MARRI LAXMAN REDDY GROUP OF INSTITUTIONS

Marri Laxman Reddy Institute of Pharmacy

(Approved by AICTE & PCI, New Delhi and Affiliated to JNTUH, Hyderabad)
Dundigal, Quthubullapur Mandal, Hyderabad-500043.
Phone Nos: 9160400039/040-29556181/9989901438

Subject: Pharmaceutical Microbiology

Unit No: I

Introduction, history of microbiology, its branches

Microbiology is the — 'scientific study of the microorganisms.

Fields of microbiology:

The entire domain of **microbiology** may be judiciously sub-divided into a plethora of diversified, well-recognized, and broadly accepted fields, namely:

Bacteriology: the study of *organism* (*bacteria*),

Mycology: the study of *fungi,* **Phycology:** the study of *algae*,

Proto zoology: the study of *protozoans,* and **Virology:** the study of viruses.

1. *Aero-Microbiology* — helps in the overall preservation and preparation of food, food-prone diseases, and their ultimate prevention.
2. *Beverage Microbiology* — making of beer, shandy, wine, and a variety of alcoholic bever-ages *e.g.,* whisky, brandy, rum, gin, vodka. etc.
3. *Exomicrobiology* — to help in the exploration of life in the outerspace.
4. *Food Microbiology* — making of cheese, yogurt.
5. *Geochemical Microbiology* — to help in the study of coal, mineral deposits, and gas forma-tion ; prospecting the deposits of gas and oil, coal, recovery of minerals from low-grade ores.
6. *Industrial Microbiology* — making of ethanol, acetic acid, lactic acid, citric acid, glucose syrup, high-fructose syrup.
7. *Medical Microbiology* — helps in the diagnostic protocol for identification of causative agents of various human ailments, and subsequent preventive measures.
8. *Pharmaceutical Microbiology* — making of life-saving drugs, **'antibiotics'** *e.g.,* penicillins, ampicillin, chloramphenicol, ciprofloxacin, tetracyclines, streptomycin.

Spontaneous Generation *Vs* Biogenesis:

The wonderful discovery of **microbes** both generated and spurred enough interest not only in the fundamental origin of **'living things'** but also augmented argument and speculation alike.

Based upon the various experimental evidences the following observations were duly made by scientists as enumerated below:

John Needham (1713-1781) : Precisely in the year 1749, while experimenting with raw meat being exposed to hot ashes, he observed meticulously the appearance of **organisms** that were not present at the initial stages; and, therefore, inferred that the bacteria virtually originated from the raw meat itself.

Germ Theory of diseases:

A plethora of observant researchers had already conceptualized and opined rather vehemently the much applauded and widely accepted **'germ theory'** of disease even before Pasteur established experimentally that **microbes** (or **bacteria**) happen to be the root cause of several human dreadful diseases. Later on various other scientists supported and proved the aforesaid **'germ theory'** in one way or the other as stated under :

Joseph Lister (1890) : made known in England the importance of **antisepsis,** which was subse-quently fully appreciated by the **medical profession all and sundry.**

Robert Koch (1843–1910) : discovered the typical bacilli having squarish ends in the blood sample of cattle that had died due to anthrax.*

Medical Microbiology:

Emil von Behring and Shibasaburo Kitasato : devised an unique technique of producing im-munity to infections caused by *C. diphtheriae* by injecting the **toxins** into healthy animals so that an **antitoxin** gets developed.

Shibasaburo Kitasato and Emil von Behring : cultivated (grown) the microorganism respon-sible for causing *tetanus* (lockjaw), *Chlostridium titani* ; and Behring prepared the corresponding **anti-toxin** for the control, prevention, treatment, and management of this fatal disease.

Emil von Behring bagged the Nobel Prize in 1901 in physiology or medicine.

Scope and importance of Microbiology

The importance of microbiology can be stated as follows:

Antibiotics/Antimicrobials - Both natural and chemically improved microbial products can be used to control the diseases caused in humans, animals, and plants. Using traditional genetics or recombinant DNA techniques, the micro-organisms can be reformed to improve the yield or action of antibiotics and other antimicrobial agents. New research guidelines are aimed at determining microbial metabolites with pharmacological activities, suitable in the treatment of diseases such as, hypertension, obesity, coronary heart disease, cancer, and inflammation.

Agriculture - Conventional, recombinant DNA and monoclonal antibody techniques are used to make improvements in microbial inoculants. These serve as fertilizer supplements by fixing atmospheric nitrogen to improve plant yields and to serve as plant pest controls. All of these require a microbiologist to insure product usefulness and worth.

Enzymes - Industrial applications of enzymes include the production of cheese, the interpretation of apple juice, the development of more efficient laundry detergents, pulp and paper production, and the treatment of sewage. These processes have been vividly enhanced by the use of recombinant DNA techniques to design enzymes and increase action, constancy, and specificity.

