxygen. Hydrogen peroxide produces hydroxyl-free radical that damages proteins and DNA.
15. **Application:** It is used at 6% concentration to decontaminate the instruments, equipments such as ventilators. 3% Hydrogen Peroxide Solution is used for skin disinfection and deodorising wounds and ulcers. Strong solutions are sporicidal.

Staining techniques-Gram stain

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

The objective of Gram Stain

This test differentiates the bacteria into Gram-Positive and Gram-Negative Bacteria, which helps in the classification and differentiation of microorganisms. The Gram stain separates bacteria into two groups: (1) Gram-positive microorganisms that retain the primary dye (Crystal violet) and (2) Gram-negative microorganisms that take the color of the counterstain (usually Safranin O).

Principle of Gram Staining

When the bacteria is stained with primary stain Crystal Violet and fixed by the mordant, some of the bacteria are able to retain the primary stain and some are decolorized by alcohol. The cell walls of gram positive bacteria have a thick layer of protein-sugar complexes called peptidoglycan and lipid content is low. Decolorizing the cell causes this thick cell wall to dehydrate and shrink, which closes the pores in the cell wall and prevents the stain from exiting the cell. So the ethanol cannot remove the Crystal Violet-Iodine complex that is bound to the thick layer of peptidoglycan of gram positive bacteria and appears blue or purple in colour.

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

Reagents

- Crystal Violet, the primary stain
- Iodine, the mordant
- A decolorizer made of acetone and alcohol (95%)
- Safranin, the counterstain

Primary stain: 2 g Crystal violet, 20 mL 95% ethyl alcohol, 0.8 g ammonium oxalate, and 100 mL distilled water.
Gram's iodine: 2 g potassium iodide, 1 g iodine crystals, and 100 mL distilled water.
Decolorizer: 50 mL acetone and 50 mL ethanol.
Counterstain: 4.0 g Safranin, 200 mL 95% ethanol, and 800 mL distilled water

Smear Preparation

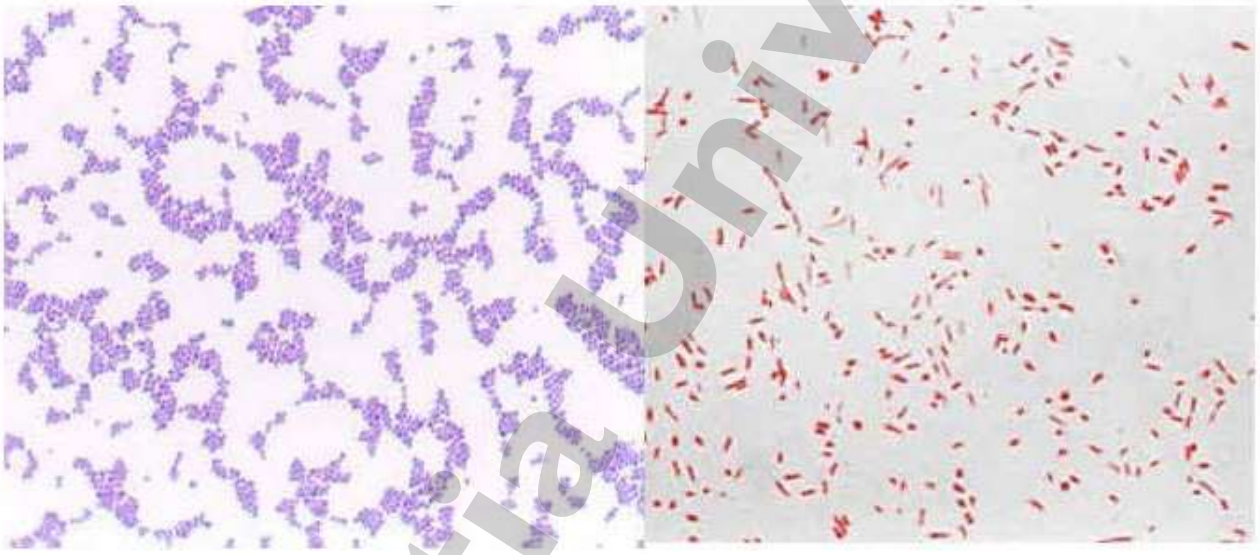
Fix material on a slide with methanol or heat. If the slide is heat fixed, allow it to cool to the touch before applying the stain.

The procedure/steps of Gram Stain

1. Prepare and fix the specimen to the microscope slide before staining.
2. Cover the smear with crystal violet, the primary stain, for 20 seconds.
3. Gently rinse off the stain with water.
4. Cover the smear with Gram's iodine, the mordant, for 1 minute.
5. Pour off the excess Gram's iodine.
6. Run the acid-alcohol decolorizer over the smear until the solution appears clear.
7. Gently rinse with water.
8. Cover the smear with safranin, the secondary or counterstain, for 20 seconds.

9. Gently rinse the stain with water.
10. Blot dry with bibulous paper.

Result Interpretation of Gram Stain



Gram +ve Bacteria

Gram -ve Bacteria

Gram-positive: Blue/Purple Color

Gram-Negative: Red/Pink Color

Limitations

1. Over-decolorization may result in the identification of false gram-negative results, whereas under-decolorization may result in the identification of false gram-positive results.
2. Smears that are too thick or viscous may retain too much primary stain, making the identification of proper Gram stain reactions difficult. Gram-negative organisms may not decolorize properly.
3. Cultures older than 16 to 18 hours will contain living and dead cells. Cells that are dead will be deteriorating and will not retain the stain properly.

4. The stain may form a precipitate with aging. Filtering through gauze will remove excess crystals.
5. Gram stains from patients on antibiotics or antimicrobial therapy may have altered Gram stain reactivity due to the successful treatment.
6. Occasionally, pneumococci identified in the lower respiratory tract on a direct smear will not grow in culture. Some strains are obligate anaerobes.
7. Toxin-producing organisms such as Clostridia, staphylococci, and streptococci may destroy white blood cells within a purulent specimen.
8. Faintly staining Gram-negative organisms, such as *Campylobacter* and *Brucella*, may be visualized using an alternative counterstain (e.g., basic fuchsin).

Examples

Gram-Positive: *Streptococcus, Staphylococcus, Corynebacterium, Listeria, Bacillus, Clostridium,* etc.

Gram-Negative: *E. coli, Salmonella Typhi, Shigella spp, Pseudomonas aeruginosa, Neisseria gonorrhoeae, Chlamydia trachomatis, Yersinia pestis*, etc.

Quality Control

Always check new batches of stain and reagents for correct staining reactions using a smear containing known Gram-positive and Gram-negative organisms.

Staining techniques- Acid-Fast Stain

It is the differential staining techniques which was first developed by Ziehl and later on modified by Neelsen. So this method is also called ***Ziehl-Neelsen staining*** techniques. Neelsen in 1883 used Ziehl's carbol-fuchsin and heat then decolorized with an acid alcohol, and counter stained with methylene blue. Thus Ziehl-Neelsen staining techniques was developed.

The main aim of this staining is to differentiate bacteria into acid fast group and non-acid fast groups.

This method is used for those microorganisms which are not staining by simple or Gram staining method, particularly the member of genus *Mycobacterium*, are resistant and can only be visualized by acid-fast staining.

Objective of Acid Fast Stain

The main aim objective of this stain is to differentiate bacteria into acid fast group and non-acid fast groups.

Principle

Acid-fast mycobacteria contain mycolic acid in their outer membrane, making the cells waxy and resistant to staining with aqueous based stains such as the Gram stain. The primary stain, carbolfuchsin is applied to the cells, and heat and phenol are used to allow the stain to penetrate into the waxy surface of acid-fast microorganisms. The excess stain is removed with treatment by acid alcohol (ethanol and hydrochloric acid). A secondary stain, methylene blue, is then applied to the cells.

Ziehl-Neelsen Stain Reagents

Primary Stain: 0.3% Carbol-fuchsin. Dissolve 50 g phenol in 100 mL ethanol (95%) or methanol (95%). Dissolve 3 g Basic fuchsin in the mixture and add distilled water to bring the volume to 1 L.

Decolorization Solution: Add 30 mL hydrochloric acid to 1 L of 95% denatured alcohol. Cool and mix well before use. Alternate decolorizing reagent (without alcohol): Slowly add 250 mL sulfuric acid (at least 95%) to 750 mL distilled water. Cool and mix well before using. **Counterstain:** 0.3% nethylene blue. Dissolve 3 g nethylene blue in 1 L distilled water.

Procedure of Acid Fast Stain

A. Smear Preparation

1. Add one loopful of sterile water to a microscope slide.
2. Make a heavy smear of *Mycobacterium smegmatis*. Mix thoroughly with your loop. Then transfer a small amount of *Staphylococcus epidermidis* to the same drop of water. You will now have a mixture of *M. smegmatis* and *S. epidermidis*.
3. Air dry and heat fix.

B. Procedure

1. Prepare and fix the specimen smear prior to staining.
2. Place a small strip of blotting or filter paper over the top of the specimen, and place the slide over a boiling hot water bath on a mesh surface.
3. Cover the filter paper with the primary stain, carbolfuchsin. Leave the slide on the water bath for 3 to 5 minutes. Continue to apply stain if the filter paper begins to dry.

4. Remove the filter paper and rinse the slide with water until the solution runs clear.
5. Run acid-alcohol decolorizer over the slide for approximately 10 to 15 seconds.
6. Rinse the slide with water.
7. Cover the smear with the secondary or counterstain, methylene blue, for 1 minute.
8. Gently rinse the slide with water.
9. Blot the slide dry with bibulous paper.

Result Interpretation of Acid Fast Stain

Acid fast: Bright red to intensive purple, Red, straight or slightly curved rods, occurring singly or in small groups, may appear beaded

Non-acid fast: Blue color; In addition, background material should stain blue.

Limitations

1. The filter paper must remain moist and in contact with the specimen during heating to allow for proper penetration of the primary stain.
2. Organisms cultivated on blood agar may experience nutrient deprivation, resulting in a lower lipid content in the outer membrane resulting in poor staining.

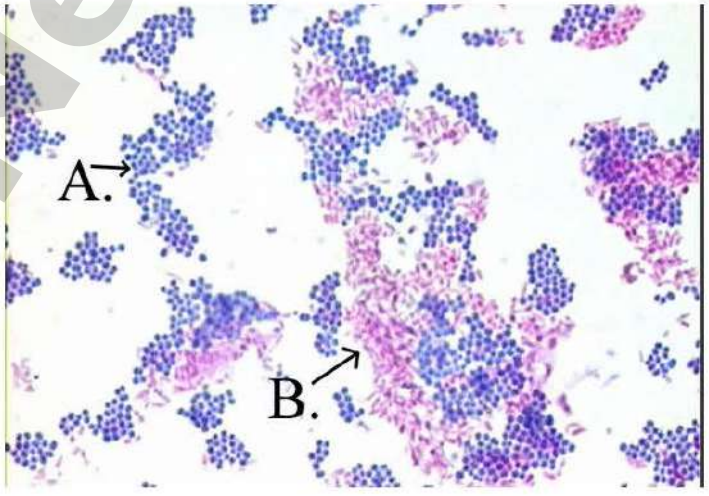
Examples:

Acid-fast: *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*.

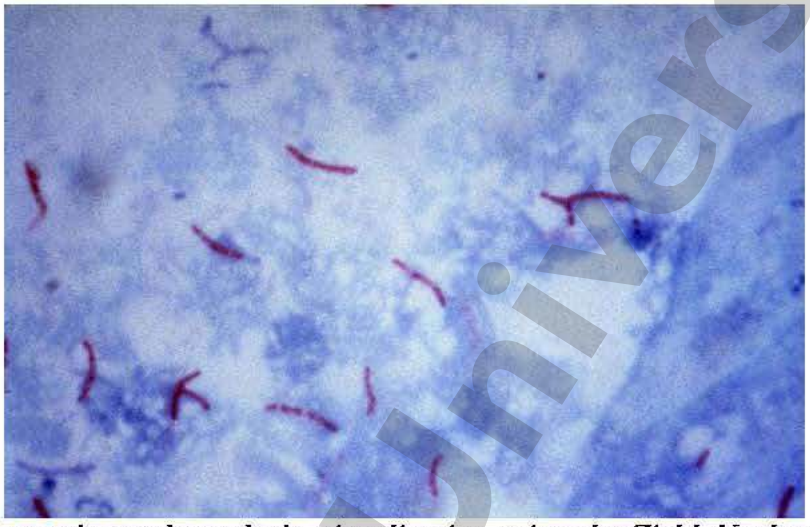
Non-Mycobacterial bacteria: *Nocardia*

Coccidian Parasites: *Cryptosporidium*

Interpretation of Acid-Fast Stain



Acid fast: Bright red to intensive purple (B), Red, straight or slightly curved rods, occurring singly or in small groups, may appear beaded
Non-acid fast: Blue color (A)



Mycobacterium tuberculosis *visualization using the Ziehl–Neelsen stain.*

Staining techniques- Albert Stain

Albert stain is a type of differential stain used for staining high-molecular-weight polymers of polyphosphate known as **metachromatic granules or volutin granules** found in *Corynebacterium diphtheriae*. Metachromatic granules are also found in Yersinia pestis, and *Mycobacterium* species.

