UNIT – 1
Fundamentals of Microbiology

This includes eukaryotes (organisms with a nucleus), such as fungi and protists, and prokaryotes (organisms without a nucleus), such as bacteria.

A fundamental understanding of how a cell works has come through the study of microorganisms. Microbiologists study microbes at the level of the community (ecology and epidemiology), at the level of the cell (cell biology and physiology) and at the level of proteins and genes (molecular biology).

Microorganisms are extremely important in our everyday lives. Some are responsible for a significant proportion of the diseases. Still others play an essential role in industry, where their unique properties have been harnessed in the production of food, beverages and antibiotics. Scientists also have learned how to exploit microorganisms in the field of molecular biology, which makes an enormous impact both industrially and medically. Microbiology also encompasses immunology, the study of the body's ability to mount defences against infectious microbes.

Because microbiology, by definition, studies organisms not visible to the naked eye, we can consider late-17th-century Dutch scientist Antony van Leeuwenhoek the father of the discipline. Leeuwenhoek was the first person to describe tiny cells and bacteria, and he invented new methods for grinding and polishing microscope lenses that allowed for curvatures providing magnifications of up to 270 diameters, the best available lenses at that time. But while van Leeuwenhoek is cited as the first microbiologist, the first recorded microbiological observation — the fruiting bodies of molds — was made earlier, in 1665, by English physicist Robert Hooke.

Other notable people in the history of science who made fundamental discoveries about microorganisms are 19th-century scientists Louis Pasteur and Robert Koch, who are considered the founders of medical microbiology. Pasteur is most famous for his series of experiments designed to disprove the then-widely held theory of spontaneous generation, which solidified microbiology's identity as a biological science. Pasteur also designed methods for food preservation (pasteurization) and vaccines against several diseases, such as anthrax, fowl cholera and rabies. Koch is best known for his contributions to the germ theory of disease, proving that specific diseases were caused by specific pathogenic microorganisms. He developed a series of criteria that have become known as Koch's postulates. Koch was one of the first scientists to focus on the isolation of bacteria in pure culture, resulting in his description of several novel bacteria, including Mycobacterium tuberculosis, the causative agent of tuberculosis.

Finally, some of the most important discoveries affecting public health occurred in the 20th century, such as the discovery of penicillin by Alexander Fleming, which started a rush to find other natural, and eventually synthetic, antibiotics; the development of vital vaccines, including those for polio and yellow fever; and the birth of molecular biology, which happened in the 1940s with the study of bacteria.

Louis Pasteur

Louis Pasteur is also known as father of microbiology. He has many contributions to microbiology:

1. He has proposed the principles of fermentation for preservation of food
2. He introduced sterilization techniques and developed steam sterilizer, hot air over and autoclave.

Robert Koch

Robert Koch provided remarkable contributions to the field of microbiology:

1. he used of solid media for culture of bacteria-Eilshemius Hesse, the wife of Walther hesse, one of Koch's assistants had suggested the use of agar as solidifying agent.
2. Koch's phenomenon: Robert Koch observed that guinea pigs already infected with tubercle bacillus developed a hypersensitivity reaction when injected with tubercle bacilli or its protein. This reaction is called Koch's phenomenon.

Paul Ehrlich

1. He was the first to report the acid-fast nature of tubercle bacillus.
2. He developed techniques to stain tissues and blood cells.
3. He proposed a toxin antitoxin interaction called as Ehrlich phenomenon and also introduced methods of standardising toxin and antitoxin.

MICROSCOPY

A good microscope should have three properties:

1. Good resolution: Resolution power refers to the ability to produce separate images of closely placed objects so that they can be distinguished as two separate entities. The resolution power of :

- Unaided human eye is about 0.2 mm (200 μm)
- Light microscope is about 0.2 μm
- Electron microscope is about 0.5 nm

Resolution depends on refractive index. Oil has a higher refractive index than air.

2. Good contrast: This can be further improved by staining the specimen.
3. Good magnification: This is achieved by use of concave lenses.

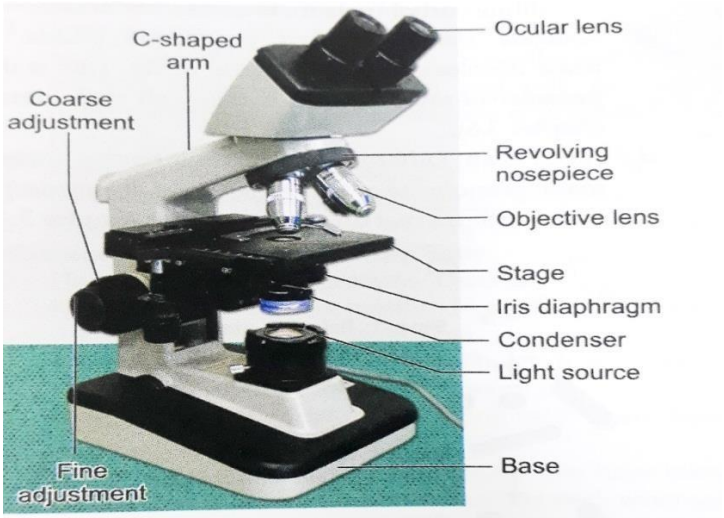


Fig. 1.1.2: Bright-field microscope

Source: Nikon Alphaphot (*with permission*).

Bright-Field or Light Microscope

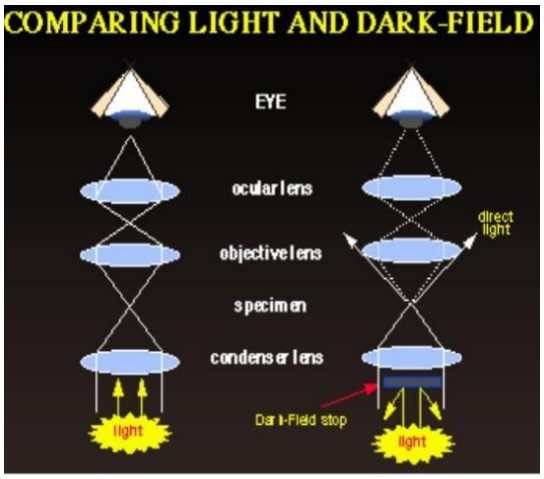
The bright-field or light microscope forms a dark image against a brighter background. Microbiology and bacteriology have always relied on the bright field technique. Different stains and staining techniques are used depending upon the type of specimen and cell structure being examined.

For example:

- Fuchsin is used to stain smooth muscle cells
- Gram stain is used on bacteria and gives rise to the name gram-negative or gram-positive bacteria based on the reaction of the bacteria to the stain.

Dark Field Microscope

- Principal: In dark field microscope, the object appears bright against a dark background. This is made possible by use of a special dark field condenser.
- Applications: It is used to identify the living, unstained cells and thin bacteria like spirochetes which cannot be visualized by light microscopy.
- In 1830, J.J. Lister invented the dark field microscope in which the standard bright field condenser is replaced with a single or double reflecting dark field condenser. In 1906 in Vienna, Karl Landsteiner and Viktor Mucha were the first to use dark field microscopy to visualize T.pallidum from syphilis lesions.



Comparison between Bright Field and Dark Field Microscope

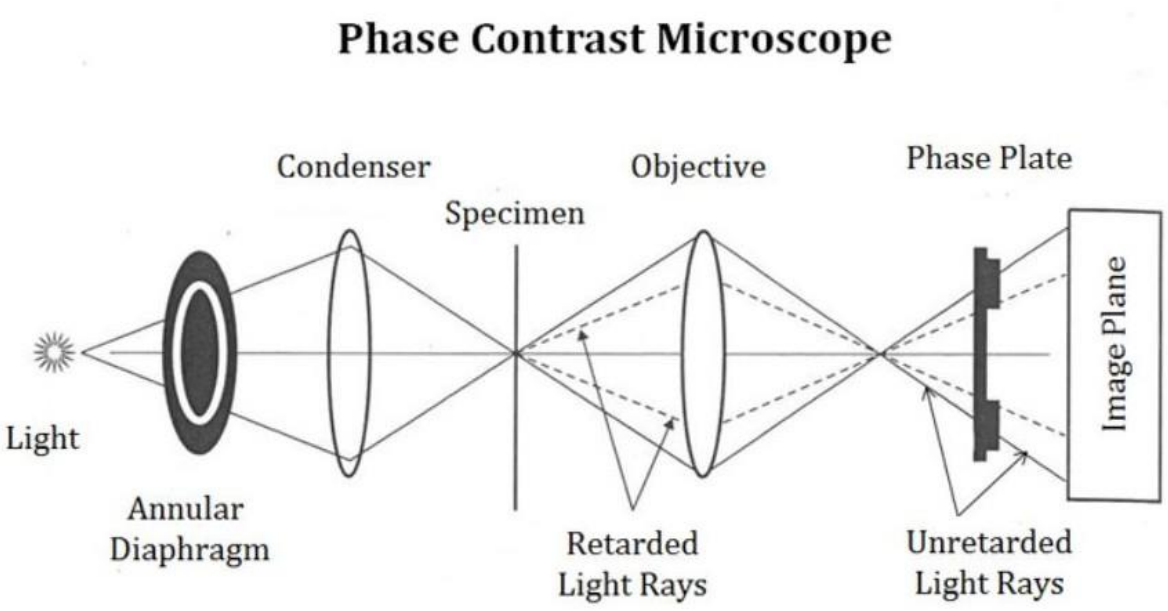
BRIGHT FIELD MICROSCOPE	DARK FIELD MICROSCOPE
Light from a plane wave source is focused through an object by a condenser.	An opaque disc is put between the source and the condenser blocking out the middle of the beam.
Some light is blocked (absorbed) by opaque parts of the object, or reflected away at the boundaries between components or materials of different refractive indices.	The condenser focuses the beam onto the sample.
The remainder passes through the objective lens to the observer.	No light enters the objective directly from the source. Light from the beam is scattered by the sample- some scattered on to the objective.
This produces a bright background with object details appearing darker in the image than their surroundings. The brightness of the image is determined by the source brightness and block size.	Only light scattered by the object enters the objective. This produces a dark background with sample details appearing brighter than surroundings. The brightness of the brightest parts of the image is determined by the amount of light scattered by the object.
This result in poorer contrast compared to dark field as the dark areas are generally grey rather than black.	This results in superior contrast to bright field as dark areas may be completely black while increasing the brightness of the source brightens the areas.

