Practical-A

1. Microbiology Laboratory Practices and Biosafety.
2. To study the principle and applications of important instruments (biological safety caabinets, autoclave, incubator, BOD incubator, hot air oven, light microscope, pH meter).
3. Preparation and sterilization of culturre media for bacterial cultivation.
4. Study of different shapes of bacteria, fungi, algae, protozoa using permanent slides/pictographs.
5. Staining of bacteria using Gram stain.
6. Isolation of pure cultures of bacteria by streaking method.
7. Estimation of CFU count.

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LECTURE PLAN

DEPARTMENT OF MICROBIOLOGY

Microbiology Lab Practices and BioSafety

1. Wash your hands with disinfectants when you arrive at the lab and again before you leave.
2. Absolutely no food, drinks, chewing gum or smoking is allowed in the laboratory.
3. Do not put anything in your mouth such as pencil, pens, labels or fingers.
4. Do not store food in areas were microorganisms are stored.
5. To be admitted to laboratory, each student should wear fresh, clean, knee-length laboratory coat and laboratory footwear.
6. Tie back your hair neatly, away from the shoulders.
7. At the start and end of each laboratory session, students should clean their assigned bench-top area with 70% ethanol.
8. Label everything clearly to avoid contamination and unnecessary activities.
9. Replace caps on reagent, solution bottles and bacterial cultures. Do not open petri plate dishes in the lab unless absolutely necessary.
10. Inoculating loops and needles should be followed flamed sterilize in a Bunsen burner before you lay them down.
11. Turn off bunsen burner when not in use.
12. Treat all microorganisms as potential pathogens with appropriate care and do not take culture out of the lab.
13. Wear disposable gloves when working with potential infectious microbes of all samples (example: sewage). If you are working with that sample, that may contain a pathogen.
14. Sterilize equipment and materials properly as instructed.
15. Laminar air flow are equipped with a UV lamp that should be turned on about 10-20 minutes before and after being used to sterilize the shell.
16. Never pipette by mouth to avoid accidents and infections.
17. Consider everything a bio hazard. Do not pour anything (cultures, contaminated utensils) down the sink or garbage can without proper decontamination.
18. Dispose all chemicals in the biohazard bag.

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19. Familiar yourself with the location of safety equipment in the lab. (Example: eye wash station, shower, sinks, fire extinguisher, biological safety cabinet, first aid kit, emergency gas valve).
20. Dispose razor blades, syringe needles and sharp metal objects in the appropriate container.
21. Report spills or any drop of culture or if any type of accident occurs immediately to your instructor.
22. Report if any injury caused during your laboratory session immediately to your instructor. No matter how small it is.
23. Keep a faithful record of all the experiments and observations. Update it regularly and submit it for evaluation at the end of each exercise.

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2. TO STUDY THE PRINCIPLE AND APPLICATIONS OF IMPORTANT INSTRUMENTS(BIOLOGICAL SAFETY CABINETS, AUTOCLAVE, INCUBATO R, BOD INCUBATOR, HOT AIR OVEN, LIGH T MICROSCOPE, PH METER)

Biological Safety Cabinets:

Laminar air flow chamber can maintain a working area devoid of contaminants many medical and research laboratory require sterile working environment in order to carry out specialized work. Laminar air flow cabinets create particles free filtration system and exhausting it across a working surface in a laminar or unidirectional air stream. They provide an excellent clear air environment for a number of laboratory requirements.

The process of laminar air flow can be described as air flow where an entire body of air flows steadily with uniform velocity. Laminar air flow consist of cabinets work by the use of inflow laminar air drawn through one or more HEPA filter designed to create a particle, working environment and provide product protection. Air is taken through a filtration system and then exhausted across the work surface as a part of the laminar flow process. Commonly the filtration system comprises of a pre filter and HEPA filter. The laminar flow consists of cabinet is enclosed on the sides and constant positive air pressure is maintained to prevent the intrusion of contaminated room air.

Types of Laminar Flow cabinets:

Laminar flow cabinets are used and can be produced as both horizontal and vertical cabinets. There are many types of cabinets with a variety of air flow patterns for different purposes.

1. Vertical Flow Laminar Chamber
2. Horizontal Flow Laminar Chamber
3. Laminar flow cabinets and hoods.
4. Laminar Flow benches and booths

All ensure that work space is devoid of contaminants and may be tailored to the lab requirements.

Horizontal Laminar Air Flow Cabinets:

Horizontal Laminar flow cabinets receive their name due to action of air flow which comes from above but then it changes direction and is provided across the work in a horizontal direction that constant flow of filtered air provider materials and product production.

Figure

Vertical Laminar Flow Cabinets:

Vertical laminar flow cabinets function equally well as horizontal flow cabinets with the laminar air directed equally vertically downwards enter the working area the air can leave the working area via noses in the base vertically flow cabinets can provide greater product production.

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AUTOCLAVE:

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AUTOCLAVE:

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Autoclave is an instrument used for the sterilization. It works under the principle of moist heat, sterilization. It was first discovered by Charles Chamberland. Pressure is used to produce high temperature stream. It is based on the principle that when water is boiled at an increased pressure, the temperature at which it boils and the stream rapidly penetrates and gives up its extent heat when it condenses, the object ensures the destruction of bacterial endospores as well as vegetative cell by coagulating and denaturizing microbial proteins and enzymes.