Carbohydrates **-** Some molecular sieves for purification and separation processes e.g., dextran and thickening agents e.g., xanthan used in salad dressings, which are steady at high temperatures, are examples of microbial carbohydrates. The latter are also used for secondary oil recovery in oil fields and as lubricants in drilling oil wells, gelling agents in foods, and thickeners in both paints and food products.

Organic Chemicals **-** Compounds such as, acetone, methanol, butanol, and ethanol have numerous applications in industrial settings. They are frequently used as raw materials for industrial manufacturing and production processes. The microbiologist is involved in research on improvements in the production and detection of new metabolic passageways. Microbes will

progressively be used to supplant or replace those processes, which rely on petroleum and natural gas for the production of these compounds.

Oil Recovery/Mining - Oil recovery may be facilitated by the development of distinctive bacteria, which produce a surfactant that forces trapped oil out of the rocks. Extraction of minerals from low-grade ores is augmented by some bacteria, i.e. microbial leaching. In addition, selective binding of metals by bio-hydrometallurgical processes is important in recycling of metals, such as silver and uranium. Research and developments in these areas also make provision of career paths for industrial microbiologists and biotechnologists.

Prokaryotes and Eukaryotes

Characteristic Distinguishing Features of Prokaryotic and Eukaryotic Cells.

S.No.	Characteristic Features	Prokaryotic Cells	Eukaryotic Cells
	Structure and Size		
1	Groups occurring as unit of structure.	Bacteria	Animals, algae, fungi, protozoa, and plants.
2	Size variants in organism.	1 – 2 × 1 – 4 µm or less.	More than 5 µm in diameter or width.
	Genetic System		
3	Location	Chromatin body, nucleoid, or nuclear material.	Chloroplasts, mitochondria, nucleus.
4	Structure of nucleus	One circular chromosome — not bound by nuclear membrane.	More than one chromosome — bound by nuclear membrane.
		Absence of histones in chromosome, no mitotic division. Nucleolus absent, clustering of functionally related genes may occur.	Chromosomes have histones — mitotic nuclear division. Nucleolus present — no clustering of functionally related genes.
5	Sexuality	Zygote is partially diploid *viz*, merozygotic in nature.	Zygote is diploid.
	Cytoplasmic Nature and Structures		
6	Cytoplasmic streaming	Absent	Present
7	Pinocytosis*	Absent	Present
8	Gas vacuoles	May be present	Absent
9	Mesosome**	Present	Absent
10	Ribosomes***	70S**** — distributed in the cytoplasm.	80S — arrayed on membranes akin to endoplasmic reticulum — 70S in mitochondria and chloroplasts.
11	Mitochondria	Absent	Present
12	Chloroplasts	Absent	Can be present
13	Golgi bodies	Absent	Present
14	Endoplasmic reticulum	Absent	Present
15	Membrane-bound vacuoles *i.e.*, true-vacuoles	Absent	Present

Morphological classification of bacteria:

Shape & Size of Bacteria

Bacteria are classified by shape into three basic groups: **cocci, bacilli,** and **spirochetes** . The cocci are round, the bacilli are rods, and the spirochetes are spiralshaped. Some bacteria are variable in shape and are said to be **pleomorphic** (many-shaped). The shape of a bacterium is determined by its rigid cell wall. The microscopic appearance of a bacterium is one of the most important criteria used in its identification. In addition to their characteristic shapes, the arrangement of bacteria is important. For example, certain cocci occur

in pairs (**diplococci**), some in chains (**streptococci**), and others in grapelike clusters (**staphylococci**).

Ultra-structure of Bacteria

Capsules and Slimes

Capsule is the outer most layer of the bacteria (extra cellular). It is a condensed well defined layer closely surrounding the cell. It is a viscous material that essentially forms a covering layer or a sort of envelope around the cell wall. It is visualized by the aid of light microscopy employing highly sophisticated and specialized staining techniques.

Flagella

(i) Monotrichous
– Single flagella on one side
(ii) Lophotrichous – tuft of flagella on one side
(iii) Amphitrichous – single or tuft on both sides
(iv) Peritrichous – surrounded by lateral flagella

Various types of mobility is observed because of the presence of the flagella as Serpentine motility is seen with Salmonella, Darting motility with Vibrio and Tumbling motility with Listeria monocytogenes.

Functioning of Flagella: The *modus operandi* of *flagella* are as given under :

(1) Flagella are fully responsible for the bacterial motility.

(2) Deflagellation by mechanical means renders the motile cells immotile.

On the basis of distribution of flagella, bacteria are classified as follows:

1. **Atrichous:** Flagella are absent.

2. **Monotrichous:** There is a single flagellum only.

3. **Amphitrichous:** There are two flagella, one at both the ends of bacterium.

4. **Cephalotrichous:** There are many flagella that occur at one end only.

5. **Lophotrichous:** There are many flagella that occur at both the ends of the bacterium.

6. **Peritrichous:** There are many flagella that occur equally distributed all over the surface of bacterium.