Phase Contrast Microscope

The phase contrast microscope is used to visualise unstained living cells. Most of the stains or staining procedures will kill the cells. Phase contrast microscopy enables the visualization of living cells and life events.

The phase contrast microscope was developed by Dutch physicist Frits Zernike in early 1930s. the invention of this microscope enables us to visualise live cells and cellular processes. The inventor was awarded by Noble prize in physics in 1953.

Principle: When light passes through cells, small phase shifts occur, which are invisible to the human eye. In a phase-contrast microscope, these phase shifts are converted into changes in amplitude, which can be observed as differences in image contrast.



Working of Phase contrast microscope:

1. Partially coherent illumination produced by the tungsten-halogen lamp is directed through a collector lens and focused on a specialized annulus (labeled condenser annulus) positioned in the substage condenser front focal plane.
2. Wavefronts passing through the annulus illuminate the specimen and either pass through undeviated or are diffracted and retarded in phase by structures and phase gradients present in the specimen.
3. Undeviated and diffracted light collected by the objective is segregated at the rear focal plane by a phase plate and focused at the intermediate image plane to form the final phase-contrast image observed in the eyepieces.

Parts of Phase contrast Microscope:

Phase-contrast microscopy is basically a specially designed light microscope with all the basic parts in addition to which an annular phase plate and annular diaphragm are fitted.

The annular diaphragm

1. It is situated below the condenser.
2. It is made up of a circular disc having a circular annular groove.
3. The light rays are allowed to pass through the annular groove.
4. Through the annular groove of the annular diaphragm, the light rays fall on the specimen or object to be studied.
5. At the back focal plane of the objective develops an image.
6. The annular phase plate is placed at this back focal plane.

The phase plate

1. It is either a negative phase plate having a thick circular area or a positive phase plate having a thin circular groove.
2. This thick or thin area in the phase plate is called the conjugate area.
3. The phase plate is a transparent disc.
4. With the help of the annular diaphragm and the phase plate, the phase contrast is obtained in this microscope.
5. This is obtained by separating the direct rays from the diffracted rays.
6. The direct light rays pass through the annular groove whereas the diffracted light rays pass through the region outside the groove.
7. Depending upon the different refractive indices of different cell components, the object to be studied shows a different degree of contrast in this micro•scope.

Applications of Phase contrast Microscopy:

To produce high-contrast images of transparent specimens, such as

- living cells (usually in culture),
- microorganisms,
- thin tissue slices,
- lithographic patterns,
- fibers,
- latex dispersions,

- glass fragments, and
- subcellular particles (including nuclei and other organelles).

Applications of phase-contrast microscopy in biological research are numerous.

Advantages:

- Living cells can be observed in their natural state without previous fixation or labeling.
- It makes a highly transparent object more visible.
- No special preparation of fixation or staining etc. is needed to study an object under a phase-contrast microscope which saves a lot of time.
- Examining intracellular components of living cells at relatively high resolution. eg: The dynamic motility of mitochondria, mitotic chromosomes & vacuoles.
- It made it possible for biologists to study living cells and how they proliferate through cell division.
- Phase-contrast optical components can be added to virtually any brightfield microscope, provided the specialized phase objectives conform to the tube length parameters, and the condenser will accept an annular phase ring of the correct size.

Limitations:

- Phase-contrast condensers and objective lenses add considerable cost to a microscope, and so phase contrast is often not used in teaching labs except perhaps in classes in the health professions.
- To use phase-contrast the light path must be aligned.
- Generally, more light is needed for phase contrast than for corresponding bright-field viewing, since the technique is based on the diminishment of the brightness of most objects**.**

Electron Microscope

Electron microscopy (EM) is a technique for obtaining high resolution images of biological and non-biological specimens. It is used in biomedical research to investigate the detailed structure of tissues, cells, organelles and macromolecular complexes. The high resolution of EM images results from the use of electrons (which have very short wavelengths) as the source of illuminating radiation. Electron microscopy is used in conjunction with a variety of ancillary techniques (e.g. thin sectioning, immuno-labeling, negative staining) to answer specific questions. EM images provide key information on the structural basis of cell function and of cell disease. It was invented by Ernst Ruska in 1931.

There are two main types of electron microscope – the transmission EM (TEM) and the scanning EM (SEM)

1. Transmission EM (MC type, examine the internal structure, resolution 0.5 nm, gives 2-dimensional view)
2. Scanning EM (examine the surfaces, resolution 7 nm, gives 3-dimensional view)

Working Principle: An electron microscope uses an ‘electron beam’ to produce the image of the object and magnification is obtained by ‘electromagnetic fields’; unlike light or optical

microscopes, in which 'light waves' are used to produce the image and magnification is obtained by a system of 'optical lenses'.

Transmission Electron Microscope (TEM): In this microscope, an electron beam from an electron gun is transmitted through an ultra-thin section of the microscopic object and the image is magnified by the electromagnetic fields. It is used to observe finer details of internal structures of microscopic objects like bacteria and other cells.

The specimen to be examined is prepared as an extremely thin dry film or as an ultra-thin section on a small screen and is introduced into the microscope at a point between the magnetic condenser and the magnetic objective.(fig 4.13)

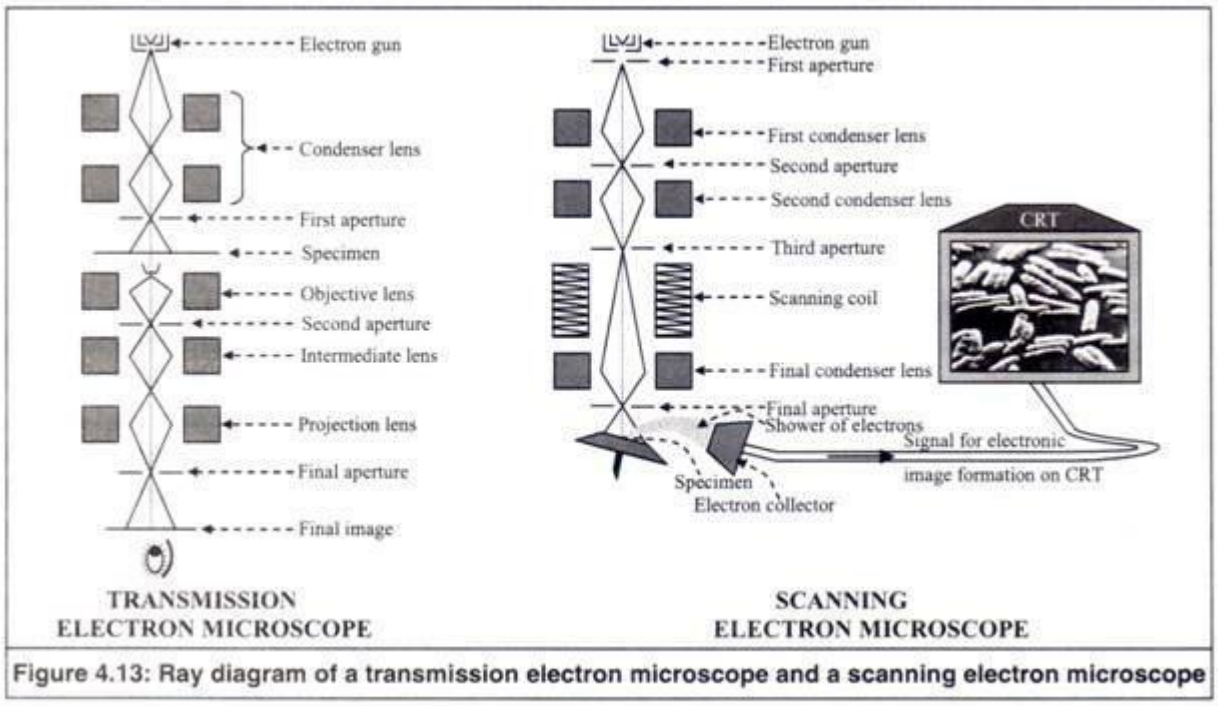


Figure 4.13: Ray diagram of a transmission electron microscope and a scanning electron microscope

The point is comparable to the stage of a light microscope. The magnified image may be viewed on a fluorescent screen through an airtight window or recorded on a photographic plate by an in-built camera. Modern variants have facility to record the photograph by digital camera.