It is named metachromatic because of its property of changing color i.e when stained with blue stain they appear red in color. When grown in Loffler's slopes, *C. diphtheriae* produces a large number of granules.

Objective

To stain and observe metachromatic granules from a *Corynebacterium diphtheriae* culture.

Principle:

The bacterial cell of Corynebacterium diphtheriae & Yersinia pestis has volutin granules in their cytoplasm which are highly acidic while the cytoplasm is neutral. Albert stain I have two dyes

'Toluidine blue O' and 'Malachite green' both of which are basic dyes with high affinity for neutral tissue components like cytoplasm and the pH of Albert stain I is adjusted to 2.8 by using 'Glacial acetic acid', which is acidic for cytoplasm (as it is neutral) but basic for volutin granules (as the pH of volutin granules are highly acidic).

Therefore, When Albert stain I applied to the cell the volutin granules stain by Toluidine blue O while cytoplasm is green by Malachite green. Due to the metachromatic property of volutin granules when stained with Toluidine blue O dye they appear Red in color.

When Albert stains II i.e. the Iodine solution is applied due to the effect of Iodine the metachromatic property is not observed and Granules appear blue-black in color.

REQUIREMENTS :

- Specimen/Bacterial culture
- Tissue paper
- Glass slides
- Inoculating loop
- Spirit lamp/Bunsen burner
- Staining tray
- Microscope
- Wash Bottle
- Albert's stain A
- Albert's Stain B

The composition of Albert stain:

Albert's A Solution consist of

- Toluidine blue 0.15 gm
- Malachite green 0.20 gm
- Glacial acetic acid 1 ml
- Alcohol (95% ethanol) 2ml

Dissolve the dyes in alcohol and add to the distilled water then add the acetic acid to the thus obtained solution. Allow the stain to stand for one day and then filter. Add Distilled water to make the final volume 100ml.

Albert's B Solution consists of

- Iodine 2gm

- Potassium iodide (KI) 3 gm

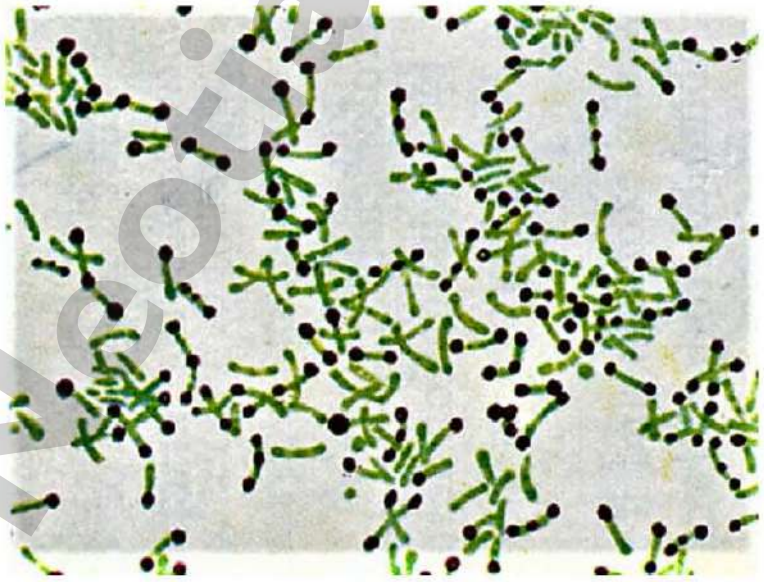
Dissolve the KI in water and then add iodine. Dissolve iodine in thus formed potassium iodide solution.

PROCEDURE OF ALBERT STAINING

- Take an inoculum of bacterial culture or sputum sample with help of inoculating loop.
- Prepare a thin smear of the specimen by mixing the cell with a drop of water / Normal saline on the glass slide.
- Heat fix the smear onto the slide by gently passing it over the flame.
- Flood the smear with Albert's Stain A solution and wait for 5 min.
- Remove the dye and flood it with Albert's Stain B for 1 min.
- Wash the smear with tap water / Distilled water.
- Air dry the slide and observe under the microscope at 100X objective.

INTERPRETATION OF THE ALBERT STAINING

Metachromatic / Volutin granules appeared as a bluish-black in the green colored bacterial body arranged at an angle to each other, resembling English letter 'L', 'V' or Chinese letter pattern.



Dissolve the dyes in alcohol and add to the distilled water then add the acetic acid to the thus obtained solution. Allow the stain to stand for one day and then filter. Add Distilled water to make the final volume 100ml.

Hanging Drop Method for Bacterial Motility

Hanging drop preparation is a special type of wet mount (in which a drop of medium containing the organisms is placed on a microscope slide), often is used in dark illumination to observe the motility of bacteria.

In this method, a drop of culture is placed on a coverslip that is encircled with petroleum jelly (or any other sticky material). The coverslip and drop are then inverted over the well of a depression slide. The drop hangs from the coverslip, and the petroleum jelly forms a seal that prevents evaporation. This preparation gives good views of microbial motility.

One characteristic that is useful in helping to identify an unknown organism is whether or not the organism is motile.

Described below are three methods which can be used to determine motility. Organisms to be studied: *Escherichia coli*, *Staphylococcus aureus,* or whatever your instructor assigns.

Note: Be especially careful when handling slides with live culture as this poses a risk of contamination.

OBJECTIVES

- To demonstrate bacterial motility by microscopic and macroscopic techniques.
- To observe flagella in prepared slides stained by specific flagellar stains.

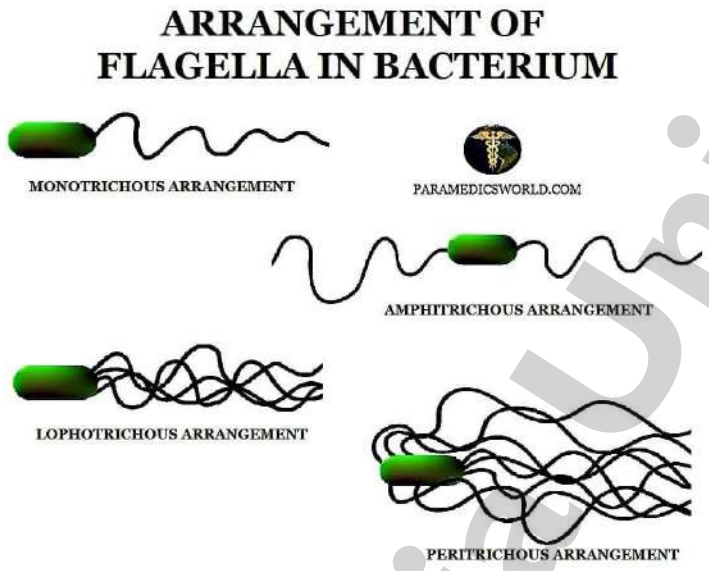
(1) Motile Bacteria:

A bacteria, which has the intrinsic ability of movement in the surrounding medium, in which it remains suspended, is a motile bacteria.

(2) Non-motile Bacteria:

- A bacteria, which does not have the intrinsic ability of movement in the surrounding medium, in which it remains suspended, is a non-motile bacteria. Non-motile bacteria may show apparent motility, resulting from their brownian movement caused by the bombardment of the water molecules in the surrounding medium, on the bacteria cells.

- In wet mount, though the shape and size of bacteria can be observed, motility may be hampered, as the suspension is pressed between the slide and the cover slip. That is why; hanging drop preparation or motility test is performed for clear observation of the motility of bacteria, besides their shape and size. It is useful in the identification of bacteria.



PRINCIPLE OF HANGING DROP PREPARATION

The Hanging drop preparations is a special type of Wet mount in which a drop of broth culture of microorganism or bacterial suspension to be analyzed is placed on a glass cover slip which is encircled with a stick substance, preferably the Petroleum jelly and this cover slip containing the specimen is promptly inverted over the special type of Glass slide known as Cavity slide or depression slide containing a well (depression or cavity) in order that the drop hangs freely on the cover slip in the concavity of slide and the Petroleum jelly forms a seal that prevents the evaporation of the specimen and preserves it temporarily.

The hanging drop preparation is then examined under the microscope to check the motility of the organism, preferably under reduced light to enhance the visibility and for better contrast. This method is ideally used in the laboratory to check the motility of Bacteria.

Materials Required

1. Glass slides (glass slide with depression) or normal glass slide with adhesive or paraffin ring
2. Paraffin wax
3. Loop
4. Coverslip
5. Microscope
6. Bunsen burner
7. Young broth culture of motile bacteria (e.g. *Proteus mirabilis*)

The Wet Mount

Materials: Clean slides, cover slips, nutrient broth cultures, inoculating loop.

Procedure:

- Use the vortex mixer to mix the broth culture.
- Place several loopfuls of a pure bacterial culture on a clean slide and cover with a cover slip.
- Observe under the microscope. Reduce lighting to a minimum for better contrast.
- Begin focusing with the lowest power objective, as always, and work up to the oil immersion objective.
- If the organism is motile, you should see some of the bacteria darting about. In some cases, only a few bacteria will be moving, while the others are still. The organism must still be considered motile.

  Caution: A common mistake is to confuse Brownian motion (or movement) with motility. Brownian movement is a continuous vibrating motion caused by invisible molecules striking the bacteria. If the bacteria are truly motile, their movement will be over greater distances and will be multi-directional, not just back and forth.

  Advantages: This method is the simplest and quickest way to determine motility. It is also useful for determining cellular shape and arrangement which is sometimes destroyed during the staining process.

  Disadvantages: This method is far too risky to use with highly pathogenic organisms.
  Disposal: Slide and coverslip should be placed in the broken glass biohazard box on the biohazard table.

The Hanging Drop Method

1. Clean and flame the hanging drop slide/cavity slide and place it on the table with concavity/depression side up & apply a thin layer of vaseline or Petroleum jelly around the concavity.
2. If you are using a routine microscopic glass slide then apply the paraffin ring or a ring of adhesive tape to make circular concavity on the slide. This step is not needed if a glass slide with depression is available.
3. Now, Clean a coverslip and apply petroleum jelly or vaseline on each of the four corners of the coverslip, using a matchstick.
4. Place the jelly coated coverslip on a clean paper with the petroleum jelly side up.
5. Transfer one loopful of broth culture or Bacterial suspension in the center of the coverslip.

Note: *The drop should not be too large or too small and the size of the drop should be adjusted as per the dimensions and depression of the coverslip and Cavity slide, respectively*.

6. Now, Place the depression slide onto the coverslip, with the cavity facing down so that the depression covers the suspension drop.
7. Press the slide gently to form a seal between the coverslip and the slide to prevent the evaporation of specimen.
8. Lift the preparation and quickly turn the hanging drop preparation coverslip up so that the culture drop is suspended in the concavity of Depression slide.
9. Examine the preparation under low power objective lens, with reduced light and close the diaphragm of the microscope. Focus the edge (appeared as irregular lines crossing the field) of the slide using the coarse adjustment knob.
10. Without moving the microscope tube, switch to high power objective lens and examine the preparation again. Adjust the focus with fine adjustment know until the edge of the drop can be seen.
11. Place a drop of immersion oil on the coverslip and examine the preparation under the oil immersion objective lens (100X).

OBSERVATIONS OF HANGING DROP PREPARATION

Observe under the microscope by Focusing the edge of the drop and carefully find the tiny objects which are bacteria. The bacterial cells will appear as either a dark or slightly greenish tiny bodies, very small rods (bacilli) or spheres (round or cocci).

The cells will appear larger and larger as you increase the power of objective lens but remember to carefully observe the edges of the drop for tiny organisms.

You can also adjust the light of the microscope by using the diaphragm lever to maximize the visibility of the cells & to develop better contrast. Observe the bacterial cells carefully and note down their morphology & grouping and also determine whether the organisms are truly motile or not.

Brownian movements and passive drifting off of all the organisms (motile & non-motile) are usually visible in the Hanging drop slides but on precise and accurate examination of the Hanging drop preparation, you will be able to differentiate the Brownian movement with true motility of bacterial cell.

Brownian movements: *These are the oscillatory movement at a nearly fixed point and possessed by all the small bodies which are suspended in fluid and due to irregularities in their bombardments by molecules of water*.

Observations (Under Oil-immersion Objective):

1. Motility:

Motile or non-motile

2. Shape of bacteria:

Spherical (coccus)

Rod-shaped (bacilli)

Comma-like (vibrio)

Spiral (spirochetes)

3. Arrangement of bacteria:

Pairs (diplobacillus/diplococcus)

In fours (tetrads)

In chains (streptococcus/streptobacillus)

Grape-like clusters (staphylococcus)

Cuboidal (sarcinae or octet).