Method of Use:

1. Add correct volume of water to the autoclave.
2. Place all the materials to be sterilized in the inner chamber of the autoclave
3. Do not over load the autoclave.
4. Secure the lid as directed by the manufacturer. Open the air lock (air outlet) and close the draw off knob.
5. Apply heat electrically as the water boils air and steam will merge through the air lock.
6. When all the water droplets have been expelled and only steam emerging, wait for 1 minute and then close the air lock. This will cause the pressure for rise.
7. When the required pressure has been reached and the excess steam begins to be released from the safety value reduce the heat….
8. Hold all the materials at 121℃ for 15 minutes with 15 psi pressure (Holding time will vary depends on materials).
9. All the end of sterilization time, turn off the heat and allow the autoclave to cool naturally. This usually takes few hours.
10. Check that the pressure gauge is showing zero. When at Zero, open the airlock and then wait for few minutes before the opening of lid and to allow time for autoclave to become fully inverted.
11. Remove all sterilized material from the autoclave and tighten the bottle cap.

Control of Autoclave:

A biological method is available to control the performance of an autoclave. It is called bioindicator for sterilization. Spores of *Bacillus stearothermophillus* acts as bioindicator system. The spore will be destroyed at 121 ℃ for 10 minutes. So people set autoclaving temperature at 121 ℃ for 15 minutes.

INCUBATOR:

In microbiology, an incubator is a device for controlling temperature, humidity and other conditions in which microbe under controlled condition. The simplest incubator is insulated boxes with adjustable heaters, typically rising up to 60 ℃ to 65 ℃. More elaborate incubators can also include the ability to control or lower the temperature or the ability to control humidity or CO2 levels. Most incubators include a timer, some can be programmed to cycle through different temperatures, humidity level etc.

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Incubators can vary in size from table top to the size of small rooms. Incubator can also contains heat uses such as shakers, measured by revolution per minutes common incubating temperatures, for the cultivation of bacteria are approximately 36-37 ˚C and for the cultivation of bacteria or fungus are 25-30 ℃. Incubator may vary with the type of microorganism.

BOD INCUBATORS: (Low Temperature Incubator)

All the aquatic animals can vary on the oxygen present in the water. Aquatic microbes use the organic matter include plant decayed leaf fall. Bacteria will break down this organic matter using oxygen and produce less complex organic matter increased disposal of waste materials, utility of oxygen by microbes will also increase so water will be the BOD low temperature incubator which can maintain temperature from 50 ˚C to as low as 2-3 ˚C is used for incubation in such cases. The constant desired temperature is set by rotating the knob of the thermostat. Rotation of the thermostat knob finely by trial and error and the temperature on the incubator is noted.

The BOD of a water sample is generally measured by incubating the sample at 20 ˚C for 5 days in the dark room under aerobic condition. In the water sample where more than 70% of the initial oxygen is consumed, it is necessary to aerate or oxygenated and dilute the sample with deionized water and dilute the sample with deionized water and passes through the column of activated carbonates re distilled to avoid a stress.

Working Principle:

Under the alkaline condition, the manganese sulphate produce a white precipitate of manganese hydroxide. This reacts with the dissolve oxygen present in the sample to form brown precipitate on acidic conditions, manganese diverts to its divalent state and release iodine. This released iodine is titrated against sodium thiosulphate using starch as an indicator.

HOT AIR OVEN:

An oven is based on the principle where dry heat or hot air accomplishes sterilization. The sterilization process in an oven is longer than autoclaving. Dry heat removes water from microorganism while moist heat has greater penetration power than dry heat.

It is used for sterilizing glassware like petridishes, test tubes, pipettes, metal instruments, oils, powders, waxes. Commonly used temperature for hot air oven sterilization is 10 minutes at 180 ℃, 40 minutes at 170 ˚C, 60 minutes at 160 ˚C, 150 minutes at 150 ℃ , 180 minutes at 140 ℃, 480 minutes at 120 ˚C. An oven consists of insulated cabinet which is held at a constant temperature of thermostat.

MICROSCOPE:

AIM:

A microscope is an optical device having lenses arranged in such a manner to enlarge an magnify a minute object.

PRINCIPLE:

There are two principle involved in the development of microscope.

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A) To magnify the object at the extreme capacity
B) The magnification showed such that all details should be seen clearly and closely.

There are 2 types of microscopes:

1) Simple Microscope
2) Compound Microscope

The early microscope is called as simple microscope and had a single lens.

RESOLVING POWER:

Resolving power of microscope is its ability to distinguish two objects close to one another to make separate and distinct objects. The resolving power of microscope is governed by the wavelength of light used to illuminate object and also the numerical aperture of the objective lens.

NUMERICAL APERTURE:

The numerical aperture of a lens is a measure of its light gathering capacity. So the numerical aperture can be defined as

$NA = n\sin\Theta$

Where,

n = refractive index of the medium between the objective and the object.

Θ = half the angle (one of the light entering in to the objective from center of the object).

Subsequently, oil immersion technique can give high resolution of the image, so the limit of resolution can be,

$$d = \frac{\lambda}{2NA}$$

Where,

λ – Wavelength of light used

NA – Numerical aperture

Working Types of Microscope:

The microscope consists of two parts

(i) Mechanical part
(ii) Optical part

MECHANICAL PART:

Mechanical parts are necessary for the operation of microscope. There is a base which is mostly horse shoe shape. From the base, the pillar arises at the top of the inclination joint. A nob is attached to the loop of the pillars. The stage is with a central opening called stage operation. There are two stage clips to hold the slide. The body tubes moves up and down by two adjustments. These adjustments are needed to focus the system upon the specimen. The coarse adjustments move the body tube over a greater vertical distance and being the specimen with the body tube slowly for fine focusing.

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There is a sub stage below the stage carrying condenser which forms the light rays upon the object. Iris diaphragm attached to the condenser helps in the regulation of light passing through the mirror into the condenser. The mirror has two surfaces, flat and concave.