Scanning Electron Microscope (SEM): In a scanning electron microscope, the specimen is exposed to a narrow electron beam from an electron gun, which rapidly moves over or scans the surface of the specimen (Figure 4.13). This causes the release of a shower of secondary electrons and other types of radiations from the specimen surface.

The intensity of these secondary electrons depends upon the shape and the chemical composition of the irradiated object. These electrons are collected by a detector, which generates electronic signals. These signals are scanned in the manner of a television system to produce an image on a cathode ray tube (CRT).

The image is recorded by capturing it from the CRT. Modern variants have facility to record the photograph by digital camera. This microscope is used to observe the surface structure of microscopic objects.

The limitations of electron microscopes are as follows:

(a) Live specimen cannot be observed.

(b) As the penetration power of electron beam is very low, the object should be ultra-thin. For this, the specimen is dried and cut into ultra-thin sections before observation.

Sterilization:

It is a process by which an article, surface or medium is made free of all micro-organisms either in the vegetative or spore form.

Disinfection means the destruction or removal of all pathogenic organisms or organisms capable of giving rise to infection. Antisepsis, term used to indicate the prevention of infection usually by inhibiting the growth of bacteria in wounds and tissues.

Table 1.2.1: Classification of sterilization/disinfection methods
A. Physical methods
1. Heat Dry heat: Flaming, Incineration and Hot air oven Moist heat: a. Temperature <100^0C, e.g. pasteurization, water bath and inspissation b. Temperature at 100^0C, e.g. boiling, steaming and tyndallisation c. Temperature >100^0C, e.g. membrane filters 2. Filtration: Depth filters and membrane filters 3. Radiation Ionizing radiation: Y-rays, X-rays and cosmic rays Non-ionizing radiation: Ultraviolet (UV) and infrared rays 4. Ultrasonic vibration
B. Chemical methods
1. Alcohols: Ethyl alcohol, isopropyl alcohol 2. Aldehydes: Formaldehyde, glutaraldehyde, Ortho-phthaladehyde hexachlorophene 3. Phenolic compounds: Cresol, Lysol, chlorhexidine, chloroxylenol, hexachlorophene 4. Halogens: Chlorine, iodine, iodophors 5. Oxidising agents: Hydrogen peroxide, Peracetic acid 6. Salts: Mercuric chloride, copper salts 7. Surface active agents: Quaternary ammonium compounds and soaps 8. Dyes: Aniline dyes and acridine dyes

METHODS OF STERILISATION

1. Physical method

i) Sunlight: has an active germicidal effect due to its content of UV rays. It is natural method of sterilisation in cases of water tanks, rivers and lakes.

ii) Heat: most reliable and commonly employed method of sterilisation. Two types of heat – dry and moist heat.

DRY HEAT	MOIST HEAT
Kills the organism by denaturation proteins, oxidative damage and by toxic effect of elevated levels of electrolytes.	Kills the micro-organisms by denaturation and coagulation of proteins.

- Bacterial spores are killed by moist heat at 121 C for 15 min. most vegetative bacteria, fungi and viruses are killed in 30 min at 65 C by moist heat.
- Materials containing organic substances require more time for sterilisation. Proteins, sugars, fats and starch are some of the organic substances.

DRY HEAT STERILISATION:

- Red heat: inoculating loops, tips of forceps and needles are held in the flame of a Bunsen burner till they become red hot.
- Flaming: glass slides, scalpels and mouth of culture tubes are passed through Bunsen flame without allowing them to become red hot.
- Incineration: by this method, infective materials are reduced to ashes by burning. Soiled dressings, animal carcasses, bedding and pathological materials are dealt with this method.
- Hot air oven.

MOIST HEAT STERILISATION: this method of sterilisation may be used at different temperatures as follows-

- At temperature below 100 C.

i) Pasteurisation of milk: two types of method, holder method (63 C for 30 min) and flash method (72 C for 20 sec followed by quickly cooling to 13 C or lower)are used. All non sporing pathogens such as mycobacteria and Brucella are killed except Coxiella burnetti which being relatively heat resistant may survive in holder method.

ii) Inspissation: some serum and egg media such as LOwerstein Jensens and Loefflers serum, are rendered sterile by heating at 80-85 C temperature for half an hour daily on three consecutive days.

- A**t a temperature of 100 C.**

i) Boiling:10-30 min may kill most of the vegetative forms but many spores withstand for a considerable time.

ii) Tyndallisation: steam at 100 C for 20 min on three consecutive days is used.

iii) Steam steriliser at 100 C for 90 min: Kochs or Arnolds steam steriliser is usually used for media which are decomposed at high temperature of autoclave.

- **At temperature above 100 C.**

i) Filtrations: this method is used for substances which get damaged by heat process eg sera, sugars, antibiotic solution etc.

Uses:

- to sterile sera sugars and antibiotic solution
- Separation of toxins and bacteriophages.
- To obtain bacteria free filtrates of clinical samples of virus isolation.
- Sterilisation of hydatid fluid.
- Purification of water.

RADIATIONS

1. Ionising Radiations: include gamma rays , X- rays and cosmic rays. They are highly lethal to all cells including bacteria. They damage DNA. Gamma radiations from cobalt 60 source are commercially used for sterilisation of disposable items such as swabs, cultures, catheters, cannulas plastic syringes. This method is known as cold sterilisation because3 there is no appreciable increase in temperature.

2. Non ionising radiations: include UV radiation. Ultraviolet rays of wavelength 240- 280 nm have marked bactericidal activity. It acts by denaturation of proteins and interfere with DNA replication.

CHEMICAL METHODS OF STERILISATION

ALCOHOLS: ethyl alcohol and isopropyl alcohol are the most frequently used. They act by denaturing bacterial proteins. They rapidly kill bacteria including tubercle bacilli but they have no sporicidal or virucidal activity.
HIV (human immunodeficiency virus) is susceptible to 70% ethyl alcohol and 35% isopropyl alcohol in the absence of organic matter.

ALDEHYDES: markedly bactericidal, sporicidal and virucidal. Formaldehyde – it is used as both aqueous solution and gaseous solution. Used to prevent tissue for histological examination.. To sterile bacterial vaccines, to prepare toxoids from toxins.

Glutaraldehyde- effective against bacteria, fungi and viruses (including HIV , hepatitis B viruses and enteroviruses), it also kills spores. Used for sterilisation of cystoscopes, endoscopes and bronchoscopes. To sterilise plastic endotracheal tubes, face masks, corrugated rubber anaesthetic tubes and metal instruments.

Ortho-phthalaldehyde (OPA)- is a high level disinfectant. More stable during storage and more rapidly mycobactericidal than glutaraldehyde.

HALOGENS: chlorine and iodine are two commonly used disinfectants. These are bactericidal and are effective against sporing bacteria and viruses. Chlorine is used in water supplies, swimming pools and dairy industries. Iodine in alcoholic and aqueous solutions is used as skin disinfectant. It is actively bactericidal with moderate action against viruses. Like chlorine it is inactivated by inorganic matter.

SALTS: salts of heavy metals have toxic effect on bacteria. The salts of copper, silver and mercury are used as disinfectants. They are protein coagulants and act by combining with sulphydryl groups of bacterial proteins and other essential intracellular compounds.
Copper salts are used as fungicides. Mercuric chloride once used as disinfectant, is highly toxic.

DYES: two groups of dyes, aniline dyes and acridine dyes have been used extensively as skin and wound antiseptics. Both are bacteriostatic in high dilution but low bactericidal action. Aniline dyes include crystal violet, brilliant green and malachite green –they are more active against Gram positive bacteria than Gram negative bacteria.

Acridine dyes include acriflavine, euflavine, proflavine and aminacrine. They are affected very little by the presence of organic material. They are also more active against Gram positive bacteria than Gram negative bacteria but are not used as selective as the aniline dyes.

OXIDISING AGENTS:

i) Hydrogen peroxide: is effective against most organisms at concentration of 3-6%, while it kills all organisms including spores at higher concentration. Mode of action is by liberation of free hydroxyl radical on decomposition of H_2O_2. These free radicals are the active ingredient in the disinfection process. H_2O_2 is used to disinfect contact lenses, surgical prostheses and plastic implants. It is used for high level disinfection and sterilisation.

ii) Peracetic Acid: is an oxidising agent. It is one of the high level disinfectants. It is effective in the presence of organic matter. It is a more potent germicidal agent than hydrogen peroxide. The end products of this agent are non-toxic. It is also used in plasma sterilisation procedure.

SURFACE ACTIVE AGENTS:

Quaternary ammonium compounds are the most important cationic surfactants. Although these compounds are bactericidal for a wide range of organisms, Gram positive species are more susceptible. They have no action on spores and tubercle bacilli. They are active against viruses with liquid envelopes and much less against non-enveloped viruses.