Pili / Fimbriae

Hair-like proteinaceous structures that extend from the cell membrane to external environment are pili which are otherwise known as fimbriae. They are thinner, shorter and more numerous than flagella and they do not function in motility. The fimbriae is composed of a subunit called pilin.There are two types pili namely Non-sex pili (Common pili) eg. fimbriae or type IV and the sex pili. The fimbriae are antigenic and mediate their adhesion which inhibits phagocytosis. The sex pili help in conjugation.

Cell wall

Beneath the external structures is the cell wall. It is very rigid & gives shape to the cell. Its main function is to prevent the cell from expanding & eventually bursting due to water uptake. Cell Wall constitutes a significant portion of the dry weight of the cell and it is essential for bacterial growth & division.

Chemically the cell wall is composed of peptidoglycan. Mucopeptide (peptidoglycan or murien) formed by N acetyl glucosamine & N acetyl muramic acid alternating in chains, cross linked by peptide chains. Embedded in it are polyalcohol called Teichoic acids.

The functions of Teichoic acid are

- gives negative charge
- major antigenic determinant
- transport ions
- anchoring

- external permeability barrier

Outer Membrane

Outer membrane is found only in Gram-negative bacteria, it functions as an initial barrier to the environment and is composed of lipopolysaccharide (LPS) and phospholipids Lipopolysaccharide (LPS).

The LPS present on the cell walls of Gram-negative bacteria account for their endotoxic activity and antigen specificity.

CytoplasmicMembrane

Just inside the peptidoglycan layer of the cell wall lies the cytoplasmic membrane, which is composed of a phospholipid bilayer similar in microscopic appearance to that in eukaryotic cells. They are chemically similar, but eukaryotic membranes contain sterols, whereas prokaryotes generally do not. The only prokaryotes that have sterols in their membranes are members of the genus *Mycoplasma.* **Cytoplasm**

The cytoplasm has two distinct areas when seen in the electron microscope:
(1) An amorphous matrix that contains ribosomes, nutrient granules, metabolites, and plasmids.
(2) An inner, nucleoid region composed of DNA.

Ribosomes

Bacterial ribosomes are the site of protein synthesis as in eukaryotic cells, but they differ from eukaryotic ribosomes in size and chemical composition. Bacterial ribosomes are 70S in size, with 50S and 30S subunits, whereas eukaryotic ribosomes are 80S in size, with 60S and 40S subunits. The differences in both the ribosomal RNAs and proteins constitute the basis of the selective action of several antibiotics that inhibit bacterial, but not human, proteinsynthesis

Nucleoid

The nucleoid is the area of the cytoplasm in which DNA is located. The DNA of prokaryotes is a single, circular molecule that has a molecular weight (MW) of approximately

Classification of Bacteria:

In 1923, David Bergey and colleagues set out to publish a definitive book on the identification and classification of bacteria. A Survey of Bacterial Phylogeny and Diversity - based on the 2ndedition of Bergey's Manual.

Macronutrients

- These include Calcium, Oxygen, Hydrogen, Nitrogen, Sulphur, Phosphorus, Potassium, Calcium, Magnesium and Iron (C, O, H, N, S, P, K, Ca, Mg, and Fe).
- They constitute over 95% of cell dry weight and are needed in relatively large quantities.
- C, O, H, N, S, and P are components of carbohydrates, lipids, proteins, and nucleic acids while the remaining four elements (K, Ca, Mg, F) exist in the cell as cations and play a variety of roles

Micro nutrients.

- These include Manganese, Zinc, Cobalt, Molybdenum, Nickel and Copper (Mn, Zn, Co, Mo, Ni, and Cu). They are used in very small amounts. In nature, they are ubiquitous and probably do not usually limit growth.

Requirements for Carbon, Hydrogen, Oxygen and Electrons

- All organisms require a source of carbon, hydrogen, oxygen, and electrons.
- Carbon is needed for the skeleton of all the organic molecules from which organisms are built.
- Hydrogen and oxygen are also important elements in organic molecules.
- The movements of electrons through the electron transport chain and during oxidation-reduction reactions provide energy for cellular work.

Nutritional types:

There are two sources of energy available to microorganisms, and based on this they are of two types:

- **Phototrophs** – Energy for growth is derived from sunlight.

- **Chemotrophs** – Energy for growth is derived from the oxidation of either organic and inorganic chemical compounds.
- Microorganisms also have two sources for hydrogen atoms and electrons and based on that they can be grouped as:
- **Lithotrophs** : Source of electrons is reduced inorganic compounds
- **Organotrophs**: Source of electrons and hydrogen is organic compounds.

Although microorganisms show great metabolic diversity, yet most of them can be categorized in one of the four nutritional types based on energy, electrons or hydrogen and carbon sources. These types are:

1. **Photolithotrophic autotrophs**

These are also called photoautotrophs as they use light as their source of energy and CO2 as their source of carbon. Some examples of this nutritional group are the eukaryotic algae and cynobacteria, purple sulphur bacteria and green sulphur bacteria.