OPTICAL PART:

Optical part governs the magnification of the image it includes a) mirror, b) objective and c) eye piece. All of them should be reception of optical axis of the best results objectives are of three types.

(i) Low power
(ii) High power
(iii)Oil immersion

The total magnification of system is determined by the multiple magnification power of microscope of one eye piece. It is called monocular microscope, where binocular microscope has two eye pieces.

HANDLING OF MICROSCOPE:

Place the slide on the storage with the specimen. The central part of the specimen to be examined is placed on the position above the stage.

Adjust the mirror until it reflects maximum amount of light through the specimen and place the objective in lower power position.

Move the body table with the help of coarse adjustment until the objective is 1-2cm from the slide took through the eye piece and slowly rises to the objective until the specimen is in appropriate flows. Never move the coarse adjustments downward by looking through the eye piece. Bring the specimen for sharp focus with fine adjustment look for sharp focusing.

Raise the body tube and rotate the position and place one or drops of immersion oil on the lower slide and lower the body tube carefully till the oil immersion objective touches the oil drop and then focus with fine adjustment.

Each time after using oil immersion objective clean the oil drop from the lens with the help of

While carrying the microscope, the right hand should hold the arm, the left hand should hold the base of microscope.

Immersion oil should be used only for oil immersion.

Microscope should be kept covered and in dust free cabinets after use.

pH METER:

A pH meter is an electronic instrument used to measure the pH of a liquid. A typical pH meter consists of a special measuring probes connected to a electronic motor that has a motor that measures and says the pH reading. The first commercial pH meter was built around by Radio meter in Denmark and by Dr. Arnold Orville Beckman in the United States. The Danish biochemist Sorensen invented pH scale in 1969. Standard pH meter has two electrodes, one glass electrode and another one is

a reference electrode. The voltage produced by one pH unit is typically about 60mv. Present pH meter contain microprocessors that makes the correction for temperature and calibration. The reference electrode which traditionally use silver chloride and has been suppressing by the calomel (mercurous chloride). A pH meter measure essentially the electrochemical potential between a known liquid inside the glass electrode and an unknown liquid outside the calomel reference electrode consists of a glass tube with a potassium chloride electrolyte which is in contact with silver chloride element. It is fragile condition joined by a liquid function tip made up of ceramic or similar material. This kind of electrode is not easily "poisoned" by heavy metals and sodium. The glass electrode consists of the glass tube with a thin glass bulb welded to it. Inside is the known solution of potassium chloride buffered at a pH of 7.0. A silver electrode with a silver chloride tip makes contact with the inside solution.

3. MEDIA PRE PARATION AND CULTIVATION OF BACTERIA

INTRODUCTION:

To establish the role of a microbial agent to a disease process, it is essentiaal to demonstrate the organism or its component in the diseased tissue. To accomplish this, the organism must be cultivated from the tissue. Though both in vitro and in vivo cultivation methods can bee used to isolate the organisms, the easiest way is to groww them in vitro on an artificial culture medium.

ARTIFICIAL CULTURE MEDIA :

A medium is an environment which supplies the ingredients necessary for the grrowth of an organism. Various kinds of media have been prepared in the laboratory to isolate, grow and identify an organism. Depending on the need to isolate and identify an organism from a particular sa mple or environment, different kinds of media are formulated.

KINDS OF MEDIA:

1) Basal or supportive media:

Basal medium is one that contains nutrients that allow the growth of most non-fastidious organisms without affording growth advantage to any organism over others.

Example: nutrient agar, trypticase soy agar, brain heart infusion agar.

2) Enriched medium:

Enriched medium is one that contains extra nutrients in addition to that preesent in basal medium which enables it to support the growth of fastidious organisms.

Example: blood agar, chocolate agar.

3) Differential medium:

The differential medium is one, which enables one to differentiate two types of organisms by their characteristic growth.

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Example: Blood agar : Haemolytic and non-heamolytic colonies can be differentiated.

Mac Conkey agar : Lactose fermenters and non-lactose fermenters can be differentiated.

Many different kinds of media can act as differential medium too.

4) Selective Medium:

Selective medium is onne that contains ingredients that afford growth advantage to particular organism over others by allowing the growth of the particular organism and inhibiting the others.

Example : Salmonella *shigella* agar : Allows the growth of *Salmonella* and *shigella* sp. And inhibits *E.coli*

PREPARATION OF NUTRIENT AGAR

Bacteriological media come an a wide range of types. Nutrient Agar is a complex medium because it contains ingredients with contain unknown amounts or types of nutrients.Nutrient Agar contains Beef Extract (0.3%), Peptone (0.5%) andd Agar (1.5%) in water. Beef extract is the coommercially prepared dehydrated form of autolysed beef and is supplied in the form of a paste. Peptone is casein (milk protein) that has been digested with the enzyyme pepsin.Peptone is dehydrated and supplied as a powder. Peptone and Beef Extract contain a mixture of amino acids and peptides. Beef Extract also contains water soluble digest products of all other macromoolecules (nucleic acids, fats, polysaccharides) as well as vitamins and trace minerals. Although we know and can define Beef Extract in these terms, each bach can not be chemically defined. There are many media ingrredients which are complex: yeast extract, tryptone, and others. The advantage of complex media is that they support the growth of a wide range of microbes.