UNIT 2

MORPHOLOGY OF BACTERIA

Table 1.1.6: Shape of bacteria and their Gram staining property	
Cluster	Staphylococcus
Chain	Streptococcus
Pairs, lanceolate shaped	Pneumococcus
Tetrads	Micrococcus
Octate	Sarcina
Pair or inj short chain, spectacle eyed shaped	Enterococcus
Gram-positive bacilli arranged in	
Chain (bamboo stick appearance	Bacillus anthracis
Chinese letter arrangement	c. diphtheriae
Pallisade arrangement	Diphtheroids
Branching and filamentous form	Actinomycetes
Pairs, lens shaped	Meningococcus
Pairs, kidney shaped	Gonococcus
Gram-negative bacilli arranged in	
Pleomorphic (various shapes)	Haemophilus, Proteus
Thumb print appearance	Bordetella pertussis
Curved	Campylobacter (Gull-wing shaped) and Helicobacter
Spirally coiled, rigid	Spirillum
Spirally coiled, flexible	Spirochetes
Comma shaped (fish in stream)	Vibrio cholerae
Chain	Streptobacillus

Bacterial Cell Wall

The cell wall is a tough and rigid structure, surrounding the bacterium. Apart from providing protection and conferring rigidity, certain parts of cell wall (e.g. LPS) are immunogenic and act as virulence factor.

- Peptidoglycan is the main component of the cell wall which makes it rigid. It is composed of alternate units of N-acety1 muramic acid (NAM) and N-acetyl glucosamine (NAG) molecules; cross linked to each other via tetrapeptide side chains and pentaglycine bridges.
- Gram-positive bacteria has a thicker peptidoglycan and contains teichoic acid.
- Gram-negative bacteria-peptidoglycan layer is thin, and it contains additional parts such as (1) Outer membrane, (2) Lipopolysaccharide (LPS) which in turn consists of (i) Lipid A or endotoxin, (ii) core polysaccharide, (iii) O side chain

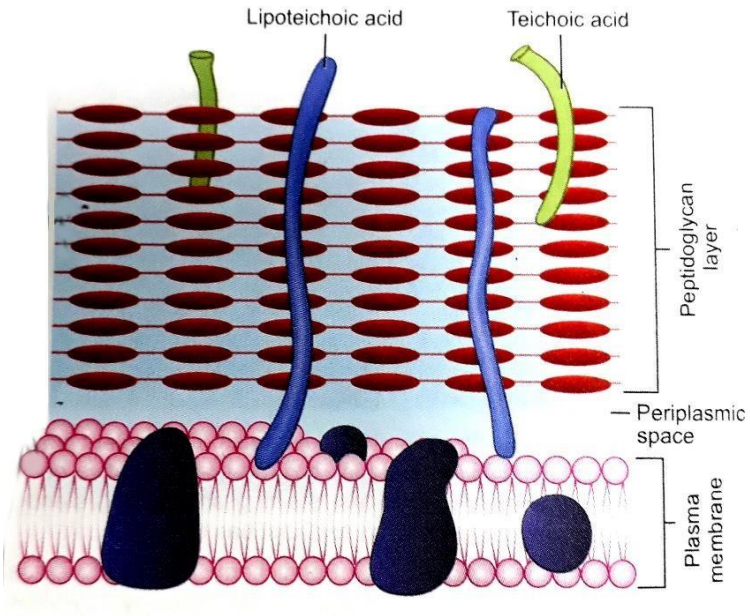


Fig. 1.1.5: Structure of Gram-positive cell wall

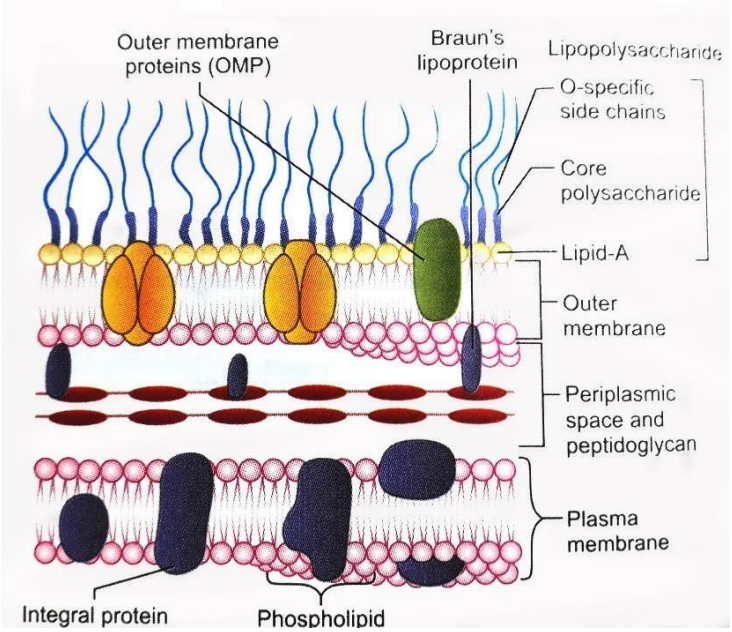


Fig. 1.1.5: Structure of Gram-positive cell wall

Nucleoid

Bacteria do not have a true nucleus; but the genetic material is located in an irregularly shaped region called the nucleoid.

- There is no nuclear membrane or nucleolus and lacks basic proteins.
- Possess a single haploid chromosome, comprising of super coiled circular ds DNA (except two chromosomes in vibrio)
- Bacterial DNA divides by simple binary fission.
- The nucleoid can be seen by electron microscopy or on staining with the Feulgen stain
- Bacteria also possess extra-chromosomal DNA called plasmids.

Capsule and Slime Layer

Some bacteria possess a layer of amorphous viscid material lying outside the cell wall called glycocalyx; which may be well organized (capsule) or unorganized loose material (slime layer). Some bacteria may possess both capsule and slime layer as in streptococcus salivarius.

The capsule has various functions

- Acts by inhibiting phagocytosis and complement-mediated lysis
- Biofilm formation and thereby helps in adherence to damaged tissues and plastic surfaces (e.g. medical devices
- Source of nutrients and energy for the bacteria
- Capsules as vaccine, e.g. Pneumococcus, Meningococcus and haemophilus nfuenzae serotype-b and S. typhi Vi vaccine.

Flagella

Flagella are thread-like appendages, protruding from the cell wall, composed of protein subunits called flagellin. It has three parts: filament, hook and basal body.

- Size-They measure 5-20 μm in length and 0.01-0.02μ in thickness
- They are organs of locomotion, confer motility to the bacteria.

Arrangement of flagella

- Size-They measure 5-20 μm in length and 0.01-0.02 □n in thickness
- They are organs of locomotion, confer motility to the bacteria.

Arrangement of flagella

- Monotrichous (single polar flagellum) e.g. Vibrio cholerae, Pseudomonas and Campylobacter
- Lophotrichous (multiple polar flagella) e.g. spirillum.
- Peritrichous (over the entire cell surface e.g. Salmonella Typhi, Escherichia coli.
- Amphitrichous (single flagellum at both the ends) e.g. Alcaligenes faecalis, Spirillum

(Note: Spirillum has tuft of flagella at one or both the ends (Amphi>lophotrichous)

Fimbriae or Pili

Many Gram-negative and some Gram-positive bacteria possess short, fine, hair-like appendages that are thinner than flagella and not involved in motility, called fimbriae or pili. They measure 0.5 □m long and 10nm in thickness:

- Pili are made-up of protein called pilin.
- They are antigenic; however, the antibodies against fimbrial antigens are not protective.
- Functions: fimbriae are called the organ of adhesion. This property enhances the virulence of bacteria.
- Certain fimbriae called sex pili also help in bacterial gene transfer.
- Fimbriae are not related to motility, can be found both in motile as well as in nonmotile organisms.

Bacterial Spores

Spores are highly resistant resting (or dormant) stage of the bacteria formed in unfavourable environmental conditions as a result of the depletion of exogenous nutrients.

ACTINOMYCETES

Actinomycetes are considered to be transitional forms between bacteria and fungi. Like fungi they form a mycelial network of branching filaments but like bacteria they are thin, possess cell walls containing muramic acid, have prokaryotic nuclei and are susceptible to antibacterial antibiotics. Actinomycetes are related to mycobacteria and Corynebacterium. They are gram positive, non-motile, non sporing, non-capsulated filaments that break up into bacillary and coccoid elements. Most are free living particularly in the soil.

Actinomycetes Variants

There are two Actinomycetes variants such as, Actinomyces and Nocardia.

Characteristics and Functions of Actinomycetes

Characteristics	
Structure	Prokaryotic
Size	1–2 μm diameter
Morphology	Filamentous lengths of cocci
Gram stain	Gram positive
Respiration	Mostly aerobic, can be anaerobic
Habitat	Soil or marine
Abundance, marine isolates	5–40 CFU/ml
Abundance, soils	10^6–10^8/g

Functions

- Source of natural products and antibiotics, e.g., streptomycin
- Produce geosmin, the compound which gives soil and water a characteristic earthy odor
- Capable of degradation of complex organic molecules
- Capable of biological nitrogen fixation with species of the non-legume-associated *Frankia*

Classic Actinomycetes have well-developed radial mycelium. According to the difference of morphology and function, the mycelia can be divided into substrate mycelium and aerial mycelium (fig 1.). .Some actinobacteria can form complicated structures, such as spore, spore chain, sporangia, and sporangiospore.