2. **Chemo-organotrophic heterotrophs**

These are also called as chemoheterotrophs. They use organic compounds like sugars and amino acids as source of energy, hydrogen and carbon. The vast majority of pathogenic microorganisms are chemoheterotrophs.

3. **Photo – organotrophic heterotrophs**

Some phototrophic bacteria like purple non sulphur and green non sulphur bacteria use organic compounds as electron donors and carbon sources. For example Rhodospirillum rubrum use succinate as an electron donor.

4. **Chemolithotrophic autotrophs**

They oxidise inorganic compounds like iron, sulphur or nitrogen to derive both energy and electrons for biosynthesis. Carbon dioxide is the carbon source for them. Few chemolihotrophs have been recognized to derive their carbon from organic sources and are called chemolithotrophic heterotrophs.

Growth curve & isolation of bacteria

Growth Curve:

The increase in cell number or growth in population is studied by analyzing the growth curve of a microbial culture. Bacteria can be grown or cultivated in a liquid medium in a closed system or also called as batch culture. In this method, no fresh medium is added and hence with time, nutrient concentration decreases and an increase in wastes is seen. As bacteria reproduce by binary fission, the growth can be plotted as the logarithm of the number of viable cells verses the time of incubation. The curve plotted shows four basic phases of growth; the lag, log, stationary, and death phase .

l Growth curve of a typical bacterial cell

Lag Phase: As the cells are introduced into the new medium, no immediate increase in cell number occurs. During this phase, the cells are undergoing a period of intense metabolic activity involving synthesis of enzymes and various other molecules required to divide in the coming phase.

Log Phase: In this phase the cell starts dividing in a logarithmic way and this is also called as exponential phase and the growth is balanced. Cellular reproduction is high during this period and the plot during this phase is a straight line.

Stationary Phase: Exponential growth cannot be continued forever in a **batch culture** (e.g. a closed system such as a test tube or flask). Population growth is limited by one of three factors: 1. exhaustion of available nutrients; 2. accumulation of inhibitory metabolites or end products; 3. exhaustion of space, in this case called a lack of "biological space".During the stationary phase, if viable cells are being counted, it cannot be determined whether some cells are dying and an equal number of cells are dividing, or the population of cells has simply stopped growing and dividing.

Secondary metabolites, such as antibiotics, do so during the stationary phase of the growth cycle (Secondary metabolites are defined as metabolites produced after the active stage of growth). It is

during the stationary phase that spore-forming bacteria have to induce or unmask the activity of dozens of genes that may be involved in sporulation process. Starving bacteria frequently produce a variety of starvation proteins, which make the cell much more resistant to damage. They increase peptidoglycan cross-linking and cell wall strength. The Dps (DNA- binding proteins from starved cells) protein protects the DNA. Bacterial pathogens like *Salmonella typhymurium* become more virulent when starved.

Death Phase: Due to the conditions during the stationary phase, the death phase is seen as there is a decline in the number of viable cells. This phase also is like the log phase where the cell number is declining in a logarithmic way. The cell is said to be dead if it does not revive itself and reproduce when incubated again in a fresh medium. In this phase, the number of live cells decreases at a logarithmic rate, as indicated by the straight downward sloping diagonal line. The duration of this phase is as highly variable as the duration of log phase. Both depend primarily on the genetic characteristics of the organism.

The population is doubling every generation; hence the increase in population is always 2^n where n is the number of generations. The resulting population increase is exponential or logarithmic.

When growing exponentially by binary fission, the increase in a bacterial population is by geometric progression. If we start with one cell, when it divides, there are 2 cells in the first generation, 4 cells in the second generation, 8 cells in the third generation, and so on. The **generation time** is the time interval required for the cells (or population) to divide.

Preservation Of Bacteria

Periodic Transfer to Fresh Media

Strains can be maintained by periodically preparing a fresh culture from the previous stock culture. The culture medium, the storage temperature, and the time interval at which the transfers are made vary with the species and must be ascertained beforehand.

Refrigeration

Pure cultures can be successfully stored at 0-4°C either in refrigerators or in cold-rooms. This method is applied for short duration (2-3 weeks for bacteria and 3-4 months for fungi) because the metabolic activities of the microorganisms are greatly slowed down but not stopped. Thus their growth continue slowly, nutrients are utilized and waste products released in medium. This results in, finally, the death of the microbes after sometime.

Paraffin Method/ preservation by overlaying cultures with mineral oil

This is a simple and most economical method of maintaining pure cultures of bacteria and fungi. In this method, sterile liquid paraffin is poured over the slant (slope) of culture and stored upright at room temperature. The layer of paraffin ensures anaerobic conditions and prevents dehydration of the medium. This condition helps microorganisms or pure culture to remain in a dormant state and, therefore, the culture can be preserved form months to years (varies with species).The advantage of this method is that we can remove some of the growth under the oil with a transfer needle, inoculate a fresh medium, and still preserve the original culture. The simplicity of the method makes it attractive, but changes in the characteristics of a strain can still occur.