Agar is purified from red algae in which it is an accessory polysaccharide (polygalacturonic acid) of their cell walls. Agar is added to microbioological media only as a **solidification agent**. Agar for most purposes has no nutrient value. Agar is an excellent solidification agent because it dissolves at near boiling but solidifies at 45oC. Thus, one can prepare molten (liquid) agar at 45oC, mix cells with it, then allow it to solidify thereby trapping living cells. Below 45oC agar is a solid and remains so as the temperature is raised melting only when >95oC iss obtained. In this experiment each student wiill prepare 200 ml of Nutrient Agar to be used in

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MATERIALS

1. Electronic or beam balances.

Weigh boats, tongue depressors
2. Tripods, asbestos wire-gauze, asbestos gloves.

3. 10 ml nonsterile pipettes.

4. pH paper or pH meter with standard buffers.

5. 4 13x100 mm screw capped culturre tubes.

6. Graduated Cylinder, 250 ml.

7. 2 500ml Erlenmeyer Flasks

8. Beef Extract, Peptone, Agar.

10. 3 N HCl, 3 N KOH.

11. 16 x 150 mm screw cap culture tubes.

12. Nonabsorbent cotton and gauze to make cotton stoppers.

Nutrient Agar

Beef Extract: 0.3%

Peptone: 0.5%

Agar: 1.5%

PROCEDURE

1. To make 200 ml of Nutrient Agar.

2. Tare a weigh boat and weigh out enough Peptone and add that to the flask.

3. Add 200 ml of distilled water and swirl to dissolve the peptone and beef extract. Check the pH, it should be 7.0.
4. Tare a weigh boat and weigh out enough Agar and add that to the flask.

5. With a bunsen burner, tripod, asbeestos wire-gauze, heat the medium to boiling to dissolve the agar. CAREFUL: 1) keep the rotating the flasks to prevent the agar from cooking onnto the bottom of the flask and 2) watch out: boiling agar can froth and boil out all over the lab bench. As soon as it begins to boil take it offf the heat and put it on to the bench. Allow it to cool a few minutes.

6. While the agar is still warm, but not hot, pipette 3 ml each into 4 13x100 mm screew cap culture tubes.
7. Label the flask and your tubes with your name.

8. After preparation of your medium, the instructor will take you to the autoclave.

9. Place your media in the autoclave with those of the rest of the class.

10. After discussion of the parts of thhe autoclave, autoclave the medium for 20 minutes.

11. The media will be saved and used in other Experiment.

4. STUDY OF DIFFERENT SHAPES OF BACTERIA, FUNGI, ALGAE, PROTOZOA USING PERMANENT SLIDES/PICTOGRAPHS

BACTERIA:

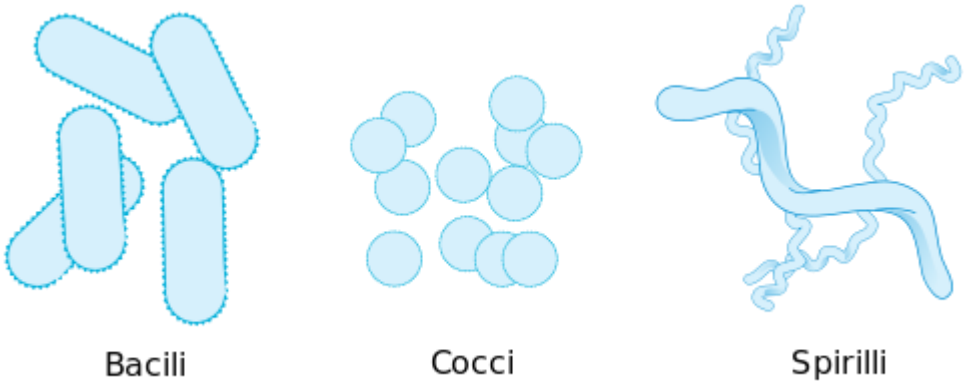
Bacteria are single-celled and are classified under the domain Prokaryota. As such, they lack membrane-bound organelles like those found in eukaryotes.

Although they are all microscopic organisms that can be found in various environments in nature, bacteria widely vary in size, shape, and arrangement.

There are several types based on their general appearance (shape) including:

Coccus Bacteria

Cocci (coccus) bacteria are some of the most common bacteria. They are spherical (or ovoid at times) in shape and are divided into; diplococcus (occur in pairs such as Neisseria spp) streptococcus (occur as a long chain or cells such as Streptococcus pneumoniae) and staphylococcus where they occur in clusters (e.g. staphylococcus saprophyticus). Cocci may also occur in tetras or in packets of 8 to form a structure that appears like a cube such as the sarcina bacteria.



simple diagram that shows the 3 main morphologic forms of bacteria by Mariana ruiz (LadyofHats)

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Bacillus

Unlike coccis bacteria, bacillus will appear as elongated rods (rod-like) when viewed under the microscope. In most cases, the bacilli occur as single cells (e.g. Mycobacterium tuberculosis), but may occur in pairs (diplobacillus) or form chains commonly refered to as streptococcus (e.g. Bacillus cereus).

Spirilla

vibrio bacteria appear comma shaped, spirilla are the type that appear spiral in shape. While some people may confuse the two when viewed under the microscope, they are different when students compare them under high magnification.

Example of vibrios includes vibrio vulnificus and Vibrio harveyi while some examples of Spirilla include members of the Campylobacter species.

***** Spirilla have flagella (on both ends), which they use to move in water or aquatic environment. This allows them to move faster in such environments compared to other bacteria. On the other hand, Vibrio have a flagellum on one end that allows them to move about.

FUNGI:
Fungi belong to their own kingdom (Kingdom Fungi). Compared to higher plants and animals, they obtain their nutrition through a range of ways including degradation of organic material and symbiosis (as lichen) among others.