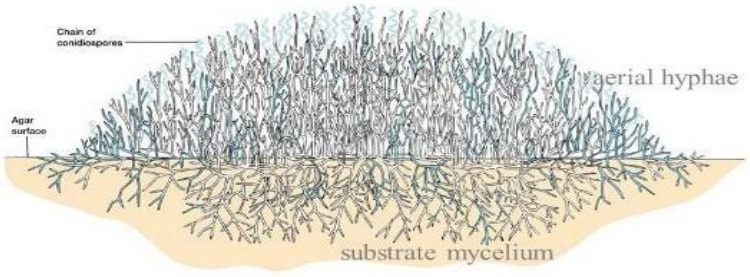


Fig: An actinomycete colony growing on agar - note the subterranean and aerial hyphae....

FUNGI

The word fungus comes from the Latin word for mushrooms. Indeed, the familiar mushroom is a reproductive structure used by many types of fungi. However, there are also many fungi species that don't produce mushrooms at all. Being eukaryotes, a typical fungal cell contains a true nucleus and many membrane-bound organelles. The kingdom Fungi includes an enormous variety of living organisms collectively referred to as Ascomycota, or true Fungi. While scientists have identified about 100,000 species of fungi, this is only a fraction of the 1.5 million species of fungus probably present on earth. Edible mushrooms, yeasts, black mold, and the producer of the antibiotic penicillin, Penicillium notatum, are all members of the kingdom Fungi, which belongs to the domain Eukarya.

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.

As such, they are categorized as heterotrophic because they are unable to synthesize their own food (they lack chlorophyll). They can reproduce sexually or asexually with a majority of fungi being spore producers. Examples include:

- Molds
- Penicillin
- Yeast
- Truffles
- Mushrooms

Morphology and Structure of Fungi

There are a wide variety that range from the smallest unicellular fungi such as yeast to larger multicellular capable of forming hyphal threads or false roots. For this reason, fungi are also classified according to their morphologies.

The following are classifications of fungi based on morphology:

Yeast

Yeast are single celled fungi that can be found in a variety of environment from soil and plants to animal and aquatic environments. Unlike bacteria, yeasts are also eukaryotic, which means that they have different types of organelles that are common in the cells of higher animals.

The Saccharomyces cerevisiae is a good example of yeast that ranges between 1 and 7 micrometers in size. When viewed under the microscope, these organisms may be pigmented on their surface.

As with the cells or higher organisms, the S. cerevisiae contains such organelles as a membrane bound nucleus, a vacuole, mitochondria and the Golgi apparatus as well as the E.R (endoplasmic reticulum). The cell wall of these yeast is composed of glucan (a polysaccharide compound) and mannoproteins.

As for the genome, research has shown these yeast to carry a single, linear double stranded DNA that consists of several repeated sequences. For yeast, the primary mode of reproduction is through budding.

Following the copying of the genetic material, a bud is formed on the surface of the cell that ultimately breaks off with its genetic material and grows to form a new cell.

Yeast-like Fungi

Yeast-like fungi are yeast that partly grow like normal yeast. However, they also attach to each other to form what is known as a pseudo hyphae (not a true hyphae).

Candida albicans is one of the most common. When viewed under the microscope, these organisms have been shown to consist of several layers that make up the cell wall.

As with yeast, the wall of C. albicans contains layers of mannoproteins, lipids and a beta glucon, a chitin inner layer that strengthen the cell wall. Like yeast, C. albicans also appear spherical or ovoid in shape and measure between 4 to 8 micrometers.

Since they also reproduce through budding, like yeast, C. albicans may end up creating an elongated chain of cells as they continue dividing to form the pseudohyphae. However, some studies suggest that some of the yeast-like fungi tend to form true hyphae in the process.

Yeast-like fungi such as C. albicans are also described as being polymorphic fungus. This is because they present four types of morphology including the yeast cell, pseudohyphae, hyphae as well as chlamydospores. As such, they are likely to be seen having varying appearance when viewed under the microscope depending on such conditions as the availability of nutrition, pH and temperature among others.

The type of morphological appearance of these cells has also been associated with the pathogenicity of the organism. Given that they are not bacteria, some of these organisms (yeast-like fungi) have characteristics associated with eukaryotic cells in that some have been shown to contain a nucleus and other essential organelles.

MOLDS

Mold (Mould) are a type of fungi that often grow well in favorable environments with warmth and moisture. They can be found growing on various surfaces such as food surfaces from which they obtain their nutrients.

Compared to yeast, molds are multicellular organisms. As such, they can be seen with the naked eye without using a microscope. However, when viewed under the microscope, it is possible to observe numerous filaments (Hyphae) that are collectively referred to as Mycelium.

While these organisms are microscopic, it is their numerous hyphae (that form the mycelium) that make it possible to see mold as it grows on food surface (bread, oranges etc). There are two main types of mycelium depending on their functions.

These include:

Vegetative mycelium - Vegetative mycelium include the hyphae that penetrates into the substrate and absorbs nutrients for the continued growth and development of the organisms. These hyphae therefore act like plant roots.

Aerial mycelium - Aerial mycelium are the hyphae that are located above the food substance. When viewed closely under the microscope, aerial mycelium contain a spherical structure at the top of the hyphae. These are known as the conidia and sere as the reproductive part of the mold. This part produces spores (asexual reproduction) that can grow in favorable conditions.

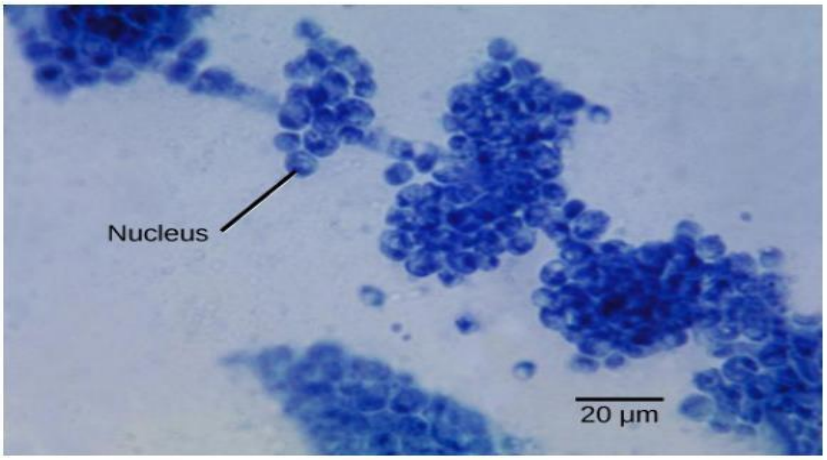
Cell Structure and Function

Fungi are eukaryotes and have a complex cellular organization. As eukaryotes, fungal cells contain a membrane-bound nucleus where the DNA is wrapped around histone proteins. A few types of fungi have structures comparable to bacterial plasmids (loops of DNA). Fungal cells also contain mitochondria and a complex system of internal membranes, including the endoplasmic reticulum and Golgi apparatus.

Unlike plant cells, fungal cells do not have chloroplasts or chlorophyll. Many fungi display bright colors arising from other cellular pigments, ranging from red to green to black. The poisonous Amanita muscaria (fly agaric) is recognizable by its bright red cap with white patches. Pigments in fungi are associated with the cell wall. They play a protective role against ultraviolet radiation and can be toxic.

The poisonous Amanita muscaria: The poisonous Amanita muscaria is native to temperate and boreal regions of North America.

The rigid layers of fungal cell walls contain complex polysaccharides called chitin and glucans. Chitin, also found in the exoskeleton of insects, gives structural strength to the cell walls of fungi. The wall protects the cell from desiccation and predators. Fungi have plasma membranes similar to other eukaryotes, except that the structure is stabilized by ergosterol: a steroid molecule that replaces the cholesterol found in animal cell membranes. Most members of the kingdom Fungi are nonmotile.



Example of a unicellular fungus: *Candida albicans* is a yeast cell and the agent of candidiasis and thrush. This organism has a similar morphology to coccus bacteria; however, yeast is a eukaryotic organism (note the nucleus).

CULTURE MEDIA

The basic constituents of culture media are:

- Peptone: Mixture of partially digested proteins
- Agar: It is used for solidifying the culture media. It has no nutritional property.
- It is prepared from seaweeds (red algae of species Gelidium and Gracilaria).
- Agar is preferred over gelatine, as it is bacteriologically inert, and it melts at 98^0C and usually solidifies at 42^0C
- For solid agar: 1-2% (Japanese ager 2% or New Zealand agar 1.2%)
- For semisolid agar:0.5%
- For solid agar to inhibit proteus swarming: 6%

Others: Meat extract, Yeast extract, Blood and serum, Water and Electrolytes (NaCl).

Simple/basal Media

They contain minimum ingredients that support the growth of non-fastidious bacteria. Examples include:

1. Peptone water: it contains peptone (1%)+NaCl(0.5%)+water
2. Nutrient broth: It is made-up of peptone water + meat extract (1%)
3. Nutrient agar: It is made-up of nutrient broth +2% agar the basal media are used for:

- Testing the non-fastidiousness of bacteria
- They serve as the base for the preparation of any other media
- Nutrient broth is used for studying the bacterial growth curve
- Nutrient agar is the preferred medium for:
- Performing the biochemical tests such as oxidase, catalase and slide agglutination
- To study the colony character and Pigment demonstration.

Enriched Media

When a basal medium is added with additional nutrients such as blood, serum or egg, it is called enriched medium. They also support the growth of fastidious bacteria, e.g.:

1. Blood agar: Prepared by adding 5-10% of sheep blood to the molten nutrient agar at 45^0C. It is used to test the haemolytic property of the bacteria.
2. Chocolate agar: It is the heated blood agar; blood is added to the molten nutrient agar at 70^0c. It is more nutritious than blood agar, and even supports Haemophilus influenzae.
3. Loeffler's serum slope is used for isolation of Corynebacterium diphtheriae.
4. Blood culture media: used for blood culture. They are of two types.