Cryopreservation

Cryopreservation (i.e., freezing in liquid nitrogen at -196°C or in the gas phase above the liquid nitrogen at -150°C) helps survival of pure cultures for long storage times.In this method, the microorganisms of culture are rapidly frozen in liquid nitrogen at -196°C in the presence of stabilizing agents such as glycerol or Dimethyl Sulfoxide (DMSO) that prevent the cell damage due to formation of ice crystals and promote cell survival. This liquid nitrogen method has been successful with many species that cannot be preserved by lyophilization and most species can remain viable under these conditions for 10 to 30 years without undergoing change in their characteristics, however this method is expensive.

Brewer's anaerobic culture plate:

In 1942, J.H. Brewer. Introduced a specially designed Petri dish cover to used with an anaerobic medium for the surface cultivation of anaerobes. It is a relatively simple device and gives

satisfactory results. The cover is provided with a circular ridge that rests on the medium, providing very rare space above the medium. An anaerobic condition in the space results from the presence of reducing agents in the medium.

After streaking on the agar surface, the continental petri dish cover is replaced by the sterile brewer cover. The medium should be deep enough so that the Brewer cover does not rest on the edge of the bottom dish. The weight if the cover will cause the cover ridge to settle somewhat into the medium.

The Gas Pak jar:

The anaerobic system is composed of the Gas Pak generator envelope and the anaerobic indicator strip kept inside the anaerobic jar.Medium plates are inoculated and kept inside the jar. Water is added to the Gas Pak generator envelope, causing the evolution of H_2 and CO_2.

Vacuum and Gas displacement method

Ideal atmospheric conditions for strict anaerobes can be achieved by displacing the air in a closed container with a mixture of nitrogen and carbon dioxide. A vacuum pump is used to withdraw the air from the chamber containing the culture plates to be incubated. A mixture of the two gasses is then allowed to fill the chamber. The CO_2 serves the same purpose as in Gas Pak system. Many bacteria have difficulty starting growth in the absence of CO_2.

The candle method:

Although this method is an old one,it is still used for culturing some bacteria such as ***Neisseria gonorrhoeae***. For many organisms,however, it is considered too toxic because of the amount of carbon monoxide produced by the burning candle. When a lighted candle is placed in a container that is tightly sealed, the O_2 will be used up, CO_2 will be produced, and the candle will quite burning after the O_2 has been exhausted

Quatitative Measurement Of Bacterial Growth

Measurement of Microbial Growth:

A number of techniques are available in order to measure growth of microbial populations. Either population number of mass may be calculated ad growth leads to increase in both.

Direct measurement of cell numbers:

Bacteria or microorganisms can be counted directly on the plate and also called as plate counting. Advantage of this method is that it measures the number of viable cells. Disadvantage is that, it is time consuming and expensive as one needs media and other conditions need to be maintained. Bacteria counted on plate counts are referred to as colony forming units as a single cell or a clump of bacterial cells can lead to a colony which contains many cells. The colonies when they are counted in plate count method are to be present sparsely for accurate counting as overcrowding can lead to incorrect counting. To solve this, one has to adapt the serial dilution method in order to get an accurate count.

Serial dilution and pour and spread plate: Supposing one has to accurately count the number of cells given in a solution, then serial dilution needs to be performed. A 1ml of the sample is taken and transferred to a tube containing 9ml of sterile water and this process can be repeated until we reach a considerable dilution (say 10^6 to 10^7). Once the original inoculum is diluted one needs to perform a pour plate or a spread plate technique in order to count the number of bacteria present in the diluted sample and then the original sample.

In pour plate method the diluted sample is poured into the petriplate and then the medium which is at nearly 50^0C is poured over the inoculum and mixed by gentle agitation. With this method, colonies grow within the nutrient agar as well as on the surface of the agar plate. As certain disadvantages are encountered in this method like heat sensitive microorganisms might not grow and also bacteria when they grow within the nutrient medium might not be useful for diagnostic purposes. In order to avoid these problems, spread plate method is mostly used (Fig. 3). A 0.1ml of the diluted sample is added to the surface of the nutrient medium and spread uniformly with the help of a glass
spreader and after incubation, the colonies can be counted and the concentration of the bacterial cells in the original sample is calculated as follows:

Number of bacteria/ml = Number of colonies on plate x reciprocal of dilution of sample

Membrane Filtration: This method can be used in order to study if the quantity of the bacteria is

very small as in aquatic samples like lakes, streams etc. Membranes with different pore sizes are used to trap different microorganisms. The sample is drawn through these special membrane filters and placed on an agar medium or on a pad soaked with liquid media. After incubation, the number of colonies can be counted and the number determined in the original sample. Selective media or differential media can be used for specific microorganisms. This is mostly used for analyzing aquatic samples.

Microscopic count: The Petroff-Hausser counting chamber or slide is easy, inexpensive and relatively quick method and also gives information about the size and morphology of the microorganisms.

Indirect methods of measurement of cell mass:

Population growth leads to increase in the total cell mass, as well as in cell numbers. The following methods can be used.