Examples include:

- Molds
- Penicillin
- Yeast
- Truffles

- Mushrooms

The structure of fungi can be explained in the following points:

1. Almost all the fungi have a filamentous structure except yeast the cells.
2. They can be either single-celled or multicellular organism.

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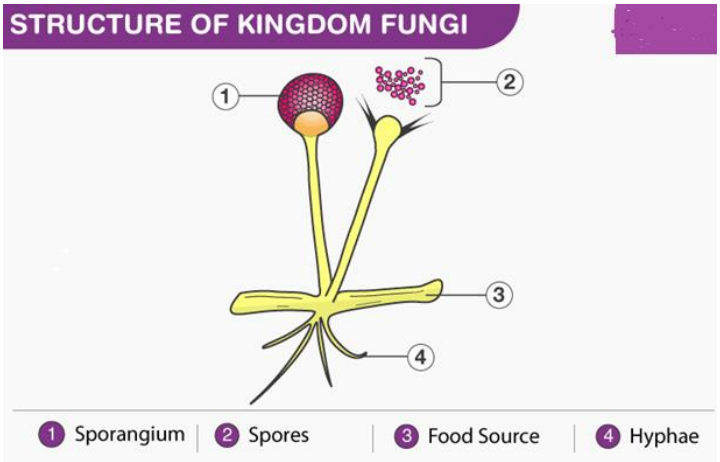
3. Fungi consist of long thread-like structures known as hyphae. These hyphae together form a mesh-like structure called mycelium.
4. Fungi possess a cell wall which is made up of chitin and polysaccharides.
5. The cell wall comprises of protoplast which is differentiated into other cell parts such as cell membrane, cytoplasm, cell organelles and nuclei.
6. The nucleus is dense, clear, with chromatin threads. The nucleus is surrounded by a nuclear membrane.



ALGAE:

Algae is a term that describes a large and incredibly diverse group of eukaryotic, photosynthetic life forms which includes multicellular seaweeds and unicellular diatoms. These organisms do not share a common ancestor and hence, are not related to each other (polyphyletic).

- Most algae require a moist or watery environment, hence, they are very common near or inside water bodies. Anatomically, they are similar to another major group of photosynthetic organisms – the land plants. However, that is where the differences end as algae lack many structural components typically present in plants, such as true stems, shoots, and leaves. Furthermore, they also do not have vascular tissues to circulate essential nutrients and water throughout their body.

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- Algae are photosynthetic organisms.
- Algae can be either unicellular or multicellular organisms.
- Algae lack a well-defined body, so, structures like roots, stems or leaves are absent.
- Algaes are generally found in the tissues of other plants or on the rocks in the moist places.
- Reproduction in algae occurs in both asexual and sexual forms. Asexual reproduction occurs by spore formation.
- Most of them are generally found in water and are aquatic in nature, especially plankton. These are of a free-floating type.

Examples of Algae

Prominent examples of algae include:

- Nostoc
- Fucus
- Diatoms
- Spirogyra

PROTOZOA:

Protozoa are one-celled animals found worldwide in most habitats. Most species are free living, but all higher animals are infected with one or more species of protozoa. Infections range from asymptomatic to life threatening, depending on the species and strain of the parasite and the resistance of the host.

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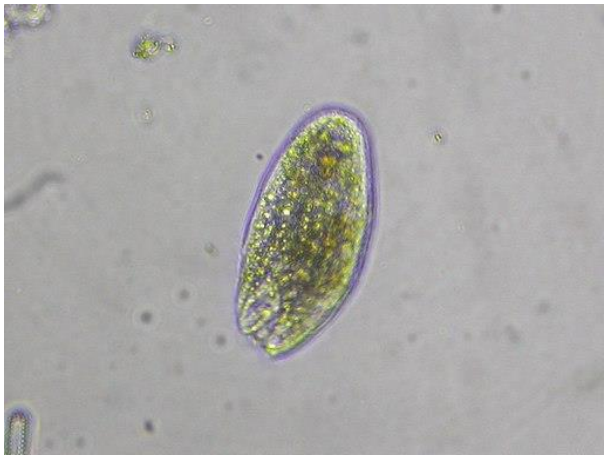
Structure



Protozoa are microscopic unicellular eukaryotes that have a relatively complex internal structure and carry out complex metabolic activities. Some protozoa have structures for propulsion or other types of movement.

Examples include:

- Giardia
- Trypanosoma
- Trichonympha
- Plasmodium
- Paramecium

Results:

5. STAINING OF BACTERIA USING GRAM STAIN

Objectives:

- To differentiate between the two major categories of bacteria: Gram positive and Gram negative.
- To understand how the Gram stain reaction affects Gram positive and Gram negative bacteria based on the biochemical and structural differences of their cell walls.

Principle:

Staining is an auxiliary technique used in microscopic techniques used to enhance the clarity of the microscopic image.Stains and dyes are widely used in the scientific field to highlight the structure of the biological specimens, cells, tissues etc.

The most widely used staining procedure in microbiology is the Gram stain, discovered by the Danish scientist and physician **Hans Christian Joachim Gram** in 1884. Gram staining is a differential staining technique that differentiates bacteria into two groups: gram-positives and gram-negatives. The procedure is based on the ability of microorganisms to retain color of the stains used during the gram stain reaction. Gram-negative bacteria are decolorized by the alcohol, losing the color of the primary stain, purple. Gram-positive bacteria are not decolorized by alcohol and will remain as purple. After decolorization step, a counterstain is used to impart a pink color to the decolorized gram-negative organisms.

. Gram positive bacteria: Stain dark purple due to retaining the primary dye called Crystal Violet in the cell wall.