- Monophasic medium is made-up of brain heart infusion (BHI) broth
- Biphasic medium has a liquid phase (BHI broth) and a solid agar slope (BHI agar).

Selective Media

Solid media that allow certain organism (pathogens) to grow and inhibit others (normal flora):

1. Lowenstein Jensen (LJ) medium is used for isolation of mycobacterium tuberculosis
2. Thiosulphate Citrate Bile salt Sucrose (TCBS) agar used for isolation of Vibrio species
3. For the isolation of enteric pathogens such as salmonella and shigella from stool.
   - DCA (Deoxycholate Citrate Agar)
   - XLD (Xylose Lysine Deoxycholate) agar
4. Potassium tellurite agar (PTA) is used for isolation of Corynebacterium diphtheriae.
5. Wilson Blair bismuth sulphite medium: It is used for isolation of salmonella typhi.

Differential Media

These media differentiate between two groups of bacteria by using an indicator.

1. MacConkey agar: It differentiates organisms into LF or lactose fermenters (produce pink colonies, e.g. E. coli and Klebsiella) and NLF (produce color less colonies, e.g. shigella and Salmonella)
2. CLED agar (Cysteine lactose electrolyte-deficient agar) This is similar to MacConkey agar, differentiates between LF and NLF. It is used as an alternative to combination of blood agar and MacConkey agar, for the processing of urine specimens.:

- Advantages over MacConkey agar: It is less inhibitory than MacConkey agar, supports gram positive bacteria (except β haemolytic Streptococcus) and Candida.
- Advantage over blood agar: It can prevent the swarming of Proteus.

7. Pour-plate culture: Quantitative culture method, used to estimate viable bacterial count.

Synthetic or defined Media

These media are prepared from pure chemical substances and the exact composition of the medium is known. These are used for various special studies such as metabolic requirements. Simple peptone water medium, 1% peptone with 0.5% NaCl in water, may be considered a semi defined medium since its composition is approximately known.

Indicator Media

These media contain an indicator which changes colour when a bacterium grows in them, for example incorporation of sulphite in Wilson and Blair medium. S. typhi reduces sulphite to sulphide in the presence of glucose and the colonies of S. typhi have a black metallic sheen. Potassium tellurium by the diphtheria bacillus to produce black colonies.

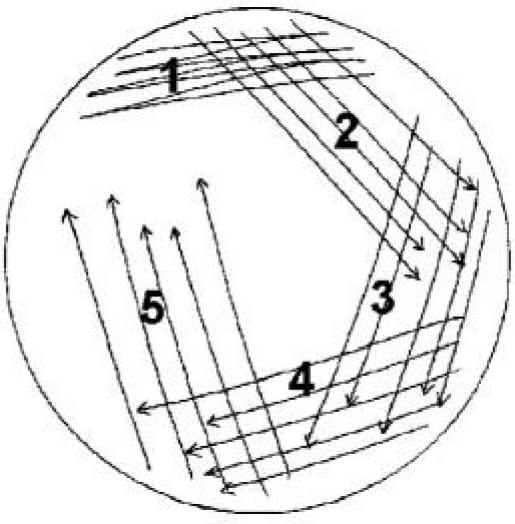
Methods of Isolating Pure Culture

1. Mixed Culture: A culture contain more than one species of microbes.
2. Pure culture: A culture containing only one species of microbes.
   The following methods are used to isolate pure culture.

   1. Streak plate technique.
   2. Pour plate technique.
   3. Spread plate technique.

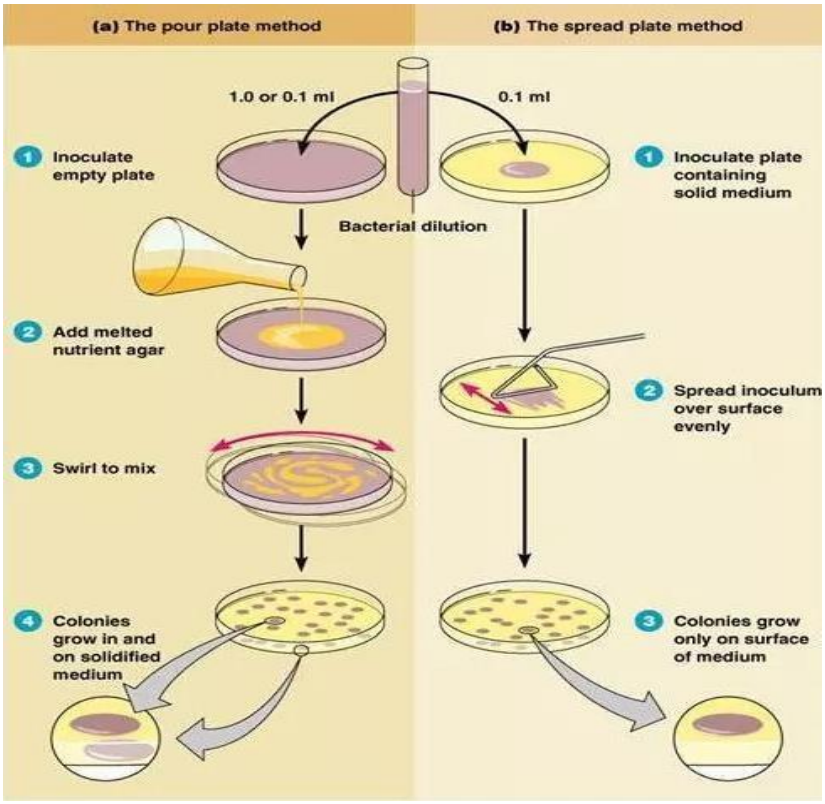
1. Streak plate technique

Streaking is the process of spreading the microbial culture with an inoculating needle on the surface of the media. Sterilizing the inoculating needle by flame to make red hot and allow it to cool for 30 seconds.



2. Pour plate technique

The bacterial culture and liquid agar medium are mixed together. After mixing the medium, the medium containing the culture poured into sterilized Petri dishes allowed solidifying and then incubate. After incubation colonies appear on the surface.



Disadvantage of pour plate method: Method is tedious, time consuming and requires skills. The micro-organisms are trapped beneath the surface of medium when it solidifies. Hence, surface as well as subsurface colonies are developed and it is very difficult to isolate and count the subsurface colonies.

The micro-organisms are subjected to hot shock because liquid medium is maintained at 45 C temperature. This method is unsuitable for isolation of psychrophilic bacteria.

Spread Plate technique

This is the best method to isolate the pure colonies. In this technique, the culture is not mixed with the agar medium. Instead it is mixed with normal saline and serially diluted.

0.1ml of sample taken from diluted mixture, which is placed on the surface of the agar plate and spread evenly over the surface by using L shaped glass rod call spreader.

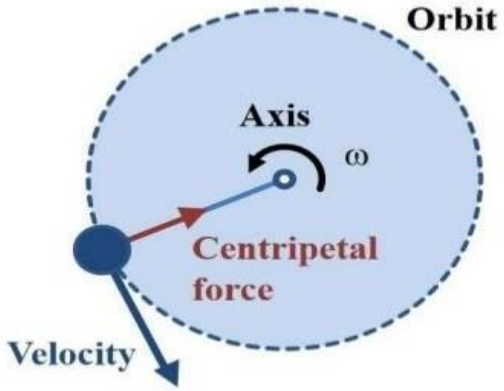
Incubate the plates. After incubation, colonies are observed on the agar surface.

Advantages of spread plate method: It is a simple method, only surface colonies are formed. Micro-organisms are not exposed to higher temperature.

MICROBIOLOGY EQUIPMENTS AND INSTRUMENTS

Centrifuge:

- Centrifugation is a technique of separating substances which involves the application of centrifugal force.
- The particles are separated from a solution according to their size, shape, density, viscosity of the medium and rotor speed.



- In a solution, particles whose density is higher than that of the solvent sink (sediment), and particles that are lighter than it float to the top.
- The centrifuge works using the sedimentation principle, where the centripetal acceleration causes denser substances and particles to move outward in the radial direction.

Incubator:

Incubator is basically the laboratory equipment which is used for the incubation of biological products under controlled conditions. This medical laboratory equipment is available with digital temperature controller with thermocouple sensor for better temperature accuracy.

They are insulated enclosures that are thermostatically regulated to maintain a constant temperature. In this type of incubator, environmental conditions, such as Temperature and humidity can be controlled for growing bacterial cultures (37°C), hatching eggs artificially/providing suitable conditions for a chemical or biological reaction.

Incubator

Autoclave:

Autoclaves provide a physical method for disinfection and sterilization. They work with a combination of steam, pressure and time. Autoclaves operate at high temperature and pressure in order to kill microorganisms and spores. To be effective against spore forming bacteria and viruses, autoclaves need to have steam in direct contact with the material being sterilized (i.e. loading of items is very important).

Create vacuum in order to displace all the air initially present in the autoclave and replacing it with steam.

Implement a well-designed control scheme for steam evacuation and cooling so that the load does not perish.