Turbidity: As bacteria grow/multiply in a liquid medium, the medium becomes turbid (Fig. 5). Spectrophotometer is used in order to measure the turbidity. A beam of light is transmitted through a bacterial suspension to a light-sensitive detector. The fact that microbial cells scatter light striking them, the amount of scattering is directly proportional to the biomass of cells present and indirectly related to cell number. The extent of light scattering can be measured and is almost linearly related to bacterial concentration at low absorbance levels.

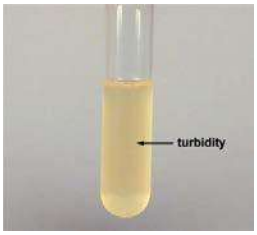


Fig. Broth culture showing turbidity

Dry weight: This method is mostly used for filamentous bacteria and moulds. The microorganism is grown in liquid medium, filtered or centrifuged to remove extraneous material, and dried in an oven and then weighted. It is time consuming and hence not very sensitive.

Different types of microscopy

DARKFIELD MICROSCOPY

Dark Field illumination is a technique used to observe unstained samples causing them to appear brightly lit against a dark, almost purely black, background.The design of the dark field microscope is such that it removes the dispersed light so that only the scattered beams hit the sample.

Bright field microscopy is the conventional technique. It is suitable for observing the natural colors of a specimen or the observation of stained samples.

on the slide.

Dark field microscopes are used in a number of different ways to view

- Living or lightly stained transparent specimens
- Single-celled organisms
- Live blood samples
- Very effective in showing the details of live and unstained samples

Disadvantages of using a dark field microscope are:

- Limited colors (certain colors will appear, but they're less accurate and most images will be just black and white)
- Images can be difficult to interpret to those unfamiliar with dark field microscopy
- Although surface details can be very apparent, the internal details of a specimen often don't stand out as much with a dark field setup.

PHASE CONTRAST MICROSCOPE

Working Principle of a Phase-Contrast Microscope

- Light passing from one object into another object of a slightly different refractive index or thickness undergoes a change in phase.In a phase-contrast microscope, this difference in phase is translated into variation in brightness of the image and hence is detectable by eye. With a phase-contrast microscope, the differences among various cells with different refractive indices or thickness can be seen in unstained condition.Unstained structures within cells, not discernible by other microscopic methods can also be observed due to the slight differences in their refractive indices or thickness.

- **construction:**

  A phase-contrast microscope is a compound microscope fitted with a phase-contrast condenser and a phase-contrast objective. An annular aperture in the diaphragm placed in the focal plane of the sub-stage condenser controls the illumination of the object.

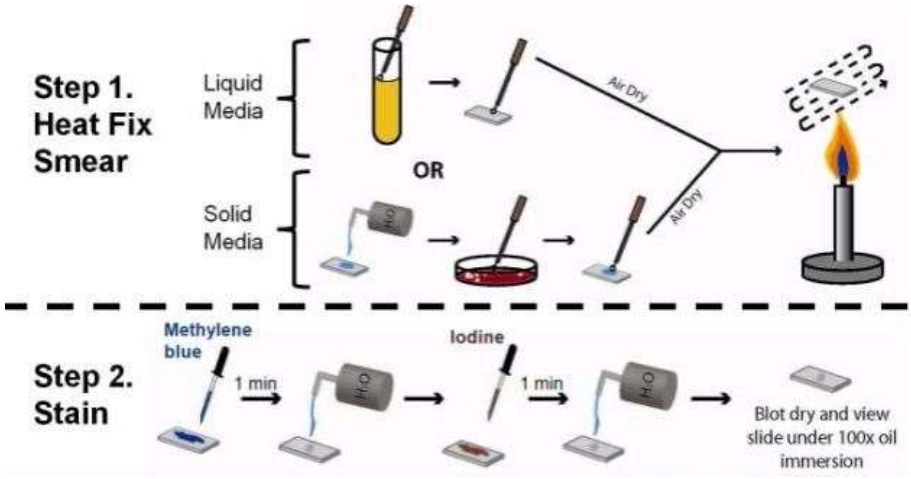
  The image of the aperture is formed at the rear focal plane of the objective. In this plane, there is a phase-shifting element or phase- plate. The phase plate also has an annular ring of phase altering pattern, which can increase the wavelength of light passing through it.