Example: *Staphylococcus aureus*

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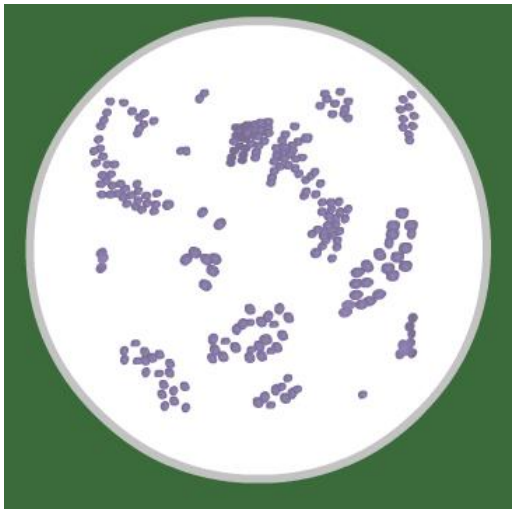

Fig: Gram positive bacteria

2. **Gram negative bacteria**: Stain red or pink due to retaining the counter staining dye called Safranin.

Example: *Escherichia coli*

Fig: Gram negative bacteria

Bacterial Morphology:

Bacteria are very small unicellular microorganisms ubiquitous in nature. They are micrometers (1µm = 10^{-6} m) in size. They have cell walls composed of peptidoglycan and reproduce by binary fission. Bacteria vary in their morphological features.

The most common morphologies are:

Coccus (pleural: Cocci):

Spherical bacteria; may occur in pairs (diplococci), in groups of four (tetracocci), in grape-like clusters (*Staphylococci)*, in chains (*Streptococci*) or in cubical arrangements of eight or more (sarcinae).
For example: *Staphylococcus aureus, Streptococcus pyogenes*

Bacillus (pleural: Bacilli):

Rod-shaped bacteria; generally occur singly, but may occasionally be found in pairs (diplo-bacilli) or chains (streptobacilli).
For example: *Bacillus cereus, Clostridium tetani*

Spirillum (pleural: Spirilla):

Spiral-shapedbacteria

For example: *Spirillum, Vibrio, Spirochete* species.

Materials Required:

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

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Reagents:

- Primary Stain - Crystal Violet
- Mordant - Grams Iodine
- Decolourizer - Ethyl Alcohol
- Secondary Stain - Safranin

Procedure:

- Flood air-dried, heat-fixed smear of cells for 1 minute with **crystal violet** staining reagent. Please note that the quality of the smear (too heavy or too light cell concentration) will affect the Gram Stain results.
- Wash slide in a gentle and indirect stream of tap water for 2 seconds.
- Flood slide with the mordant: **Gram's iodine.** Wait 1 minute.
- Wash slide in a gentle and indirect stream of tap water for 2 seconds.
- Flood slide with **decolorizing agent (Acetone-alcohol decolorizer)**. Wait 10-15 seconds or add drop by drop to slide until decolorizing agent running from the slide runs clear.
- Flood slide with a counterstain, **safranin**. Wait 30 seconds to 1 minute.
- Wash slide in a gentile and indirect stream of tap water until no color appears in the effluent and then blot dry with absorbent paper.
- Observe the results of the staining procedure under oil immersion (100x) using a Bright field microscope.

Results:

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6. ISOLATION OF PURE CULTURES OF BACTERIA BY STREAKING METHOD

Objective:

- To obtain isolated microbial colonies from an inoculum by creating areas of increasing dilution on an agar petriplate.

Principle:

The streak plate method is a rapid qualitative isolation method. The techniques commonly used for isolation of discrete colonies initially require that the number of organisms in the inoculums be reduced. It is essentially a dilution technique that involves spreading a loopful of culture over the surface of an agar plate. The resulting diminution of the population size ensures that, following inoculation, individual cells will be sufficiently far apart on the surface of the agar medium to effect a separation of the different species present. Although many type of procedures are performed, the four ways or quadrant streak is mostly done.

Materials Required:

- Mixed culture of bacteria.
- Sterile petri dish with appropriate bacterial media(such as trypticase soy agar, nutrient agar).
- Inoculating loop (usually nichrome, a nickel-chromium alloy, or platinum; it may also be a single-use disposable plastic loop, which would be discarded between sectors rather than resterilized).
- Bunsen burner.
- Marking pen.

Procedure:

All the process is done in a laminar air flow cabinet aseptically.
Petri dishes are labelled on the bottom rather than on the lid. Write close to the edge of the bottom of the plate to preserve area to observe the plate after it has incubated. Labels usually include the organism name, type of agar, date, and the plater's name or initials. Using sterile cotton swabs, remove any visible water on the agar in the plate or around the inner rim of the petri plate. Observe the plate and mentally

divide it into three sectors. The plate will then be turned clockwise (if you are right handed) with the agar side up. The second sector will then be at the top for streaking and then the plate is turned again so that the third sector can be streaked.

Sterilize the Transfer Loop before Obtaining a Specimen:

To streak a specimen from a culture tube, metal transfer loops are first sterilized by flaming the wire loop held in the light blue area of a Bunsen burner just above the tip of inner flame of the flame until it is red-hot. If a hot incinerator is available, the loop may be sterilized by holding it inside the incinerator for 5 to 7 seconds. Once sterile, the loop is allowed to cool by holding it still. Do not wave it around to cool it or blow on it. When manipulating bacteria, transfer loops are usually held like a pencil. If plastic disposable loops are being utilized, they are removed from the packaging to avoid contamination and after being used, are discarded into an appropriate container. A new loop is recommended for each sector of an isolation streak plate.