The efficiency of the sterilization process depends on two major factors. One of them is the thermal death time, i.e. the time microbes must be exposed to at a particular temperature before they are all dead. The second factor is the thermal death point or temperature at which all microbes in a sample are killed.

Water Bath:

Working principle of water bath: The Cu50 sensor transfer water temperature to resistance value, amplified and compared by integrated amplifier, then output the control signal, efficiently control the average heating power of electric heating tube and maintain water in constant temperature.

Biological Safety Cabinets:

Biological safety cabinets (BSCs) are among the most effective primary containment devices used in laboratories working with infectious agents. They act as primary barriers to prevent the escape of biological aerosols into the laboratory environment.

BSCs are designed for:

- *Personnel Protection*: Protects personnel from harmful agents inside the BSC.
- *Product Protection*: Protects the work, product, experiment, or procedure performed in the BSC from contaminants in the laboratory environment or from cross contamination inside the cabinet.
- *Environmental Protection*: Protects the environment from contaminants contained in the BSC.

Inoculating loops and Needles: Inoculating loops are used to transfer micro-organisms to grow media and staining slides. The wire forms a small loop with a diameter of about 5mm. the loop of wire at the tip may be made of platinum or nichrome. Needles are straight wires used to pick up bacteria from closely packed colonies or to inoculate in a much defined area. Needles are commonly used to inoculate semi soft media.

UNIT -3

STAINING TECHINIQUES

Staining is necessary to produce colour contrast and thereby increase the visibility of the object. Before staining, the fixation of the smear to the slide is done:

- **Heat fixation** is usually done for bacterial smears by gently flame heating an air-dried film of bacteria
- **Chemical fixation** such as ethanol, acetic acid, mercuric chloride, formaldehyde, methanol and glutaraldehyde. This is useful for examination of blood sears.

Staining Techniques Used in Microbiology

1. **Simple Stain:** basic dyes such as methylene blue or basic fuchsin are used as simple stains. They provide the colour contrast, but impart the same colour to all the bacteria in a smear. It is used to compare morphological shapes and arrangements of bacterial cells.

   i) Loffler's methylene blue:

   - ✓ Saturated solution of methylene blue in alcohol (30 ml).
   - ✓ KOH, 0.01 % in water – 100ml.
   - ✓ Dissolve the dye in water and filter.
   - ✓ For smear stain for 3 inch.
   - ✓ For section stain for 5 inch.

   ii) Dilute Carbol Foschini:

   - ✓ Made by diluting Z-N stain with 10-15 times its volume of water.
   - ✓ Stain for 20-25 seconds, wash with water.

   Use: To demonstrate the morphology of vibrio cholera.

2. **Negative staining,** e.g. India ink or nigrosine. The background gets stained black whereas unstained bacteria stand out in contrast. This is very useful in the demonstration of bacterial capsules which do not take up simple stains

   Use : To demonstrate the capsule of Cryptococcus neoformans and Streptococcus pneumoniae.

3. **Impregnation methods** (e.g. silver(: Used for demonstration of thin structures like bacterial flagella and spirochetes.

4. **Differential stain:** Two stains are used which impart different colors which help in differentiating bacteria, e.g. Grams Stain and Acid fast stain.

i) Gram's stain*:*

- ✓ *Originally devised by Hans Christian Gram in the late 19th century.*
- ✓ Differentiates bacteria into Gram-positive (appear violet) and Gram-negative (appear pink)
- ✓ groups.
- ✓ Also be used for the detection of fungal hyphae and yeast cells.

Fig. 1.1.3: Principle and procedure of Gram staining

Smear is allowed to dry and it is viewed under oil immersion objective lens.
This procedure shows Gram positive bacteria resists decolourisation and appears violet and Gram negative bacteria which are decolourised by organic solvents take up the counter stain and appear red.

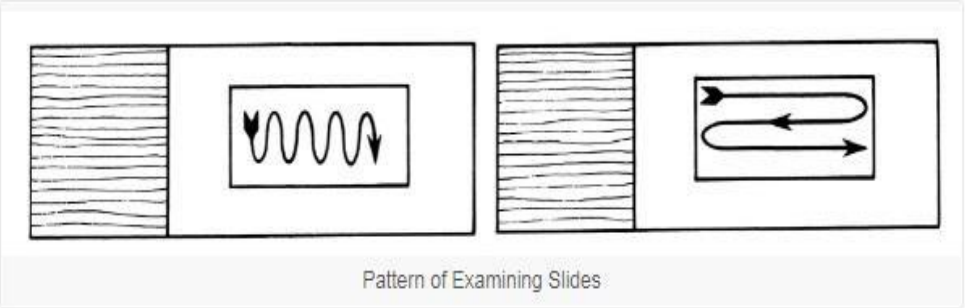
***ii)* <u>Acid-fast stain or Ziehl Neelsen stain:</u>**

- ✓ *It was discovered by Ehrlich and modified by Ziehl Neelsen.*
- ✓ *It differentiates acid fast and non-acid fast organisms.*

Procedure

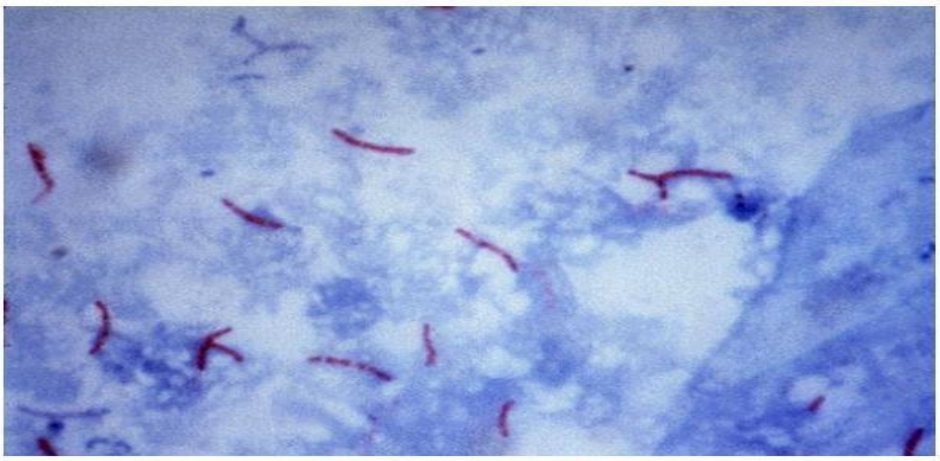
- ✓ Make a thin smear of the material for study and heat fix by passing the slide 3-4 times through the flame of a Bunsen burner or use a slide warmer at 65-75 C. Do not overheat.
- ✓ Place the slide on staining rack and pour carbol fuschin over smear and heat gently underside of the slide by passing a flame under the rack until fumes appear (without boiling!). Do not overheat and allow it to stand for 5 minutes.
- ✓ Rinse smears with water until no color appears in the effluent.
- ✓ Pour 20% sulphuric acid, wait for one minute and keep on repeating this step until the slide appears light pink in color (15-20 sec).
- ✓ Wash well with clean water.
- ✓ Cover the smear with methylene blue or malachite green stain for 1–2 minutes.
- ✓ Wash off the stain with clean water.
- ✓ Wipe the back of the slide clean, and place it in a draining rack for the smear to air-dry (do not blot dry).

✓ Examine the smear microscopically, using the 100x oil immersion objective.



Pattern of Examining Slides

Result:



Mycobacterium tuberculosis visualization using the Ziehl–Neelsen stain.

iii) <u>Albert stain</u>:

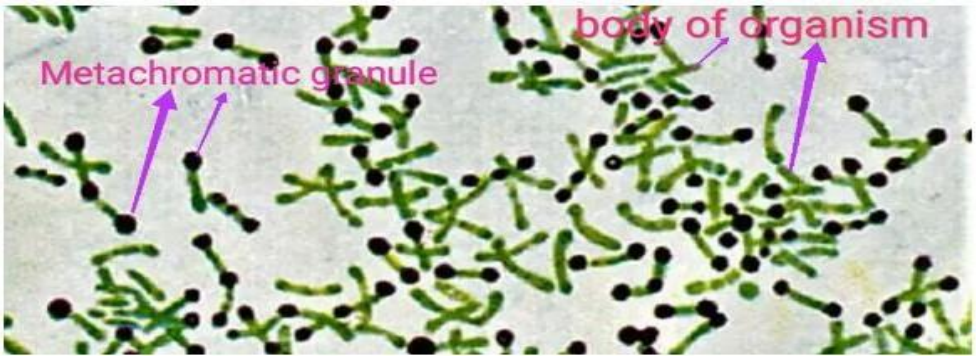
✓ Special staining for Corynebacterium diphtheria.
✓ To demonstrate the metachromatic granules.

<u>Procedure :</u>

✓ Prepare the smear, dry in air and fix by heat.
✓ Cover the slide with Alberts stain for 3-5 min.
✓ The slide is washed off with distilled water.
✓ Cover the slide with Albert's iodine for 1-2 min.
✓ Wash with water and blot dry.
✓ Examine under oil immersion objective of light microscope.

Using Alberts stain, metachromatic granules of C. Diphtheria appears blue black, while the body of the bacillus appears green or bluish green.