UNIT II

Identification of bacteria using staining techniques (simple)

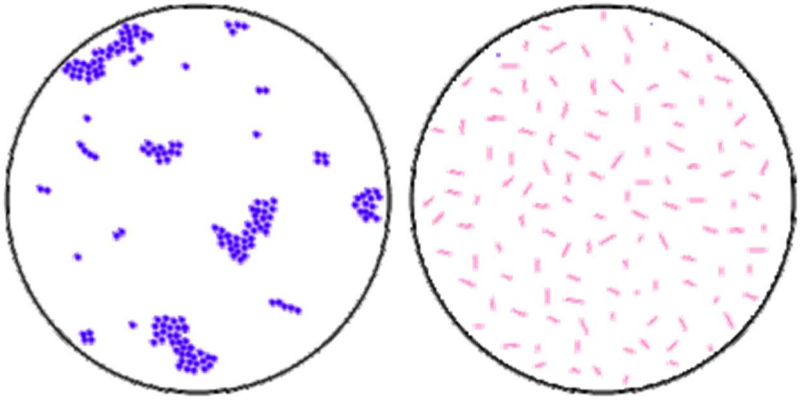
The simple stain can be used as a quick and easy way to determine cell shape, size and arrangements of bacteria. True to its name, the simple stain is a very simple staining procedure involving single solution of stain. Any basic dye such as methylene blue, safranin, or crystal violet can be used to color the bacterial cells.

These stains will readily give up a hydroxide ion or accept a hydrogen ion, which leaves the stain positively charged. Since the surface of most bacterial cells and cytoplasm is negatively charged, these positively charged stains adhere readily to the cell surface. After staining, bacterial cell morphology (shape and arrangements) can be appreciated.



Staining Procedure:

1. Cover the smear with methylene blue and allow the dye to remain in the smear for approximately one minute (Staining time is not critical here; somewhere between 30 seconds to 2 minutes should give you an acceptable stain, the longer you leave the dye in it, the darker will be the stain).
2. Using distilled water wash bottle, gently wash off the excess methylene blue from the slide by directing a gentle stream of water over the surface of the slide.
3. Wash off any stain that got on the bottom of the slide as well.
4. Saturate the smear again but this time with Iodine. Iodine will set the stain



Gram's Staining & Acid-fast staining)

Identification of bacteria by Gram Staining:

Hans Christian Gram (1884) – a Danish bacteriologist first and foremost developed the well known staining procedure called as **Gram staining**. Since, its inception earned a well-deserved recognition across the globe by virtue of the fact that it categorically divides microorganisms into *two* major categories, namely: (*a*) **Gram-positive***, and (*b*) **Gram-negative**

Methodology: The various steps involved are as follows :

(1) A **heat-fixed bacterial smear** is duly covered with the following staining reagents in a sequential manner, namely : (*a*) **crystal violet** (*i.e.*, a **basic purple dye**) which eventually imparts its colour to **all cells** ; and hence usually referred to as a **primary strain** ; (*b*) **iodine solution** *i.e.*, clearly washing off the purple dye after a short while, the smear is covered with iodine solution that serves as a **mordant***** ; (*c*) **alcohol****** *i.e.*, the iodine is washed off thereby causing a **'decolourizing effect'** ; and (*d*) **safranin** – a basic red due (or other appropriate agent) *i.e.*, to act as a **counterstrain**.

(2) The resulting **'smear'** is washed again, blotted dry, and carefully examined microscopically.

Acid-Fast Staining

Acid-Fast Stain- Principle, Procedure and Interpretation

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

Principle of Acid-Fast Stain

When the smear is stained with carbol fuchsin, it solubilizes the lipoidal material present in the Mycobacterial cell wall but by the application of heat, carbol fuchsin further penetrates through lipoidal wall and enters into cytoplasm.

Procedure of Acid-Fast Stain

1. Prepare bacterial smear on clean and grease free slide, using sterile technique.
2. Allow smear to air dry and then heat fix.
   Alcohol-fixation: This is recommended when the smear has not been prepared from sodium hypochlorite (bleach) treated sputum and will not be stained immediately. M. tuberculosis is killed by bleach and during the staining process. Heat-fixation of untreated sputum will not kill M. tuberculosis whereas alcohol-fixation is bactericidal.
3. Cover the smear with carbol fuchsin stain.
4. Heat the stain until vapour just begins to rise (i.e. about 60 C). Do not overheat. Allow the heated stain to remain on the slide for 5 minutes.
   Heating the stain: Great care must be taken when heating the carbol fuchsin especially if staining is carried out over a tray or other container in which highly fiammable chemicals have collected from previous staining.
5. de clean, and place it in a draining rack for the smear to air-dry (do not blot dry).
6. Examine the smear microscopically, using the 100 X oil immersion objective.

chemical methods of sterilization

Types of disinfectants

i) Phenol and its derivatives

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

- Phenol or carbolic acid was used by lister in 1867 for the development of antiseptic surgery
- It is used as a standard in evaluation of the other disinfectants.

Applications:

- They are bactericidal, bacteriostatic, fungicidal agents depending on the concentration used but are inactive against spores and most viruses.
- The cresols are more germicidal than phenol when they are emulsified in liquid soaps and alkalies.

3. Aldehydes

Examples: Formaldehyde (HCHO), Gluteraldehyde ($CHO.CH_2.CH_2.CH_2.CHO$)

i) Formaldehyde:

- Formaldehyde is available as a gas or as an aqueous solution.
- Formaldehyde gas is generated by heating a concentrated solution of formaldehyde. Formaldehyde in the form of aqueous solution is known as formalin. It contains 37- 40% of formaldehyde

Mechanism of action:

Formaldehyde acts through alkylation of amino-, carboxyl-, groups by combining with vital organic nitrogen compounds like proteins and nucleic acids and damages them. It is a bactericidal agent with poor penetrating power. It kills both vegetative cells and spores.