Open the culture tube and collect a sample of specimen using the sterile loop:

Isolation can be obtained from any of a variety of specimens. This protocol describes the use of a mixed broth culture, where the culture contains several different bacterial species or strains. The specimen streaked on a plate could come in a variety of forms, such as solid samples, liquid samples, and cotton or foam swabs. Material containing possibly infectious agents should be handled appropriately in the lab using bio safety procedure.
Remove the test tube cap. It is recommended that the cap be kept in your right hand (the hand holding the sterile loop). Curl the little finger of your right hand around the cap to hold it or hold it between the little finger and third finger from the back. Modern test tube caps extend over the top of the test tube, keeping the rim of the test tube sterile while the rim of the cap has not been exposed to the bacteria. The cap can also be placed on the disinfected table, if the test tube is held at an angle so that air contamination does not fall down into the tube. Insert the loop into the culture tube and remove a loopful of broth. Replace the cap of the test tube and put it back into the test tube rack.

Streak the Plate:

The lid of the agar plate has to be opened just sufficiently enough to streak the plate with the inoculation loop. Minimize the amount of agar and the length of time the agar is exposed to the environment during the streak process.

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Three Sector Streak (t streak):

- Sterilize the wire loop.
- Cool the loop by touching it on the edge of the sterile agar plate.
- Dip the loop into the broth culture containing the mixture of bacteria.
- Lift the lid of the plate just enough to insert the loop. Drag the loop over the surface of the top one-third of the plate back and forth in a "zig-zag" formation.
- The loop has picked up thousands of bacteria which are spread out over the surface of the agar.
- Sterilize the loop in the flame.
- Turn the plate 90 degrees and drag the loop through the area you have just streaked two to three times and continue to drag the loop in a "zig-zag" formation in the remaining half of the plate without touching that area again.
- Sterilize the loop in the flame.
- Turn the plate 90 degrees. Repeat the procedure. Drag the loop two to three times through the area you just streaked, and fill in the remaining area of the plate (zig-zag formation), being very careful not to touch any of the areas you previously streaked.
- Incubate the plate for 24 hours. If you streaked correctly, you will see isolated colonies in the third sector. The heaviest growth will be in the first sector. There will be less growth and some isolated colonies in the second sector. The third area should have the least growth with isolated colonies.

Four Quadrant Streak :

- Loosen the cap of the bottle containing the inoculum.
- Hold an inoculation loop in your right hand.
- Flame the loop and allow it to cool.
- Lift the test tube containing the inoculum with your left hand.
- Remove the cap/ cotton wool plug of the test tube with the little finger of your right hand.
- Flame the neck of the test tube.
- Insert the loop into the culture broth and withdraw. At all times hold the loop as still as possible.
- Flame the neck of the test tube again.
- Replace the cap/ cotton wool plug of the test tube using the little finger of your right hand. Place the test tube in a rack. For a liquid culture, dip the loop into the broth, or for solid media, lightly touch a colony with the loop.
- Partially lift the lid of the Petri dish containing the solid medium.

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Place a loopful of the culture on the agar surface on the area 1. Flame the loop and cool it for 5 seconds by touching an unused part of the agar surface close to the periphery of the plate, and then drag it rapidly several times across the surface of area1.

- Remove the loop and close the Petri dish.
- Reflame and cool the loop, and turn the petri dish 90°C then touch the loop to a corner of the culture in area1 and drag it several times across the agar in area 2, hitting the original streak a few times. The loop should never enter area 1 again.
- Remove the loop and close the Petri dish.
- Reflame and cool the loop and again turn the dish 90°C anticlockwise. streak area 3 in the same manner as area 2, hitting last area several times.
- Remove the loop and close the Petri dish.
- Flame the loop, again turn the dish 90°C and then drag the culture from a corner of a area3 across area 4, contacting area 3 several times and drag out the culture as illustrated. Using a wider streak. Do not let the loop touch any of the previously streaked areas. The flaming of the loop at the points indicated is to effect the dilution of the culture so that fewer organisms are streaked in each area, resulting in the final desired separation.
- Remove the loop and close the Petri dish.
- Tape the plate closed and incubates the plate in an inverted position in an incubator for 24-48 hours.
- Flame the loop before putting it aside.

Fig 1*: Staphylococcus aureus* colony

RESULTS

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7. ESTIMATION OF COLONY FORMING UNIT(CFU) COUNT

AIM:

To estimate the colony forming units of the given bacterial culture using spread plate and pour plate technique.

Principle:

Two methods of this examinations are differentiated: 1) In the pour–plate method, a sample from an accurate dilution of microbes/sample is pipetted onto a Petri - dish, then agar medium is poured over the liquid and mixed. 2) In the spread–plate method, generally 0.1ml of the diluted sample is pipetted onto the surface of a solidified agar medium and spread with a sterilized, bent, glass rod. The theory behind the technique of CFU establishes that a single microbe can grow and become a colony via division. These colonies are clearly different from each other, both microscopically and macroscopically. This technique allows the user to know how many CFU's are present per mL in the sample. Therefore, it enables us to know the microbiological load and the magnitude of the infection in humans and animals, or the degree of contamination in samples of water, vegetables, soil or fruits and in industrial products and the equipment.

Viable Count

The most common procedure for the enumeration of bacteria is the viable plate count. In this method, serial dilutions of a sample containing viable microorganisms are plated onto a suitable growth medium. The suspension is either spread onto the surface of agar plates (spread plate method), or is mixed with molten agar, poured into plates, and allowed to solidify (pour plate method). The plates are then incubated under conditions that permit microbial reproduction so that colonies develop that can be seen without the aid of a microscope. It is assumed that each bacterial colony arises from an individual cell that has undergone cell division. Therefore, by counting the number of colonies and accounting for the dilution factor, the number of bacteria in the original sample can be determined.