Result:



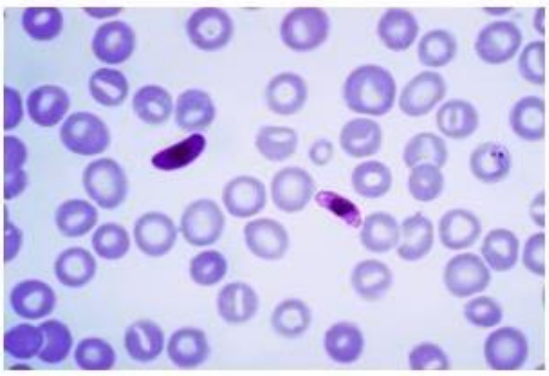
iv) <u>Leishman's stain:</u>

- ✓ Used to demonstrate blood parasites.
- ✓ Made by dissolving 0.15g of Leishman's powder in 100 ml pure methyl alcohol.

<u>Procedure :</u>

- ✓ 2ml of undiluted stain is poured on to the unfixed film and allowed to stand for 1min.
- ✓ 4 ml of distilled water is added to dilute the stain on the slide or to prevent it from drying; this is allowed to stand for 10-15 min.
- ✓ The slide is then washed with running tap water until the blood film appears bright pink in colour; the preparation is then dried in air.
- ✓ The smear is examined under the oil immersion objective lens of the light microscope.

Result:



Blood smear of Plasmodium falciparum parasite.

v) <u>Giemsa Stain :</u>

- ✓ Giemsa powder 3.8 gm.
- ✓ Glycerol 250 ml.
- ✓ Methanol 250 ml.

- ✓ Add methanol to Giemsa powder and dissolve.
- ✓ Add glycerol and place in water bath at 60 C for 3 hours with intermittent shaking.

Procedure:

- ✓ Prepare the smear.
- ✓ Fix with pure methanol for 3-5 min.
- ✓ Diluted stain (5ml) is added and allowed to dry for 30-45 min.
- ✓ Smear is then washed with running water.
- ✓ Dry it in vertical position.
- ✓ Observe under oil immersion lens.

Result:

Giemsa stained *Trypanosoma* parasites (Chagas disease pathogen)

5. Other special Staining Methods:

- o Spore staining: Acid fast stain (using 0.25% sulfuric acid) and Malachite green stain (Schaeffer and Fulton method modified by Ashby) methods are used; however, phase contrast microscope of unstained wet film is the best method.

SPECIAL STAINING

(Endospore / Capsule / Flagella/ Metachromatic granules)

Spore Staining:

If spore bearing organisms are stained with ordinary dyes, or by Gram's stain, the body of the bacillus is deeply coloured, whereas the spore remains unstained. The vegetative bacterial cells are stainable with aqueous dyes, but endospores possess permeability barrier that prevents stain/dyes from entering the spore coat unless the barrier is destroyed by heating, UV light, mechanical rupture or by treatment of acid. The tough spore coat is formed to protect the bacterial cells DNA and important proteins from adverse environmental conditions (excessive heating, short of nutrients, drying etc.)Spore coat is a complex multilayered structure containing high calcium ions and dipicolinic acid which makes the structure more tough .

Below spore coat, lies the peptidoglycan. Once the protective tough spore coat is penetrated, the stain/dye interacts with peptidoglycan to produce the desired effect of staining.

There are other staining methods to introduce dye into the substance of the spore. When thus stained, the spore tends to retain the dye and resist decolourization. Several methods such as Acid Fast Staining Method for Spores (Spores stained bright and protoplasm of the bacillus stains blue), Hansen's Method,Dorner's method and Schaeffer and Fulton's Method are widely applied methods for staining spores in proper.
Commonly used staining methods for endospores includes:

1. The Schaeffer-Fulton method – Most common method used to stain endospores.

2. Dorner method

3. Hansens method

1. Schaeffer-Fulton method for staining endospores

Malachite green stain (0.5% (wt/vol) aqueous solution) 0.5 g of malachite green
100 ml of distilled water

Decolorizer Tap water,

Safranin counterstain Stock solution (2.5% (wt/vol) alcoholic solution) 2.5 g of safranin O 100 ml of 95% ethanol Working solution 10 ml of stock solution 90 ml of distilled water

Procedure:

Schaeffer-Fulton method:

1. Fix the air dried smear by passing over the flame 2-3 times.

2. Flood smear with malachite green and heat for 5 min. Do not boil.

3. Allow to cool and wash with water.

4. Counter stain with dilute safranin (working solution) for 1 min.

5. Wash the smear and air dry it.

Spore stains:Endospore takes bright green and bacterial cells are brownish red to pink.

Dorner method for staining endospores –

Spore stains: Bacterial cells are colorless, endospores are red, and the background is black. Carbol Fuchsin – primary stain and counterstain Nigrosin .

Hansen's Method

Material: Bacterial smear, Cocn. carbol fuchsin, 5% Acetic acid, Loeffer's methylene blue.

Procedure:

- Fix the air dried smear by passing over the flame 2-3 times.
- Stain the smear as follows,
- Flood smear with Conc. carbol fuchsin and heat for 5 min. Do not boil.
- Allow to cool and wash with water.
- Decolorize with 5% Acetic acid for 1 min. and wash with water.
- Counter stain with Loeffer's methylene blue for 3 min.
- Wash the smear and air dry the smear and observe under microscope.

Spore stains: spores stain red and the vegetative cells blue.

ii)Capsular Staining

The best way to demonstrate capsules of bacterial cells is to stain them by some procedure, which differen-tiates them from the bacterial cell itself. Anthony's method (with Tyler's modification) to stain capsule is the simplest method.

The best way to demonstrate capsules of bacterial cells is to stain them by some procedure, which differen-tiates them from the bacterial cell itself. Anthony's method (with Tyler's modification) to stain capsule is the simplest method.

Anthony's method (With Tyler's modification)

Staining solution:

- Acetic crystal violet
- Crystal violet (35% dye content)——— 0.1 g
- Glacial acetic acid ————————- 0.25 ml
- Distilled water ————————————100 ml

Procedure:

Anthony's method (With Tyler's modification)

- Prepare a smear of bacterial culture on the slide.
- Dry it in the air.
- Stain for 4-7 minutes in the 'acetic crystal-violet' solution.
- Wash with 20% aqueous copper sulphate ($CuSO_4, 5H_2O$)
- Dry with blotting paper and examine.

Capsules stains blue violet; Bacterial cell stains dark blue.

iii) Flagellar Staining

Robert Koch published staining procedure for bacterial flagella in 1877. Subsequently several modifications and methods were developed for staining flagella were developed. In 1930, Leifson published a simple flagella stain. Many modifications or alternative methods includes a wet-mount procedure of Mayfield and Innis and a more traditional dried-smear preparation ,combination of the wet-mount technique of Mayfield and Innis and the stain of Ryu suggested by Kodaka et al. ,overcame most difficulties in staining flagella.**Presque Isle Cultures flagella stain** – ready made staining method available commercially.

A silver-plating stain for flagella was developed in 1958 and simplified in 1977. Recently a fluorescent protein stain, NanoOrange from Molecular Probes (Eugene, OR), is being applied to screen for bacteria possessing flagella by light microscopy .

Materials 12-16 Hrs incubated bacterial culture, Microscope slides, 95% ethanol

- Micorpipette with sterile disposable tips, Distilled water. Leifson flagella stain Solution A: Sodium chloride 1.5 g
- Distilled water 100 ml
- Solution B: Tannic acid 3.0 g
- Distilled water 100 ml
- Solution C: Pararosaniline acetate 0.9 g Paraosaniline hydrochloride 0.3 g Ethanol, 95% (vol/vol) 100 ml Take equal volumes of solutions A and B and then add 2 volumes of the mixture to 1 volume of solution C. The resulting solution may be kept refrigerated for 1 to 2 months. (Ryu method – reagent stable at room temperature)

Preparation:

Bacterial cultures incubated for 12-16 Hrs can be used for flagellar staining. Collect small quantity of growth from agar medium and emulsify in 100 ml of distilled water. Take in a micro-centrifuge tube mix by gentle vortexing. Avoid too much of inoculums.

If culture is used from incubated broth, centrifuge the culture, remove spent medium.

Resuspend in 100 ml of distilled water by gently vortexing, again centrifuge, and remove supernatant. Finally, resuspend in 200 ml of distilled water and prepare slightly cloudy emulsion to be used for staining.

Preparation of smear:

1. Take ethanol treated clean new microscope slide and flame to dry before use.

2. Cool the slide, place 5 to 10 ml of the culture emulsion on one end of the slide and spread

it with the help of pipette.

3. Dry at room temperature. Do not heat fix.(Heating destroys the flagella)

Staining procedure:

Leifson flagella staining method

1.Take a prepared slide and mark an area of 1×1.5 inch^2 with grease pencil. 2. Flood Leifson dye solution on the slide within the marked area.

3. Incubate at room temperature for 7 to 15 minutes or allow to act till formation of fine

precipitate.(Golden film develops on the dye surface).

4. Remove the stain by gentle wash with water steam and air dry.

5. Observe under oil immersion

Bacterial body and flagella will stain red.

iv) Staining of Metachromatic Granules – Albert's Method

Metachromatic granules:

Special stains (Albert, Neisser) pick out the volutin granules and give the bacilli a beaded or barred appearance; the granules are polar in short bacilli. Volutin staining reactions are best seen in young cultures .

(See Albert Staining Procedure in upper section)