4. Size of bacteria:

By eye estimation, make drawing of the field under oil-immersion objective.

Advantages: Like the wet mount, the hanging drop method preserves cell shape and arrangement. The Vaseline-sealed

depression also slows down the drying-out process, so the organisms can be observed for longer periods.

Disadvantages: The hanging drop method is also far too risky to use with highly pathogenic organisms.

Disposal: Place depression slide in the plastic beaker labeled "Depression Slides"; these will be autoclaved and reused. Place the coverslips in the broken glass biohazard box on the biohazard table.

Different Culture Media Preparation

Why bacteria have to be grown (cultured) in the laboratory on artificial culture media?

1. One of the most important reasons being its utility in diagnosing infectious diseases. Isolating an organism from sites in body normally known to be sterile is an indication of its role in the disease process. Indeed, isolating an organism from the clinical specimen is the first step in proving its role as an etiologic agent.
2. Culturing bacteria is also the initial step in studying its morphology and its identification.
3. Bacteria have to be cultured in order to obtain antigens from developing serological assays or vaccines.
4. Certain genetic studies and manipulations of the cells also need that bacteria be cultured in vitro.
5. Culturing bacteria also provide a reliable way estimating their numbers (viable count).
6. Culturing on solid media is another convenient way of separating bacteria in mixtures.

Storage and Use Requirements

1. Powders to prepare media are stored in general chemical storage.
2. Lab coats, gloves and goggles are worn during the preparation of media.
3. Use weigh paper or a plastic weigh dish for weighing media powder. Weigh all media in a fume

   hood.

4. Autoclave gloves are worn when handling hot glassware and when using the autoclaves.

General Procedures for Preparing Media Agar Petri Plates

5. Calculate the amount of media that needs to be made.
6. Each plate requires 25 – 30 mL of agar.
7. If 100 plates are needed, 2500-3000 mL of agar is needed.
   o Always add 200mL to the amount required in case of spills or miscalculation.
8. Follow package instructions for preparation.
9. Instructions are typically written for 1L (1000mL) of media. If less is desired calculate the amount needed as shown:

For example: If the instructions state 23g for 1L and 600mL is desired, use a ratio to calculate the amount needed (in this example 13.8 g is needed for preparing 600mL):

- 23g/1000mL = Xg/600mL
- 23 * 600= 1000X
- 13800 = 1000X
- 13800/1000 =X
- 13.8g=X

1. Always prepare media in a beaker with 1/3 of empty space. (i.e. prepare 600mL of media in a 1000mL beaker). If the amount of media to be prepared is greater than 1L, prepare it in 500mL aliquots or use a 2000mL beaker. Label the beaker with autoclave tape and state what media is being prepared, the date, and your initials (i.e. Nutrient Agar 8-18-08 KH).
2. Add powder to beaker first, and then fill with necessary amount of water.
3. Stir with a glass stirring rod to mix.
4. Place in microwave and heat at 3-5 minute intervals.
5. Stir between intervals, using caution and allowing the media to sit for 30 seconds in the microwave before stirring.
6. Heat for approximately 10 minutes or until boiling has been achieved.
7. Test the pH of the media to insure that it is within the acceptable range as stated on the package. If the pH needs to be adjusted, add drops of 1N Hydrochloric Acid (HCl) (to make more acidic) or 1N Sodium Hydroxide (NaOH) (to make more basic) as necessary until desired pH is achieved.

8. Cover the beaker with foil and secure with autoclave tape.
9. Autoclave for 20 minutes. Refer to the Standard Operating Procedure for Autoclave Use.
10. While the media is in the autoclave, arrange Petri plates on the counter top.
    Once sterilization is complete, open the autoclave and remove the beaker of media. You must wear the autoclave gloves when removing anything from the autoclave.
11. Allow media to cool slightly, but not for longer than 10 minutes as the agar may solidify.
12. Poke a small hole in the foil covering the top in order to pour the agar.
13. Pour approximately 10-15mL of agar into the plates. The bottom of the plate needs to be covered. If necessary, swirl the plate slightly in order to evenly disperse agar.
14. Allow plates to cool on the countertop overnight to reduce condensation.
15. When cooled, store upside-down in plastic bags in the refrigerator to prevent the agar from drying out.

- ♣ Be sure to seal the plastic bag with masking tape.
- ♣ Be sure the bag is labelled with its contents, the date it was prepared, and your initials (i.e. Nutrient Agar 8-18-08 KH).

Preparation of media and cultures

Culture media

The method for the preparation of basic microbiology media is given below. In situations where preparation is uneconomic in time, prepared, sterilized media (liquid and solid) are available from the major school science equipment suppliers. Sterilization is at 121 °C (15 lb in $^{-2}$) for 15 minutes. pH values are 7.0 unless stated otherwise.

Note: Allow 15 cm^3 of agar for each Petri dish and 5-10 cm^3 of broth for each McCartney bottle. All cotton wool plugs should be made of non-absorbent cotton wool. Plastic or metal caps may also be used.

Nutrient agar

Suspend 28 g of nutrient agar powder in 1 litre of distilled water. Bring to the boil to dissolve completely. Dispense as required and sterilize.

Nutrient broth

Add 13 g of nutrient broth powder to 1 litre of distilled water. Mix well. Dispense as required and sterilize.

Malt extract agar

Suspend 18g agar powder in 1 litre of distilled water. Bring to the boil to dissolve completely. Add 15g malt extract per litre. Mix well. Dispense as required and sterilize.

Mannitol yeast extract agar

Suspend 10 g agar in 1 litre of distilled water. Heat to dissolve. Add 0.5 g K_2HPO_4, 0.2g $MgSO_4.7H_2O$, 0.2 g NaCl, 0.2 g $CaCl_2.6H_2O$, 10 g mannitol and 0.4 g yeast extract. Dispense as required and sterilize.

Mannitol yeast extract broth

As above, without agar.

Glucose nutrient broth

Make up nutrient broth as already directed and add 10 g per litre of glucose.

Sugar peptone water

Add 10 g of peptone, 5 g of NaCl, 5 g of sugar and 20 cm^3 of Universal indicator to 1 litre of distilled water; pH should be 7.4. Dispense as required and sterilize.

Tributyrin agar

Supplied ready for use. Heat to melt and dispense aseptically. May be prepared by adding 1% tributyrin to nutrient agar.

Glucose yeast extract broth

Add 10 g of peptone, 5 g of NaCl, 3 g of yeast extract to 1 litre of distilled water. Dispense as required and sterilize.

Glucose yeast extract lemco broth

Add 10 g of Lemco (meat extract) to glucose yeast extract broth.

Milk agar

Make up nutrient agar as above but using only 900 cm^3 of distilled water. Dissolve 20 g of dried skimmed milk in 100 cm^3 of distilled water. Sterilize separately. Transfer the milk to the agar aseptically after cooling to 45-50 °C. Dispense aseptically.

Starch agar

Suspend 15 g of nutrient agar in 100 cm^3 distilled water. Bring to the boil to dissolve completely. Heat 40 g of soluble starch in 100 cm^3 of distilled water to form a suspension. Allow to cool and then mix with the nutrient agar solution. Dispense and sterilize.

Iodine solution

Dissolve 1 g of iodine crystals and 2 g of potassium iodine in 300 cm^3 of distilled water.

Cellulose broth (for *Trichoderma reesei*)

- 800 cm^3 distilled water
- 0.1 g $CaCl_2$
- 0.5 g $(NH_4)_2SO_4$
- 0.5 g yeast extract powder
- 0.5 g asparagine
- 10 g carboxymethylcellulose
- 1.0 g KH_2PO_4
- pH6.2

Mix ingredients, heat gently, and stir until dissolved.

Principle:

Using Cultural techniques in microbiology helps demonstrate the presence of organisms which may be causing disease, and when indicated, to test the susceptibility of pathogens to antimicrobial agents. If pathogens are to be isolated successfully, standard operating procedures are needed which detail for each culture medium used in the laboratory, its purpose, from where it can be obtained, its preparation, and how it is sterilized, dispensed, labelled, stored and performance tested.

Procedures for various media

(1) Blood Agar

Purpose: Blood agar is blood agar base II enriched with 5-10 % sheep blood. Blood agar is used to isolate organisms with exacting growth requirements, including streptococci. It is appropriate for sputum, throat swabs, urine, urogenital swabs, pus aspirates, eye and ear swabs, wound swabs, aspirates (for aerobes and anaerobes), and some fungi. It is appropriate for culturing almost all specimens for bacteriology.

Procedure for preparation:

i) Suspend 40.0g in 1 litre of distilled water.

ii) Heat until completely dissolved.

iii) Autoclave at 121^0C for 15 minutes.

iv) Cool to 45-50^0C.

v) Aseptically add 50 ml of defibrinated sheep blood.

vi) Pour 15-20 ml of the ready media on to petri dishes.

vii) Leave standing for thirty minutes to solidify.

viii) Perform sterility testing as described in Section 7.

ix) Label the bottom of each plate with date of preparation and batch number.

x) Store the culture media plates upside down at 2-8^{0}C sealed in plastic bags to reduce chances of contamination.

xi) Test Samples for performance, using stable, typical control cultures.

- pH of the medium 7.2-7.6 at room temperature depending on the agar base used.
- Shelf life: up to *sixteen weeks* provided there is no change in the appearance of the medium to suggest contamination, haemolysis, or deteroriation.

(2) Chocolate (Heated Blood) Agar

Purpose: Chocolate agar supplies the factors required for the proper growth of *Haemophilus influenzae*, the X and V factors. It is also used to culture nutritionally demanding pathogens such *Neisseria meningitidis* and *Streptococcus pneumoniae*. When Blood agar is heated, the red cells are lysed and the medium becomes brown in colour; it is referred to as chocolate agar. It is appropriate for isolating pathogenic bacteria in sputum, throat swabs, eye swabs, ear swabs, urogenital swabs, cerebrospinal fluid.

Procedure for preparation:

i) Prepare as described for Blood agar except after adding blood, heat the medium in a 70^{0}C water bath until it becomes brown in colour. This takes about 10-15 minutes during which time the medium should be mixed gently several times.

ii) Allow the medium to cool to about 45^{0}C, remix and dispense in sterile petri dishes as described for blood agar.

iii) Leave standing for thirty minutes to solidify.

iv) Perform sterility testing as described in Section 7.

v) Label the bottom of each plate with date of preparation and batch number.

vi) Store the culture media plates upside down at 2-8^{0}C sealed in plastic bags to reduce chances of contamination.

vii) Test Samples for performance, using stable, typical control cultures.

- pH of the medium 7.2-7.6 at room temperature depending on the agar base used.
- Shelf life: up to *sixteen weeks* provided there is no change in the appearance of the medium to suggest contamination or deteroriation.

Important: Care must be taken not to over heat or prolong the heating of the medium because this will cause it to become granular and unfit for use.

(3) Mac Conkey Agar

Purpose: Mac Conkey agar is a differential medium to distinguish lactose fermenting gram negative bacteria from non lactose fermentors; lactose fermentors are coloured pink, while non lactose fermentors are pale. It is appropriate for stool, urine, urogenital specimens, throat swabs, ear and eye swabs, wound swabs, sputum, aspirates, cerebrospinal fluid.

Procedure for preparation:

i) Suspend 51.1g of powder in 1 litre of distilled or deionized water.

ii) Heat until completely dissolved.

iii) Sterilize in autoclave at 121^{0}C for 15 minutes.

iv) Cool to 45-50^{0}C.

v) Pour 15-20 ml of the ready media on to petri dishes.

vi) Leave standing for thirty minutes to solidify.

vii) Perform sterility testing as described in Section 7.

viii) Label the bottom of each plate with date of preparation and batch number.

ix) Store the culture media plates upside down at 2-8^{0}C sealed in plastic bags to reduce chances of contamination.

x) Test Samples for performance, using stable, typical control cultures.

- Shelf life: up to *sixteen weeks* provided there is no change in the appearance of the medium to suggest contamination or deteroriation.

(4) Sorbitol MacConkey Agar

Purpose: This MacConkey medium contains the carbohydrate sorbitol instead of lactose. *E.coli 0157* produces colourless colonies on the medium because it does not ferment sorbitol. Most other *E.coli* strains and other enterobacteria ferment sorbitol, producing pink colonies. Sorbitol

MacConkey agar is therefore a useful way of screening for *E.coli 0157* (specificity 85%, sensitivity 100%).

Procedure for preparation:

This medium is best prepared from ready to use dehydrated powder.

(i) Use at a concentration of 5.15 g in every 100ml distilled water. Concentration may vary depending on manufacturer.

(ii) Prepare, sterilize, dispense and store the medium as described above for MacConkey agar.

- Shelf life: up to *sixteen weeks* provided there is no change in the appearance of the medium to suggest contamination or deteroriation.
- pH of the medium 7.2-7.6 at room temperature.