There are several drawbacks to the viable count method. The major disadvantage is that it is selective and therefore biased. The nature of the growth conditions, including the composition and pH of the medium used as well as the conditions such as temperature, determines which bacteria in a mixed population can grow. Since there is no universal set of conditions that permits the growth of all microorganisms, it is impossible to enumerate all

microorganisms by viable plating. This same disadvantage, however, becomes advantageous when one is interested in only a specific microbial population. For example, we can design selective procedures for the enumeration of coliforms and other physiologically defined microbial groups.

The viable count is an estimate of the number of cells. Because some organisms exist as pairs or groups and because mixing and shaking of the sample does not always separate all the cells, we actually get a count of the "colony forming units". One cell or group of cells will produce one colony, therefore when we record results for a viable count, it is customary to record the results as colony forming units per ml (cfu/ml) or per gram (cfu/g) of test material.

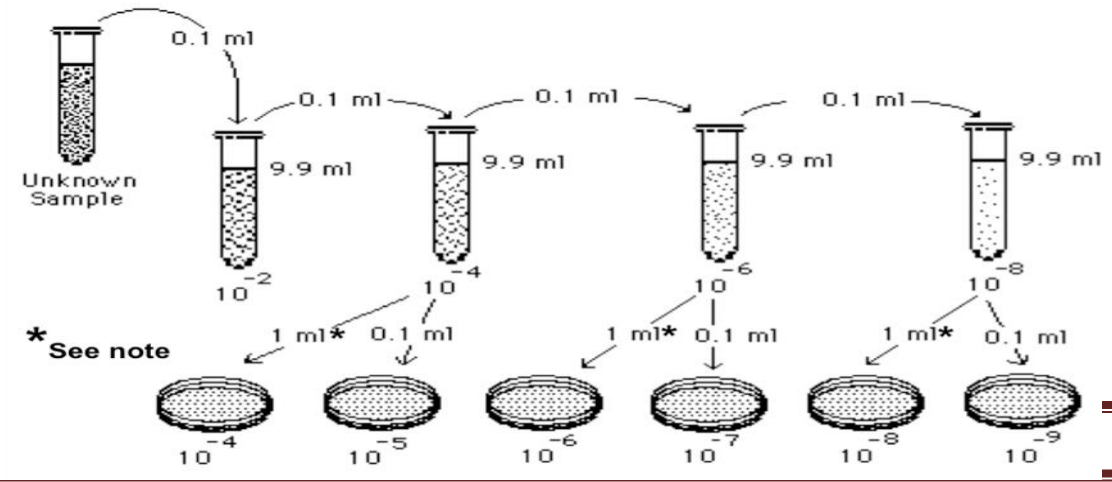
Because we generally have no idea of how many bacteria are in a sample, it is almost always necessary to prepare a dilution series to ensure that we obtain a dilution containing a reasonable number of bacteria to count. Dilutions in the range 10^{-1} (1/10) to 10^{-8} (1/100,000,000) are generally used, although with particular types of samples the range of dilutions can be restricted. For example, for water that is not turbid, the maximal dilution needed is 10^{-6} because we know that if there were 10^{7} or more bacteria per milliliter, the water would be turbid.

Viable Plate Count

1. Label four 9.9 ml saline tubes 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8}, respectively. Label six BHI plates 10^{-4} to 10^{-9}.

2. Vortex the unknown sample to ensure an even distribution of bacteria. Aseptically remove 0.1 ml of sample with a sterile pipette and transfer it to the 10^{-2} dilution tube (see diagram).

3. Vortex the 10^{-2} tube and transfer 0.1 ml to the 10^{-4} tube



4. Again vortex the 10^{-4} dilution tube and transfer 0.1 ml to the 10^{-6} tube. Vortex this last tube well.

5. Vortex the 10^{-6} tube, transfer 0.1 ml to 10^{-8} tube and vortex again.

6. Using a new sterile pipette, aseptically transfer 1.0 ml from the 10^{-4} dilution tube to the plate labeled 10^{-4} and 0.1 ml to the plate labeled 10^{-5}. Spread the inoculum on the surface of the agar in each plate using an alcohol-dipped, flamed, metal spreader. Dip the spreader into the alcohol jar and quickly take it through the flame and let the alcohol burn off after each spreading. Do not allow the spreader to get too hot. Never hold the spreader in the flame for more than a second

7. Repeat the above to transfer 1.0 and 0.1 ml from the 10^{-6} dilution tube to the 10^{-6} and 10^{-7} plates, respectively. In the same manner establish the 10^{-8} and 10^{-9} plates. Do not discard your dilution tubes. (See note above)

8. Allow the surface of the agar to dry before you move or invert the plates.
Incubate the plates at 37°C for 2 days.

9. Serial dilutions are used to calculate the concentration of microorganisms. As it would usually be impossible to actually count the number of microorganisms in a sample, the sample is diluted and plated to get a reasonable number of colonies to count. Since each colony on an agar plate theoretically grew from a single microorganism, the number of colonies or Colony Forming Units is representative of the number of viable microorganisms. Since the dilution factor is known, the number of mic ml in the original sample can be calculated.

   For the example above, the countable plate had 200 colonies, so there were 200 CFU, and the FDF was 1/4000.

   - 200 CFU x 1/1/4000 = 200 CFU x 4000 = 800000 CFU/ml = 8 x 10
   - CFU/ml in the original sample

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