(5) S.S Agar (Modified)

Purpose: A selective medium for the isolation of *Salmonellae* and *Shigellae* from faeces, foodstuffs and other materials. Brilliant green, ox bile and high concentrations of thiosulfate and citrate largely inhibit the accompanying microbial flora. Sulfide production is detected by using thiosulfate and iron ions, the colonies turn black. The presence of coliform bacteria is established by detecting degradation of lactose to acid with the pH indicator neutral red. Non-lactose fermenting colonies are colourless / pale, lactose fermenting colonies are pink to red. Colonies of microorganisms (*Salmonellae* and *Shigellae*) producing hydrogen sulfide have a black centre.

Procedure for preparation:

i) Suspend 52.0g of powder in 1 litre of distilled or deionized water.

ii) Mix well.

iii) Heat to boiling for 1 minute to completely dissolve the powder. DO NOT AUTOCLAVE and avoid over heating.

iv) Cool to 45-50^{0}C and dispense into petri dishes.

v) Pour 15-20 ml of the ready media on to petri dishes.

vi) Leave standing for thirty minutes to solidify.

vii) Perform sterility testing as described in Section 7.

viii) Label the bottom of each plate with date of preparation and batch number.

ix) Store the culture media plates upside down at 2-8^{0}C sealed in plastic bags to reduce chances of contamination.

x) Test Samples for performance, using stable, typical control cultures.

- Shelf life: up to *sixteen weeks* provided there is no change in the appearance of the medium to suggest contamination or deteroriation.
- pH of the culture medium is 5.0- 6.0 at room temperature.

(6) Mueller Hinton II Agar

Purpose: It is the medium of choice for antibiotics sensitivity testing. Fastidious bacteria like Streptococci do not grow well on Mueller Hinton.

Procedure for preparation:

i) Suspend 38g of the powder in 1 litre of purified water.

ii) Mix thoroughly.

iii) Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121^{0}C for 15 minutes.

iv) Do not over heat. Cool to 45-50^{0}C and dispense into petri dishes.

v) Pour 15-20 ml of the ready media on to petri dishes.

vi) Leave standing for thirty minutes to solidify.

vii) Perform sterility testing as described in Section 7.

viii) Label the bottom of each plate with date of preparation and batch number.

ix) Store the culture media plates upside down at 2-8^{0}C sealed in plastic bags to reduce chances of contamination.

x) Test Samples for performance, using stable, typical control cultures.

- Shelf life: up to *sixteen weeks* provided there is no change in the appearance of the medium to suggest contamination or deteroriation.
- pH of the medium 7.2-7.6 at room temperature.

(7) CLED Agar (Cystine-Lactose-Electrolyte-Deficient Agar)

Purpose: Used for isolating a wide variety of Gram positive and Gram negative bacteria from urine. In 1960, Sandys reported on the development of a new method of preventing the swarming of *Proteus* on solid media by restricting the electrolytes in the culture medium, to enable colony counts of *Proteus*. Lactose and sucrose in the medium was substituted for mannitol and the

concentration of bromothymol blue indicator in the agar increased. Mackey and Sandys further modified the medium by incorporating cystine in order to enhance the growth of cyctine-dependent "dwarf colony" coliforms and by deletion of sucrose. Hence the name Cystine-Lactose-Electrolyte-Deficient Agar, and was found to be ideal for urinary bacteriology and for colony enumeration.

Procedure for preparation (CLED):

i) Suspend 36g of the powder in 1 litre of purified water.

ii) Mix thoroughly.

iii) Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121^{0}C for 15 minutes.

iv) Cool to 45-50^{0}C and dispense into petri dishes.

v) Pour 15-20 ml of the ready media on to petri dishes.

vi) Leave standing for thirty minutes to solidify.

vii) Perform sterility testing as described in Section 7.

viii) Label the bottom of each plate with date of preparation and batch number.

ix) Store the culture media plates upside down at 2-8^{0}C sealed in plastic bags to reduce chances of contamination.

x) Test Samples of the finished product for performance, using stable, typical control cultures.

- Shelf life: up to *sixteen weeks* provided there is no change in the appearance of the medium to suggest contamination or deteroriation.
- pH of the medium is between 7.3 – 7.5 at room temperature.

(8) Brain Heart Infusion Broth (BHI)

Purpose: Brain Heart Infusion is a general-purpose liquid medium used in the cultivation of fastidious and non-fastidious microorganisms, including aerobic and anaerobic bacteria, from a variety of clinical and non-clinical specimens. It serves as a base for supplemented media containing 0.1% agar, Fildes enrichment or 6.5 % sodium chloride. A supplemented pre-reduced formulation in tubes is especially recommended for the cultivation of anaerobes. Rosenow (1919, Journal of Dental Research 1:205) described brain-heart infusion broth prepared by adding pieces of brain tissue to meat infusion or beef extract-dextrose broth. It is appropriate for blood culture and culturing specimens likely to contain anaerobes.

Procedure for preparation (BHI Broth):

i) Suspend 37.0g in 1 litre of distilled water.

ii) Heat if necessary to dissolve the medium completely.

iii) Dispense into bottles or tubes as desired. Sterilize by autoclaving at 15 Ibs (121^{0}C) for 15 minutes.

iv) Cool to 45-50^{0}C.

v) Dispense into 20ml universal tubes.

vi) Perform sterility testing as described in Section 7.

vii) Label the side of each tube with date of preparation and batch number.

viii) Store the culture medium at 2-8^{0}C sealed in plastic bags to reduce chances of contamination.

ix) Test Samples of the finished product for performance, using stable, typical control cultures.

- Shelf life: up to *eighteen months* provided there is no change in the appearance of the medium to suggest contamination or deteroriation.
- pH of the medium 7.2-7.6 at room temperature.

(9) Nutrient agar

Purpose: Nutrient agar is used for the cultivation of a wide variety of non-fastidious bacteria. It was originally developed in recognition of the need for a standardized medium for use in the examination of water and waste water, dairy products and various foods. Currently it is used as a maintenance medium for *Staphylococcus aureus, Proteus mirabilis,* and *Escherichia coli*. Tube slants are used primarily for the cultivation and maintenance of pure cultures.

Procedure for preparation:

(i) Suspend 23 g of the powder in 1 litre of purified water.

(ii) Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.

(iii) Autoclave at 121˚C for 15 minutes.

(iv) Cool to 45-50^{0}C.

(v) Pour 15-20 ml of the ready media into sterile 20ml glass universal tubes.

(vi) Leave standing for thirty minutes to solidify, resting the tubes leaning at 30˚- 60˚ to produce the slope effects in the tubes..

(vii) Perform sterility testing as described in Section 7.

(viii) Label the side of each tube with date of preparation and batch number.

(ix) Store the tubes at 2-8^{0}C sealed in plastic bags to reduce chances of contamination.

(x) Test Samples of the finished product for performance, using stable, typical control cultures.

- Shelf life: up to *eighteen months* provided there is no change in the appearance of the medium to suggest contamination or deteroriation.
- pH of the medium 7.2-7.6 at room temperature.

(10) Nutrient Broth

Purpose: Maintenance medium and enrichment medium for non fastidious bacteria.

Procedure for preparation:

Prepare from ready to use dehydrated nutrient broth powder. The contents of nutrient broth are the same as those for nutrient agar except that the agar is omitted. Its preparation and storage are the same as described previously for nutrient agar.

- Shelf life: up to *twelve months* provided there is no change in the appearance of the medium to suggest contamination or deteroriation.

(11) Semi-solid nutrient agar

Purpose: Maintenance medium for non fastidious bacteria.

Procedure for preparation:

(To make about 20 bottles)

(i) Mix 0.75g nutrient agar and 1.3g nutrient broth in 100 ml distilled water, and heat to 100˚C to dissolve the ingredients (place the flask in a boiling water bath).

(ii) Dispense the medium in 5-7 ml amounts in screw-cap bottles. Sterilize by autoclaving (with caps loosened) at 121˚C for 15 minutes. When cool, tighten the bottle caps.

(iii) Perform sterility testing as described in Section 7.

(iv) Date the medium and give it a batch number.

(v) Sore at 2-8 ˚C.

- Shelf life: up to *twelve months* provided there is no change in the appearance of the medium to suggest contamination or deteroriation.
- pH of the medium is 7.2-7.6 at room temperature.

(11) (EMB) Eosin-methylene blue agar

Purpose: EMB agar contains sucrose and lactose, utilized as fermentable carbohydrates substrates, which encourage the growth of some gram-negative bacteria, especially fecal and non-fecal coliforms. Differentiation of enteric bacteria is possible due to the presence of the sugars lactose and sucrose in the EMB agar and the ability of certain bacteria to ferment the lactose in the medium.

- Lactose-fermenting gram-negative bacteria acidify the medium, which reduces the pH, and the dye produces a dark purple complex usually associated with a green metallic sheen. This metallic green sheen is an indicator of vigorous lactose and/or sucrose fermentation ability typical of fecal coliforms.
- Organisms that are slow lactose-fermenters, produce less acid, and the colonies appear brown-pink.
- Non-lactose fermenters, increase the pH of the medium by deamination of proteins and produce colorless or light pink colonies.

Eosin Y and methylene blue are pH indicator dyes that combine to form a dark purple precipitate at low pH; they also serve to inhibit the growth of most Gram-positive organisms. Peptic digest of animal tissue serves as a source of carbon, nitrogen, and other essential growth nutrients. Phosphate buffers the medium.

Procedure:

1. Weigh and suspend 35.96 grams of dehydrated media in 1000 ml distilled water.
2. Mix until the suspension is uniform and heat to boiling to dissolve the medium completely.
3. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.
4. Cool to 45-50°C, and with frequent gentle swirling, pour the media into sterile Petri plates. *Note: frequent swirling is recommended to restore the blue color o methylene blue and to suspend the flocculent precipitate if any*.
5. Label with initials of the name of the medium, date of preparation, and store the plates upside down (lids below) in the refrigerator until use.

Culture techniques

Planning ahead is essential when embarking on practical microbiology investigations. There are five areas for consideration.

1. Incubation of cultures and sampling during growth.
2. Preparation and sterilisation of equipment and culture media.
3. Sterilisation and safe disposal of all cultures and decontamination of all contaminated equipment.
4. 4.Preparation of microbial cultures as stock culture for future investigations and inoculum for the current investigation.
5. Inoculation of the media with the prepared culture.
6. Incubation of cultures and sampling during growth.
7. Sterilisation and safe disposal of all cultures and decontamination of all contaminated equipment.

Preparation of culture media

Rehydrate tablets or powder according to manufacturer's instructions. Before sterilisation, ensure ingredients are completely dissolved, using heat if necessary. Avoid wastage by preparing only sufficient for either immediate use (allowing extra for mistakes) or use in the near future. Normally allow 15–20 cm^3 medium per Petri dish. Dispense in volumes appropriate for sterilisation in the autoclave/pressure cooker. Agar slopes are prepared in test tubes or Universal/McCartney bottles by allowing sterile molten cooled medium to solidify in a sloped position. Bottles of complete, sterile media are available from suppliers but are expensive.

Pouring a plate

1. Collect one bottle of sterile molten agar from the water bath.
2. Hold the bottle in the right hand; remove the cap with the little finger of the left hand.
3. Flame the neck of the bottle.

4. Lift the lid of the Petri dish slightly with the left hand and pour the sterile molten agar into the Petri dish and replace the lid.
5. Flame the neck of the bottle and replace the cap.
6. Gently rotate the dish to ensure that the medium covers the plate evenly.

7. Allow the plate to solidify.

The base of the plate must be covered, agar must not touch the lid of the plate and the surface must be smooth with no bubbles.

The plates should be used as soon as possible after pouring. If they are not going to be used straight away they need to be stored inside sealed plastic bags to prevent the agar from drying out.

Aseptic Transfer and Inoculation Techniques

It is important when transferring or moving bacteria from one location (or growth medium) to another to use **aseptic technique**. **Aseptic** means without contamination (**'a' = without** and **'septic' = contamination.** By using **aseptic technique**, we will (hopefully) grow only the bacteria we are studying, without contamination by other living organisms.

Inoculation is transfer of a bacterial sample onto a growth media for the purpose of growing the sample. Of course, you must use aseptic technique to avoid contamination.

Tools used for inoculation must be **sterile**. That is, there is nothing growing or alive on the tools. Tools that will be used in lab include metal "needles" and loops, cotton swabs, and wooden dowels.

- Metal and glass tools must be sterilized for 20-30 seconds with the micro-incinerator, prior to use.
- Swabs and wooden dowels can be used once and then must be disposed of in the sharps container.

Procedures

A. Streak Isolation Technique: Isolating Individual Microbe Colonies

Introduction

One of the most important techniques in microbiology is the Streak Isolation Technique. This procedure allows ***isolation*** of single bacterial **cells** that will then grow in to single **colonies**. This

technique can be used so colony morphology can be studied, and it can also allow separation of species from a mixed culture so that the different species can be identified.

To perform this technique, we drag the microbe sample (that contains billions of cells) across an agar surface (usually a Petri dish) in a sequential pattern that reduces the number of cells and allows isolation of individual cells. We won't be able see these single cells when performing the technique, but we will see the ***colonies*** that grow from those isolated cells (that underwent binary fission—See Figure 3-1) on the Petri dish after incubation.

Materials (Per student) for Streak Isolation

- Inoculating metal loops
- Test tube racks to hold tools
- Micro-incinerators
- Black Sharpie-style markers
- Appropriate personal protective gear (lab coats, gloves, face shield, hair ties)
- Medium
    - One Chocolate agar Petri plate
- Bacterial cultures
    - *Pseudomonas aeruginosa* (grown in a T-soy broth)
    - *Staphylococcus aureus* (grown on a T-soy plate)

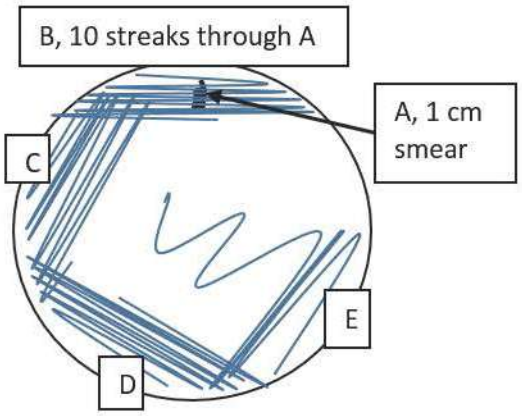
Streak Isolation methods

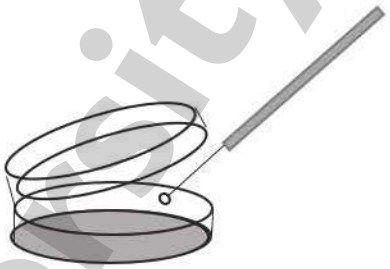
Choose ONE of the two methods described below and follow it carefully to perform a Streak Isolation.

a. Streak Isolation from a broth

1. Use ***Pseudomonas aeruginosa***.
2. Label the bottom of your Petri plate with your name, the date, the species and the type of media.
3. Sterilize your loop using the micro-incinerator or Bunsen burner. Allow it to cool for 15-20 seconds.
4. Stir the broth sample with your sterile cooled loop to mix the organism throughout the liquid.

5. Tap your loop on the inside edge of the upper part of the test tube to decrease the amount of liquid on the loop. YOU DO NOT WANT MUCH AT ALL!!!
6. Clam shell your Petri dish. Smear your loop in a vertical 1 cm smear on your plate. ("A" on Figure 3-4).
7. **Sterilize your loop using the micro-incinerator to get rid of the bacteria—we are trying to get isolation.** Allow it to cool for 15-20 seconds
8. Clam shell your Petri dish. Using the edge of the newly sterilized and cooled loop, gently streak through your smear "A", 10 times, from the outer edge of the plate inwards, as shown on the diagram. This is streak "B".
9. **Sterilize your loop using the micro-incinerator to get rid of the bacteria—we are trying to get isolation.** Allow it to cool for 15-20 seconds
10. Using the edge of the cooled loop, gently streak through your smear "B", 10 times, from the outer edge of the plate inwards. This is streak "C".
11. **Sterilize your loop using the micro-incinerator to get rid of the bacteria—we are trying to get isolation.** Allow it to cool for 15 seconds
12. Using the edge of the loop, gently streak through your smear "C", 10 times, from the outer edge of the plate inwards. This is streak "D".
13. **Sterilize your loop using the micro-incinerator to get rid of the bacteria—we are trying to get isolation.** Allow it to cool for 15-20 seconds
14. Streak through "D" and into the middle on the plate WITHOUT touching streak "A"
15. Reflame loop.
16. Place your inoculated Petri plate upside down (see Fig. 3-5) in a Petri plate rack for incubation.





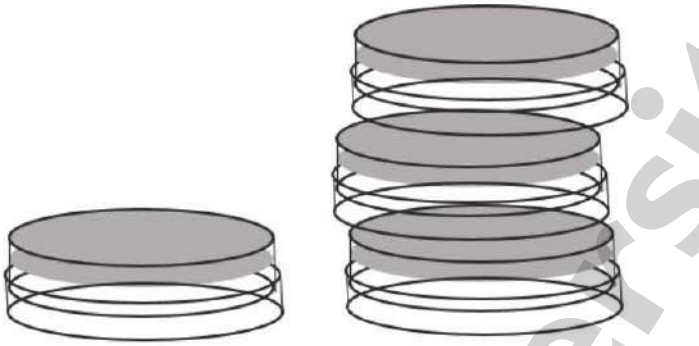
b. Streak Isolation from a plate (Petri dish)

1. Use ***Staphylococcus aureus.***
2. Label your plate with your name, the date, the species and the type of media.
3. Using a sterile, cooled loop, obtain **1-2 colonies** from the source bacteria grown on the Petri plate (that means **NOT VERY MUCH**) of identical morphology (shape) using just the **tip** of your loop. **USE A VERY SMALL AMOUNT!! THE SIZE OF A SMALL FRECKLE**! Smear those colonies in a vertical 1 cm smear on your plate with the tip of your loop ("A" on Figure 3-4).
4. Follow steps 6-16 above.

Precautions

- Make sure to cool the metal inoculation loop before transferring bacteria to the Petri plate for the first streak (A), otherwise you will kill your bacteria.
- Use VERY SMALL amounts of bacteria when sampling from a plate. A sample that is 1mm X 1mm has billions of bacteria.
- Make sure to sterilize the metal loop after the original (A) streak and each subsequent streak. The idea is to REDUCE the number of cells each streak.
- Don't gouge the surface of the agar.

Figure 3-5. Proper incubation for Petri dishes: AGAR SIDE UP. Agar side up allows you to read the label and reduces condensation in the plate.



Single plate, agar side up Stack of plates, agar side up

A. Inoculating from a Slant (solid) into a Broth (liquid)

From the slant with the *E. coli* →	using a sterilized loop →	to <u>your</u> un-inoculated, properly labelled, broth
E. coli is represented by the black squiggle on the surface of the slant. 	GENTLY draw the sterile loop over the *E. coli* on the surface of the slant to pick up a very small amount of bacteria. 	Then, put the loop with the *E. coli* into the broth and swirl gently. 

Method for inoculating from a slant to a broth

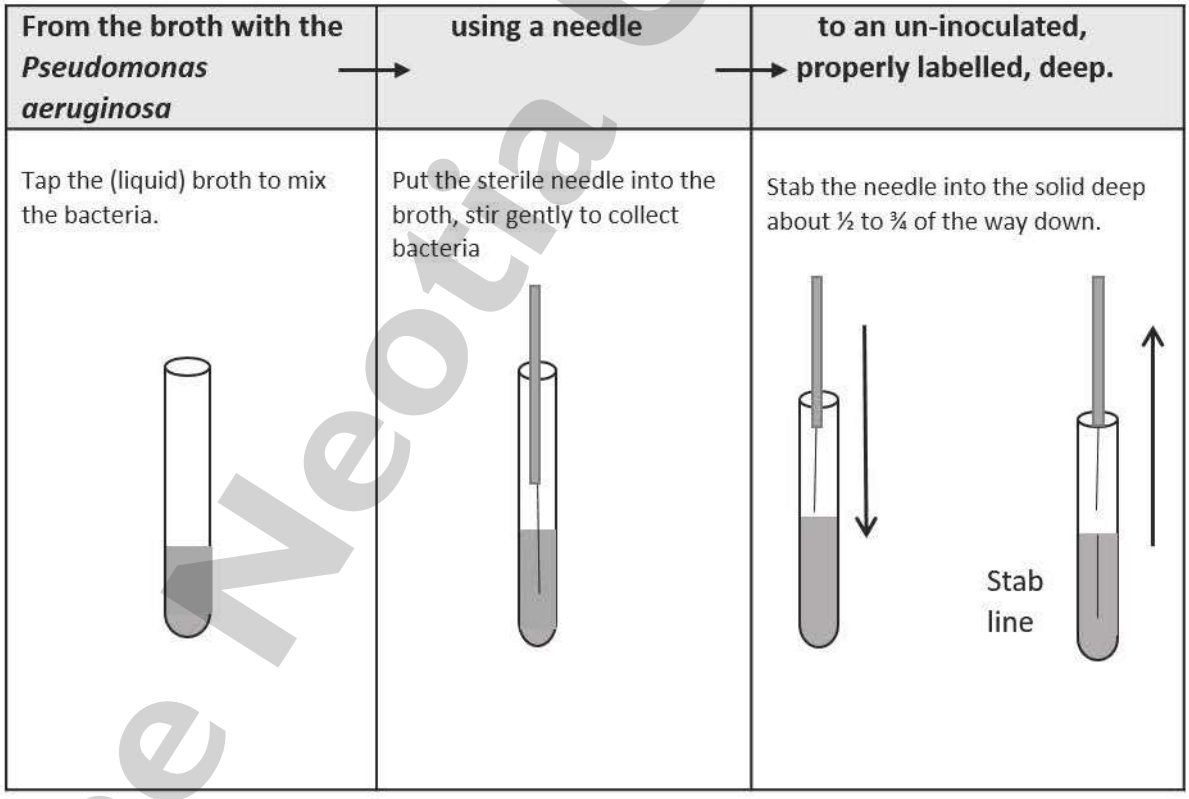
1. Properly label your uninoculated broth medium.
2. Sterilize your loop and store in the test tube rack.
3. Pick up your un-inoculated broth and your *E. coli* slant.
4. Unscrew the lids and place them with the open end facing down, on a clean Kimwipe to prevent bacteria from landing on the inside. DO NOT MIX THEM UP.
5. Pick up your cooled, sterilized loop.
6. The bacteria will resemble a whitish slime on the slanted surface of the agar.

GENTLY draw the loop over the surface of the slant to pick up a very small amount of bacteria- the area of a small freckle (about 1mm X 1mm) of the *E.coli*. A sample of this size will contain millions of bacteria cells. Do NOT dig into the agar.

7. Put the loop with the *E. coli* in the broth and gently swirl the loop. Remove the loop but keep holding it.
8. Put the lid back on the *E. coli* slant.
9. Put the lid back on your newly inoculated T-soy broth.
10. Leave the lid a bit loose on your broth so that the bacteria have access to oxygen.
11. Re-sterilize your metal tool and return to the canister.
12. Place your newly inoculated T-soy broth in the test tube rack designated for incubation.

Inoculating from a Broth (liquid) to a Deep (Semi-solid)

Figure 3-8: Inoculating from a Broth to a Deep.



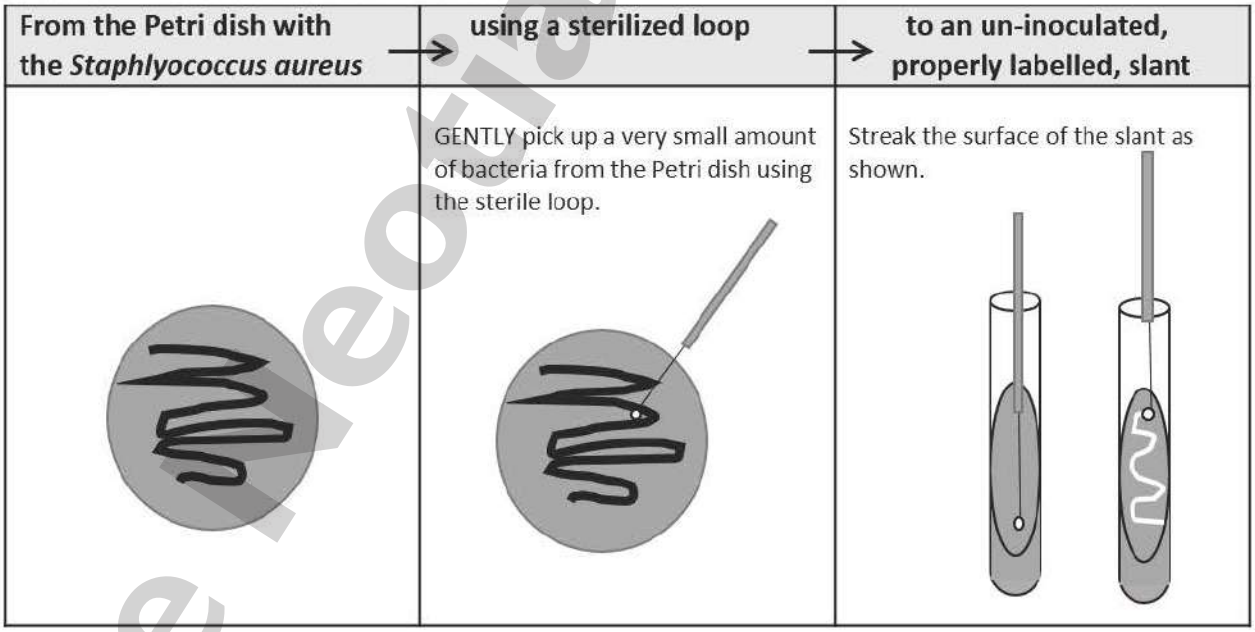
Method for inoculating from a broth to a deep

1. Label your medium.
2. Incinerate your needle and store in the test tube rack.

3. Pick up your un-inoculated deep and your *Pseudomonas aeruginosa* broth.
4. Unscrew the lids and place them with the open end facing down, on a clean kimwipe. DO NOT MIX THEM UP.
5. Pick up your cooled, sterilized needle.
6. Put the needle into the *Pseudomonas aeruginosa* broth.
7. Remove the needle from the broth and stab the needle into your solid un-inoculated deep about ½ to ¾ of the way down.
8. Remove the needle but keep holding it.
9. Put the lid back on the *Pseudomonas aeruginosa* broth.
10. Put the lid back on your newly inoculated T-soy deep.
11. Leave the lid loose on your deep so that the bacteria have access to oxygen.
12. Re-sterilize your metal tool and return to the canister.
13. Place your newly inoculated T-soy deep in the test tube rack for incubation.

Inoculating from a Petri Dish (solid) to a Slant (solid)

Figure 3-9. Inoculation from a petri dish to a slant



Method for inoculating from a Petri dish to a slant

1. Label your medium.
2. Incinerate your loop and store in the test tube rack.

3. Unscrew the lid of your un-inoculated slant and place it with the open end facing down on a clean Kimwipe.
4. Pick up your cooled, sterilized loop.
5. **Clam-shell** the lid of your *Staphlyococcus aureus* containing Petri dish and pick up only a very small amount of bacteria (about 1mm X 1mm). A sample of this size will contain millions of bacteria cells. Do NOT dig into the agar.
6. Replace the lid of the Petri dish.
7. Streak the surface of the slant as shown in Figure 3-12. Do NOT dig into the medium.
8. Remove the loop but keep holding it.
9. Put the lid back on your newly inoculated T-soy slant.
10. Leave the lid loose on your slant so that the bacteria have access to oxygen.
11. Re-sterilize your metal tool and return to the canister.
12. Place your newly inoculated T-soy slant in the test tube rack for incubation.

Biochemical reactions-Sugar fermentation test

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.

Glycolysis

Carbohydrates + Bacteria Aldehyde or acid + CO_2 + H_2

pH indicator (Colour Change)

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).

Carbohydrate Fermentation Test

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

Biochemical reactions- Oxidation-Fermentation test

The oxidative-fermentative (OF) test was developed by Hugh and Leifson in 1953. They developed OF media to differentiate between oxidative bacteria (that produces acid from carbohydrates under aerobic condition only) and fermentative bacteria (that produces acid both under aerobic and anaerobic conditions).

Saccharolytic microorganisms degrade glucose either fermentatively or oxidatively. The end products of fermentation are relatively strong mixed acids that can be detected in a conventional fermentation test medium. However, the acids formed in oxidative degradation of glucose are extremely weak and less, and the more sensitive oxidation fermentation medium of Hugh and Leifson's OF medium is required for the detection. The medium was made by increasing the amount of glucose above that found in the medium used to detect fermentation and by decreasing the amount of peptone.

The OF medium of **Hugh and Leifson** differs carbohydrate fermentation media as follows:

- The concentration of agar is decreased to 2% from 3%, making it semisolid in consistency *(This assists in the **determination the motility of the organism**).*
- The concentration of peptone is decreased from 11% to **2%**. (decreasing the amount of alkaline product produced by the metabolism of peptone; thus reducing the neutralizing effect of these products).
- Carbohydrate concentration is increased by 0.5% to **1.0%** (The increased concentration of glucose in the medium enhances the production of these weak acids to a level that can be detected by bromthymol blue indicator.)

Objective of OF Test

To differentiate microorganisms based on the ability to oxidize or ferment specific carbohydrates.

Principle

The oxidative-fermentative test determines if certain gram-negative rods metabolize glucose by fermentation or aerobic respiration (oxidatively). During the anaerobic process of fermentation, pyruvate is converted to a variety of mixed acids depending on the type of fermentation. The high concentration of acid produced during fermentation will **turn the bromthymol blue indicator in OF media from green to yellow** in the presence or absence of oxygen .

Certain nonfermenting gram-negative bacteria metabolize glucose using aerobic respiration and therefore only produce a small amount of weak acids during glycolysis and Krebs cycle. The decrease amount of peptone and increase amount of glucose facilitates the detection of weak acids thus produced. Dipotassium phosphate buffer is added to further promote acid detection.

Media Used in OF Test

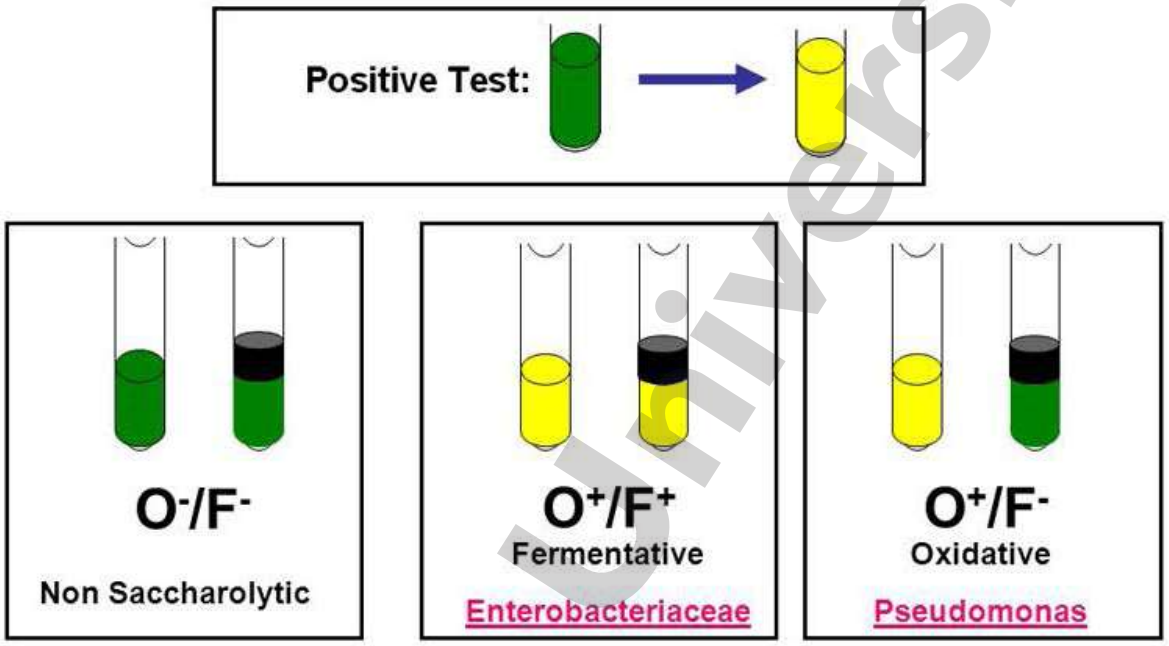
Hugh and Leifson's medium: Peptone 2.0gm/L, Sodium chloride 5.0gm/L, Dipotassium phosphate 0.30gm/L, Glucose (Dextrose) 10.0gm/L, Bromothymol blue 0.030gm/L, Agar 3.0gm/L, Final pH (at 25°C) 7.1±0.2

Procedure of OF Test

1. Inoculate two tubes of OF medium with organism by stabbing with a straight wire.
2. Pour liquid paraffin over the medium to form a layer about one cm in depth into one of the tubes.

3. Incubate the tubes at 35-37°C for 24-48 hours.
4. Examine both open and closed tubes for the color change.

Result Interpretation of OF Test



Oxidative: yellow colouration in open tube only

Fermentative: yellow colouration on both open and closed tubes

Limitations of OF Test

- OF medium is general purpose medium and may not support the growth of fastidious organisms.
- Slow-growing organisms may not produce results for several days.

Biochemical reactions urease test

Urease Test Definition

Urease test is a biochemical test that detects the alkaline fermentation of urine (urea) with the resultant production of ammonia by microorganisms.

- The fermentation of urea occurs in the presence of the enzyme 'urease', resulting in two molecules of ammonia and carbon dioxide.

- Urease activity is one of the important characteristics for the identification of *Proteus* species and allows for *Proteus* to be distinguished from non-lactose-fermenting members of the Enterobacteriaceae.
- Christensen developed the test in 1946 for the differentiation of enteric bacilli. The urea agar base used for the testing of urease activity is named Christensen Urea Agar after him.
- During the test, the organisms utilize urea as the sole source of nitrogen, producing a sufficient amount of ammonia to overcome the buffering capacity of the medium.
- The change in color of the medium as a result of the change in pH is indicative of the test result.

Objectives of Urease Test

- To test the ability of an organism to produce the enzyme urease that hydrolyses urea.
- To differentiate urease-positive *Proteus* from other Enterobacteriaceae.

Microorganisms Tested

The urea test is part of the battery of tests to identify the following:

1. Gram-negative enteric pathogens, including *Yersinia* spp.
2. Fastidious Gram-negative rods—*Brucella, Helicobacter pylori,* and *Pasteurella*.
3. Gram-positive rods—*Corynebacterium* and *Rhodococcus* spp.
4. Yeasts—*Cryptococcus* spp.

Directly, this test is performed as a rapid test on gastric biopsy samples to detect the presence of *H. pylori*.

Principle of Urease Test

Urea medium, whether a broth or agar, contains urea and the phenol red as a pH indicator. Many organisms, especially those that cause urinary tract infections, produce the urease enzyme, which catalyzes the splitting of urea in the presence of water to release two molecules of ammonia and carbon dioxide. The ammonia combines with the carbon dioxide and water to form ammonium carbonate, which turns the medium alkaline, turning the indicator from its original orange-yellow color to bright pink. This test is performed as part of the identification of several genera and species of the *Enterobacteriaceae* family, including *Klebsiella, Proteus,* and some *Citrobacter* and *Yersinia* species, as well as some *Corynebacterium* species. The test is also useful to identify *Cryptococcus*, *Brucella*, *Helicobacter pylori*, and many other bacteria that

produce the urease enzyme. Disks are available that combine urea and phenylalanine deaminase (PDA), allowing a one-disk test to identify *Proteus*,*Providencia*, and *Morganella* and to separate them from *Klebsiella* and *Yersinia enterocolitica*. The disk reactions are rapid and sensitive and allow for the rapid detection of agents of serious infections, g., *Brucella*, and *Cryptococcus*.

Media, Reagents, and Supplies Used

Media Used

- Both urea agar slants and broth media can be used for the detection of urease production. Agar media includes the Urea Agar Base (Christensen agar), and the broth includes the urea broth.
- Besides, rapid test kits are also available for the detection of urease activity.
- The composition of the urea agar base is given below:

S.N	Ingredients	Gram/liter
1.	Dextrose	1.0
2.	Peptic digest of animal tissue	1.5
3.	Sodium chloride	5.0
4.	Monopotassium phosphate	2.0
5.	Phenol red	0.012
6.	Agar	15.0
Final pH at 25°C: 6.8 ±0.2		

Supplies Used

- Sterile wooden sticks or loops
- Saline or water in a small plastic tube for the disk test
- Incubator at 35°C and 30°C

Procedure of Urease Test

A. Preparation of media

- About 24.52 grams of the dehydrated medium is dissolved in 950 ml distilled water in a beaker.

- The solution is heated to bring it to a boil in order to dissolve the medium completely.
- The prepared suspension is sterilized by autoclaving at 15 lbs pressure, 121°C for 15 minutes.
- The beaker is taken out following the autoclaving and cooled to 50°C. To the beaker, 50 ml of sterile 40% urea solution is added and mixed well.
- The medium is dispensed into tubes and set in a position to obtain agar slants.

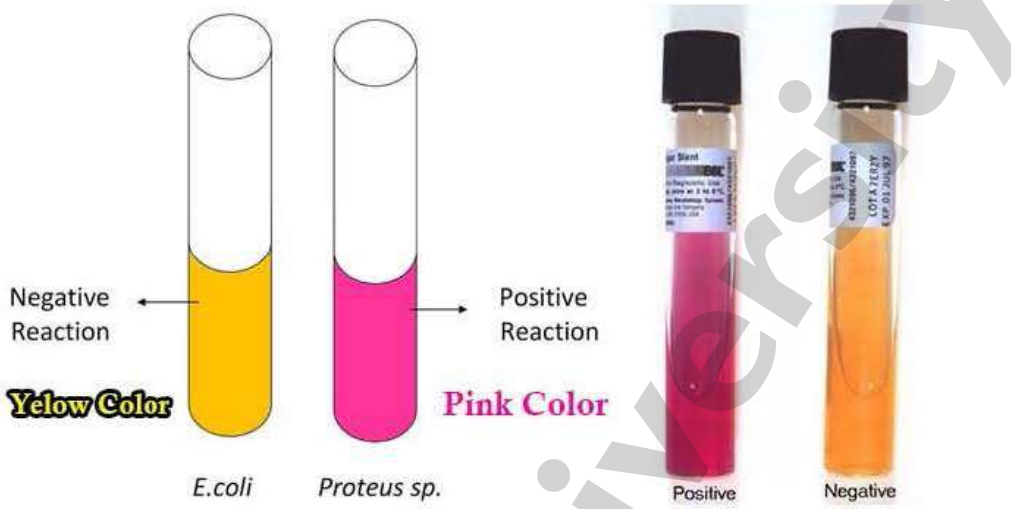
B. Urease Activity

- A loopful of a well-isolated colony is taken with an inoculating loop and inoculated on the agar slants. The inoculation should be done on just the slant, and the butt shouldn't be stabbed.
- The tubes are then incubated with loosened caps at 35 to 37°C. For non-fermenters, the tubes are incubated at 30°C.
- The tubes are observed for the development of pink color for as long as 7 days.
- If no growth is seen on the slant, further inoculation with heavy inoculum should be done.

C. Rapid Urease Test

- Rapid urease test is also called the CLO test (*Campylobacter*-like organism) and is used for the rapid identification of *Helicobacter pylori*.
- For the rapid urease test, a biopsy is taken of the mucosal layer of the antrum of the stomach and placed on the urea broth with phenol red indicator.
- The tube is then observed for the change in color from yellow to pink.

Result Interpretation of Urease Test



- A positive test is demonstrated by an intense magenta to bright pink color in 15 min to 24 hours.
- A negative test shows no color change.

Control organisms

- Positive test: *Proteus mirabilis*.
- Negative test: *Escherichia coli*.

Uses of Urease Test

- Urease test is used to identify organisms that are capable of hydrolyzing urea to produce ammonia and carbon dioxide.
- The test is particularly used for the presumptive identification of *Proteus* species for other members of the Enterobacteriaceae family.
- The test also differentiates *Proteus* from the non-lactose-fermenting bacteria.
- This test is performed as a rapid test on gastric biopsy samples to detect the presence of *H. pylori*.

Limitations of Urease Test

- Some organisms should rapid result as they rapidly split urea (*Brucella* and *H. pylori*), while others might react slowly.
- When performing overnight tests from a medium that contains peptone, the alkaline reaction may be due not to urease but to hydrolysis of peptone.
- Urea is light sensitive and might undergo autohydrolysis. The medium thus must be stored at 2 to 8°C in the dark.

- The test is less sensitive if the medium is not buffered.

Biochemical reactions 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 can be used to distinguish between coliforms such as Klebsiella (formerly Enterobacter) aerogenes (+ve) which occur naturally in the soil and in aquatic environments from fecal coliforms such as Escherichia coli (-ve) whose presence would be indicative of fecal contamination.

Objective

- to detect the ability of organisms to produce citrase enzyme.

Principle of citrate utilization test:

The basic principle of this test is to detect the ability of an organism which can utilize citrate as a sole source of carbon for their metabolism with resulting alkalinity. The ***citrase enzyme*** hydrolyses the citrate to form oxaloacetic acid and acetic acid.

Reaction:

Step I:

Citrate ———citrase enzyme———-> Oxaloacetic acid + acetic acid

Step II:

Oxaloacetic acid———-> pyruvic acid + CO2

The test organism is cultured in a medium which contains sodium citrate and indicator bromothymol blue. Change in color of indicator from light green to blue due to alkaline reaction is indication of citrate utilization by the test orgaism.

All coliforms metabolize citrate when the molecule is generated inside the bacterial cell. But not all coliforms produce transport enzyme that bring citrate from environment across the cytoplasmic membrane and into the cell. In bacteria, that utilize citrate, the cleavage of citrate involves an enzyme system without the intervention of coenzyme. This enzyme system requires a divalent cation for its activity which is supplied by the Mg++ or Mn++ ion. The product obtained from the citrate metabolism depends on the pH of the medium. In alkaline medium, mostly acetic and formic acid are produced.

Pyruvic acid———–> Formoc acid + Acetic acid + CO2

In acidic medium, acetoin and lactic acid are produced

Pyruvic acid———–> acetoin + Lactic acid + CO_2

In both cases, there is production of CO_2 which then combines with sodium present in medium to form Sodium carbonate, an alkaline product.

$Na + CO_2$ ———–>Na_2CO_3

The organisms capable of utilizing citrate as sole source of carbon can also utilize ammonium salts as sole source of Nitrogen. When ammonium salts are used by microorganisms, it breakdown into NH_3 which increases the pH of medium.

The Citrate medium contains ammonium salts as sole source of Nitrogen and Citrate as sole source of carbon. It also contains a pH indicator bromothymol blue, which is green as neutral pH and changes to deep prussian blue at alkaline pH above 7.5. Henc, the formation of green color ie. no change in color indicates negative citrate test while the formation of blue color indicates positive citrate test.

Requirements:

- Simmon's citrate agar slant
- Given bacterial samples (*E. coli* and *Klebseilla)*
- Inoculating loop

Media Composition:

In the citrate utilization test, the citrate medium most commonly used is the formula of Simmons. The medium is poured into a tube on a slant. The composition of Simmons citrate agar is as follows:

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. Prepare Simmon citrate agar or Koser's citrate medium in test tubes, taking 5 ml medium by autoclaving at 15lbs for 15 minutes.
2. tilt the test tube containing melted citrate medium to prepare distinct slant and butt.
3. Inoculate the given sample of organism were on the slant of the media using sterile wire and label the tubes
4. Incubate the tubes at 37°C for 24 -48 hours.
5. Observe the color change in the medium

Result:

- **Citrate test Positive:** color of media change to blue (*Klebsiella* spp)
- **Citrate test Negative:** No change in color of media (*E. coli*)

Quality Control

1. Perform QC on each new lot of media prior to using them. Inspect agar for evidence of freezing, contamination, cracks, dehydration, and bubbles prior to storage and before use. Discard tubes that are blue.
2. Organism
    1. *Klebsiella pneumoniae* ATCC 13883—citrate positive (growth; blue color)
    2. *Escherichia coli* ATCC 25922—citrate negative (no growth or trace of growth)

TSI (Triple Sugar Iron) test

Objective

- to check the ability of organism to ferment sugar (lactose, sucrose) and their ability to produce H2S gas.

Preparation of TSI agar medium

- Combine the ingredients, and adjust the pH to 7.3
- Boil to dissolve the agar and dispense into tubes.
- Sterilize by autoclaving at 121°C for 15 minutes
- Cool in a slanted position to give a 2.5 cm butt and a 3.8 cm slant.

TSI agar is also available commercially

Principle of TSI test:

- Triple Sugar Iron Agar (TSIA) contains 3 sugar glucose (dextrose) sucrose, lactose in the ratio of1: 1:10 as the source of carbon as its name suggest. Besides this the media also contains yeast and beef extract, peptic digest of animal tissue and casein as the source of protein.
- The presence of fetrous sulphate and sodium thiosulphate fulfills the demand of sulphur and there is the indicator phenol red for the indication of change in the invironment of the media due to the production of acid or alkali. The media is prepared in the form of slant containing, a slant and butt region.
- This test is used to differentiate gram negative enteric. Enter bacteria and other glucose fermenter first begin to metabolize glucose as glucose utilizing enzymes are present constitutively and bacteria can gain the most energy by using simplest sugar.

- All other sugar must be converted to glucose before they enter the EMP. Glucose utilization occur both aerobically on the slant where O2 is available and on the butt where there is anaerobic condition. Once the glucose fermenting bacterium has reduced all of the available glucose to pyruvate, it will further metabolize pyruvate via TCA cycle to produce acid end products. The acid in the medium cause the ph indicator phenol red, to change its colour to yellow. Thus, after 6 hours of incubation both the slant and butt of TSIA that has been inoculated with a glucose fermenter will appear yellow.
- After the depletion of limited glucose in the medium, the organism that has enzymes to degrade lactose or sucrose starts to utilize them as they are present 10 times more concentration than glucose and the organism continue to make acid end products.
- Hence, the butt and slant of TSIA will still remain yellow for lactose and sucrose fermentors (degrades) after 18 hours of incubation and the reaction is called acid/acid.
- If the organism being tasted cannot use the lactose or sucrose in the medium, it must shift to protein utilization as there is no other source or energy in the media.
- As the protein breakdown occurs only in aerobic condition, the byproduct of protein and amino acid metabolism i.e NH3 changes the pH environment of the medium from the neutral to alkaline in the slant region where the condition is aerobic. Hence, the colour of slant appears red and the reaction is called alkali/acid while the colour of the butt is still yellow due to anaerobic glucose breakdown.
- Gas production can be detected when holes are formed or the medium is broken into several fragments. H2S production by organism results in the blackening of the medium. This colour is due to the production of H2S from an ingredient of the medium, sodium thiosulphate, which then combines with another component of the medium, ferrous ammonium sulphate resulting in the formation of black, insoluble compound, ferrous sulphide.

$FeSO4 + H2S \longrightarrow H2SO4 + FeS$ (Black ppt)

Requirements

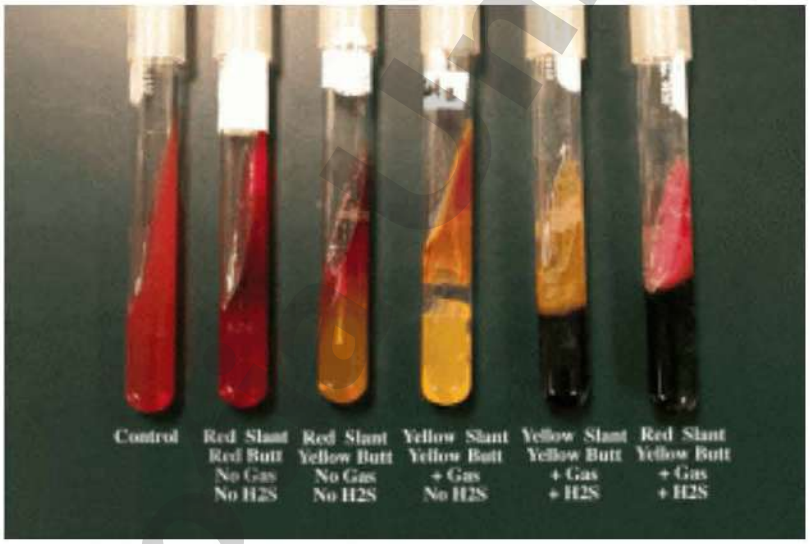
- TSIA slant
- Given sample of bacterial cultures (*E.coli* and *Klebsiella)*
- Inoculating wire
- Burner

- Incubator.

Procedure of TSI test

1. Take 5-7 ml of the autoclaved TSIA media in a sterile test tube and slant was made by tilting the media till it solidified.
2. Using a flamed inoculating loop, pick a colony from the bacterial culture.
3. Inoculate the organism in TSIA slant by stabbing in the butt and then with the same loop streaking on the whole slant surface of the medium
4. Incubate at 37°C for 18-24 hours.
5. Observe for the color change in the slant and butt, gas production and H2S production.

Result interpretation:



Color observed (Slant/Butt)	pH (Slant/Butt)	Utilization
Red/yellow	Alkali/Acid	Glucose only and peptone
Yellow/Yellow	Acid/Acid	Glucose, Lactose and sucrose
Red/Red	Alkali/Alkali	No sugar are utilized, utilize peptone

*H2S gas production is indicated by blackening of media

** Gas production is indicated by bubbles in butt

- *E. coli* gives acid/Acid with gas production without H2S production. This means E. coli utilize all sugar anaerobically with production of gas during fermentation.

*** *Pseudomonas* spp gives alkaline/alkaline (Red/Red) which means it does not utilize any sugar.

Methyl Red (MR) test determines whether an organism performs mixed acid fermentation and produces stable acid end products. MR indicator is used to determine the pH after an enteric Gram-negative rod has fermented glucose to completion.

Principle

In mixed acid fermentation, three acids (acetic, lactic, and succinic) are formed in significant amounts decreasing the pH of the medium below 4.4. This is visualized by using a pH indicator, methyl red (p-dimethylaminoazeobenzene-O-carboxylic acid) pH indicator which is red at pH ≤ 4.4, and yellow color at pH 5.8.

Fig: Methyl Red (MR) Test Reaction

The pH at which methyl red detects acid is considerably lower than the pH indicators used in bacteriologic culture media. Thus, to produce a color change, the test organism must produce large quantities of acid from the supplied carbohydrate source. Change in the color of the MR-VP broth is observed after the addition of pH indicator and the result is categorized as;

- **MR Positive**: Culture medium turns red *(because pH of the medium is at or below 4.4 from the fermentation of glucose).*

- **MR Negative**: Culture medium remains yellow *(less acid is produced from the fermentation of glucose).*

Media and Reagents

Methyl red-Voges-Proskauer (MR/VP) broth (formulated by Clark and Lubs) is used in this test. Media and reagents can be prepared in house or purchase from commercial providers.

The composition of MR/VP broth is as follows:

Ingredients	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 (0.02%) pH Indicator

1. Dissolve 0.1 g of MR in 300 ml of ethyl alcohol, 95%.
2. Add sufficient distilled water to make 500 ml.
3. Store at 4 to 8°C in a brown bottle.
4. Solution is stable for 1 year.

Quality Control

After the preparation or procurement of each lot of medium, examine the broth for signs of contamination, dehydration, and deterioration. Perform performance testing of media and reagent prior to use with one organism known to demonstrate a positive reaction and one organism known to give a negative reaction.

Organisms

1. *Escherichia coli* ATCC 25922—MR positive (red)
2. *Klebsiella pneumoniae* ATCC 13883—MR negative (yellow)

Methyl Red (MR) Test

Objective: 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 at least 48 hours.
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 color of the medium changes to red within a few minutes.

Principle: The development of a stable red color on the surface of the medium indicates sufficient acid production to lower the pH to 4.4 and constitutes a positive test. Because other organisms may produce smaller quantities of acid from the test substrate, an intermediate orange color between yellow and red may develop. This does not indicate a positive test.

1. *Escherichia coli*: MR test positive- the appearance of red color after the addition of methyl red reagent.
2. *Klebsiella* (formerly *Enterobacter*) *aerogenes*: MR test negative- the lack of color change after the addition of methyl red.

Reporting results:

- Organisms in the *E. coli* group are MR positive, and those in the *Enterobacter-Klebsiella* group are MR negative
- Most members of the family *Enterobacteriaceae* give opposite MR and VP reactions; however, certain organisms, like *H. alvei* and *Proteus mirabilis*, may give both a positive MR reaction and a positive VP reaction (often delayed)

Methyl-Red (MR) positive organisms:

1. *Escherichia coli*
2. *Shigella* species
3. *Salmonella* species
4. *Citrobacter* species

5. *Proteus* species
6. *Yersinia* species

Methyl-Red (MR) negative organisms:

1. *Enterobacter* species
2. *Hafnia* species
3. *Serratia marcescens*
4. *Klebsiella pneumoniae*

Limitations:

- Avoid overinoculation. Bacterial growth is inhibited when the inoculum exceeds approximately 10^9 viable cells per ml
- A false-positive MR result may be obtained if the tubes are not incubated for a sufficient period of time.

Voges Proskauer (VP) Test

The Voges-Proskauer (VP) test is used to determine if an organism produces acetylmethylcarbinol from glucose fermentation. Voges-Proskauer is a double eponym, named after two microbiologists working at the beginning of the 20th century. They first observed the red color reaction produced by appropriate culture media after treatment with potassium hydroxide.

It was later discovered that the active product in the medium formed by bacterial metabolism is acetyl methyl carbinol, a product of the butylenes glycol pathway.

Principle of Voges–Proskauer (VP) Test

The Voges-Proskauer (VP) test is used to determine if an organism produces **acetylmethyl carbinol** from glucose fermentation. If present, **acetylmethyl carbinol** is converted to **diacetyl** in the presence of ——————————**∝- naphthol**, strong alkali (**40% KOH**), and atmospheric oxygen. The **∝-**——————————**naphthol** was not part of the original procedure but was found to act as a color intensifier by Barritt and must be added first. The **diacetyl** and **quanidine**-containing compounds found in the **peptones** of the broth then condense to form a **pinkish red polymer**.

2 pyruvate = acetoin + $2CO_2$

acetoin + NADH + H^+ = 2,3-butanediol + NAD^+

Media and Reagents used in Voges–Proskauer (VP) Test

MRVP broth (pH 6.9)

Ingredients per liter of deionized water:

buffered peptone= 7.0 gm

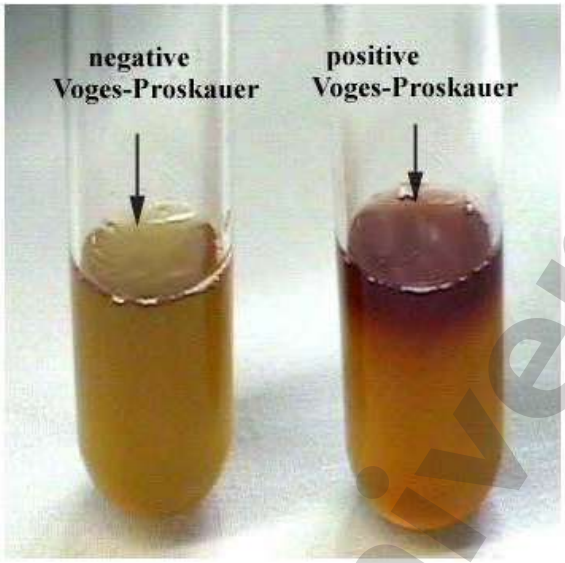
glucose= 5.0 gm

dipotassium phosphate= 5.0 gm

Voges-Proskauer Reagent A: Barritt's reagent A	
Alpha-Naphthol, 5%	50 gm
Absolute Ethanol	1000 ml
Voges-Proskauer Reagent B: Barritt's reagent B	
Potassium Hydroxide	400 gm
Deionized Water	1000 ml

Procedure of Voges–Proskauer (VP) Test

1. Prior to inoculation, allow medium to equilibrate to room temperature.
2. Using organisms taken from an 18-24 hour pure culture, lightly inoculate the medium.
3. Incubate aerobically at 37 degrees C. for 24 hours.
4. Following 24 hours of incubation, aliquot 2 ml of the broth to a clean test tube.
5. Re-incubate the remaining broth for an additional 24 hours.
6. Add 6 drops of 5% ——————————alpha-naphthol, and mix well to aerate.
7. Add 2 drops of 40% potassium hydroxide, and mix well to aerate.
8. Observe for a pink-red color at the surface within 30 min. Shake the tube vigorously during the 30-min period.



Results and Interpretation:

A positive VP test is represented by the **development of a pink-red color at the surface** within 15 minutes or more after the addition of the reagents indicating the presence of diacetyl, the oxidation product of acetoin. The test should not be read after standing for over 1 hour because negative Voges-Proskauer cultures may produce a copper-like color, potentially resulting in a false-positive interpretation.

If the result is negative, the glucose or MRVP broth can be incubated for up to 48 h and the test repeated.

Reporting results:

- Most members of the family *Enterobacteriaceae* **give opposite MR and VP reactions**; however, certain organisms, like *H. alvei* and *Proteus mirabilis*, may give both a positive MR reaction and a positive VP reaction (often delayed).
- *Streptococcus mitis* group organisms are VP negative, whereas the other viridans group streptococci are VP positive, except *Streptococcus vestibularis*, which is VP variable

Voges-Proskauer (VP) Positive Organisms of <u>Enterobacteriaceae family</u> are:

1. *Klebsiella* species
2. *Enterobacter* species
3. *Hafnia* species
4. *Serratia* species

Limitations:

1. With **prolonged incubation (>3 days)**, some VP-positive organisms can produce an acid condition in the medium, yielding **weak positive reactions or false-negative VP reactions.**
2. Do not add more than 2 drops of KOH per 2 ml of medium. **Excess amounts of KOH can give a weakly positive reaction**, which may be masked by the formation of a copper-like color because of the reaction of KOH with α-naphthol alone.
3. **Do not read the test more than 1 h after adding the VP reagents**. A copper-like color may develop, resulting in a potential false-positive interpretation.
4. Reagents must be added in the **specified order.** A reversal of order may result in a weakly positive or false-negative VP result.

Minimum Inhibitory Concentration (MIC)

In-vitro Microbiological Tests for the Characterization of Test Compounds:

- **Minimum Inhibitory Concentration (MIC) assays** determine the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism.
- **Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) assays** determine the lowest concentration of an antimicrobial agent required to achieve bactericidal and fungicidal killing, defined as a 99.9% reduction in the initial inoculum.

Emery Pharma routinely provides antibiotic susceptibility testing for the following methods according to Clinical and Laboratory Standards Institute (CLSI) guidelines. For more information, see our **MIC guide** in the resources section:

- broth micro- and macro-dilution (see illustration below)
- disk diffusion
- agar dilution

Test articles can be natural or synthetic, mixtures or purified. **Hundreds of bacterial strains**, including multi drug-resistant clinical isolates, ESKAPE pathogens, and multiple **fungal strains**, are available in our inventory for immediate testing.

ESKAPE Pathogens	
Species	**Phenotype**
E. faecium	Gram +ve, VRE
S. aureus	Gram +ve, MRSA
K. pneumoniae	Gram -ve, MDR
A. baumannii	Gram -ve, MDR
P. aeruginosa	Gram -ve, MDR
E. cloacae	Gram -ve, ESBL

Minimum Bactericidal Concentration (MBC) Test

MBC test determines the lowest concentration at which an antimicrobial agent will kill a particular microorganism. The MBC is determined using a series of steps, undertaken after a Minimum Inhibitory Concentration (MIC) test has been completed.

MBC testing is useful for comparing the germ-killing activity of several antimicrobial agents at once.

Summary of the Minimum Bactericidal Concentration Test

- A pure culture of a specified microorganism grown overnight, then diluted in growth-supporting broth (typically Mueller Hinton Broth) to a concentration between 1 x 10^5 and 1 x 10^6 cfu/ml.
- A stock dilution of the antimicrobial test substance is made at approximately 100 times the expected MIC (if known).
- Further 1:1 dilutions are made in test tubes or 96 well microtiter plates.
- All dilutions of the test product(s) are inoculated with equal volumes of the specified microorganism.
- A positive and negative control tube or well is included for every test microorganism to demonstrate adequate microbial growth over the course of the incubation period and media sterility, respectively.
- An aliquot of the positive control is plated and used to establish a baseline concentration of the microorganism used.
- The tubes or microtiter plates are then incubated at the appropriate temperature and duration.
- Turbidity indicates growth of the microorganism and the MIC is the lowest concentration where no growth is visually observed.
- To determine the MBC, the dilution representing the MIC and at least two of the more concentrated test product dilutions are plated and enumerated to determine viable CFU/ml.
- The MBC is the lowest concentration that demonstrates a pre-determined reduction (such as 99.9%) in CFU/ml when compared to the MIC dilution.

Experiment for Identification of Unknown Bacteria

Principle:

Identification of unknown bacteria is one of the major responsibilities of the microbiologists. Samples of blood, tissue, food, water and cosmetics are examined daily in laboratories throughout the world for the presence of contaminating microorganisms.

In addition, industrial organisations are constantly screening materials to isolate new antibiotic-producing microbes or microbes that will increase the yield of marketable products, such as vitamins, solvents and enzymes. Once isolated, these unknown microbes must be identified and classified.

The science of classification is called taxonomy and deals with the separation of living organisms into interrelated groups. Bergey's Manual of Determinative Bacteriology, 8th edition, is the official taxonomic key containing the orders, families, genera and species of all known classified bacteria (Appendix III).

With the fundamental knowledge in staining methods, isolation techniques, bacterial nutrition, biochemical activities and growth characteristics of bacteria, it becomes easier for identification of any unknown bacteria.

Characteristics of few bacteria have been given in Table 7.2. Other bacteria can be identified in a similar way based on the observations and results obtained following the experimental procedures.

Materials Required:

Slides, cavity slides, petri dishes, conical flasks, cotton plugs, inoculating loop, autoclave, bunsen burner, laminar flow chamber, dispose jar, incubator, nutrient broth, nutrient agar, gram staining reagents, media and reagents for biochemical tests, compound microscope, isolated colonies or pure cultures of bacteria.

Procedure:

1. Gram staining of the unknown bacteria is performed. Besides gram staining, its morphology and arrangement is also recorded. Motility test of the bacteria is determined by hanging drop preparation

Using sterile inoculating technique, the bacteria is inoculated into two nutrient agar slants, a nutrient broth tube and to a nutrient agar plate by means of streak inoculation. Following incubation, one slant culture is used to determine the cultural characteristics of the unknown bacteria.

The second is used as a stock sub-culture, should it be necessary to repeat any of the tests. Growth characteristics are also observed in the broth tube and colony characteristics on the plate.

3. Exercising care in sterile technique, so as not to contaminate cultures and thereby obtain spurious results, the bacteria is inoculated into respective media to carry out the different biochemical tests.

4. The inoculated media are incubated at the required temperatures for the required lengths of time.

Observations:

1. In gram staining, besides gram reaction, the morphology and arrangement of the bacteria are also recorded.

2. The cultural characteristics of the bacteria in nutrient broth, on agar slant and on nutrient agar plate are noted.

3. Results of the biochemical reactions are recorded.

Interpretation of Results:

1. Using the above recorded data and with the help of the Bergey's Manual of Determinative Bacteriology, the bacteria is identified as to its genus and species. It should be kept in mind that, results might vary depending on the strains of each species used and the length of time the bacteria has been maintained in stock culture.

The observed results may not be completely identical to the expected results. Therefore, the bacteria that best fits the results are chosen.