ACIDS AND ALKALIES:

Mechanism of action:

- The germicidal efficiency of acids is dependent on dissociation and release of the hydrogen-ion (H^+) in their solutions.
- Most mineral acids (HCl, H_2SO_4, HNO_3, H_3P0_4, etc.) act primarily as generators of hydrogen ions.

HALOGENS:

Eg. Chlorine, iodine, bromine and fluorine in the Free State as well as their compounds are actively germicidal.

Bromine and fluorine are irritant and carcinogenic hence difficult to handle but chlorine and iodine are of practical use.

i) Chlorine:

- Chlorine is one of the most widely used disinfectants either in the form of a gas or in certain chemical combinations

Examples of chlorine compounds: hypochlorites, chloramines

Applications:

- Chlorine gas is used to disinfect filtered water of municipal water supplies and sewage disposal plants.

Iodine:

Iodine is one of the oldest and most effective germicidal agents. It is slightly soluble in water but readily soluble in alcohol and aqueous solutions of potassium or sodium iodide.

Applications:

- Iodine is used as a skin disinfectant (antiseptic) and for cold sterilization of surgical sutures.
- Iodine compounds are also effective for disinfection of water, air, and sterilization of food utensils.

DYES:

- Basic dyes and acidic dyes both inhibit bacterial growth. Basic dyes are more effective bactericides than acidic dyes.

Acidic dyes: eg., acridine dyes like acriflavine, proflavine,aminacrine and enflavine.

Mechanism of action: They are more active against gram positive bacteria than gram negative bacteria. They impair the DNA complexes of micro-organisms and destroy the reproductive capacity of the cell. Thus they inhibit the bacterial growth.

Applications: They are used for the treatment of burns, in opthalmics and in bladder irrigation

HEAVY METALS:

- Most of the heavy metals, either alone or in certain chemical compounds, are germicidal.
- The most widely used compounds of heavy metals are those of mercury, silver and copper.

Mechanism of action:

- Heavy metals and their compounds act antimicrobially by combining with cellular proteins/ enzymes, coagulates cytoplasmic proteins, resulting in inactivation or damage of the cell.

Active enzyme mercuric chloride inactive enzyme

Applications:

8. Quaternary ammonium compounds:

Mechanism of action: The primary mode of action is disruption of cell walls and membranes.

Applications:

- They are used for control of micro-organisms on floors, walls, nursing homes and other public places.
- They are used as skin antiseptics and as sanitizing agents in diary, egg and fishing industries.

7. Detergents and soaps:

- They are widely used as surface active agents, wetting agents and emulsifiers. They are classified into four groups such as anionic, cationic, nonionic and amphoteric.

➢ Anionic detergents- those which ionize, with the detergent property resident in the anion

➢ Cationic detergents - those which ionize with the detergent property resident in the cation e.g., $(R\text{-}N(CH_3)_3)\ Cl^-$ quaternary ammonium compounds

➢ Non-ionic detergents - those which do not ionize.

➢ Amphoteric detergents – they have detergent properties of both cationic and anionic detergents. eg., Tego compound.

Mechanical methods of sterilization

FILTRATION:

Filtration does not kill microbes, it separates them out. Membrane filters with pore sizes between 0.2-0.45 μm are commonly used to remove particles from solutions that can't be autoclaved. It is used to remove microbes from heat labile liquids such as serum, antibiotic solutions, sugar solutions, urea solution.

Different types of filters:

1. Earthenware filters: These filters are made up of diatomaceous earth or porcelain. They are usually baked into the shape of candle. Different types of earthenware filters are:

A .**Pasteur-Chamber land filter:**These candle filters are from France and are made up of porcelain (sand and kaolin).

B**. Berkefeld filter:**These are made of Kieselguhr, a fossilized diatomaceous earth found in Germany. Quality of V grade filter is checked using culture suspension of *Serrtia marcescens* (0.75 μm).

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

2. **Asbestos filters**: These filters are made from chrysotiletype of asbestos, chemically composed of magnesium silicate.

3. **Sintered glass filters**: These are made from finely ground glass that are fused sufficiently to make small particles adhere to each other. They are usually available in the form of disc fused into a glass funnel.

Pharmaceutical Sterilizers

Sterilizers are special equipments used to make a product free from live bacteria or other microorganisms. The sterilizer process is used to kill all microbes. The industries such as pharmaceuticals and health care have become more concerned about safety issues and thus sterilizers become an essential component for controlling microbial contamination. Sterilization can be achieved through application of heat, chemicals, irradiation, or filtration.

- or other items or to disassemble those with sliding or multiple parts. In addition, instruments and other items can be placed in closed containers.
- Place instruments and other items in the oven, and heat to the designated temperature. The oven must have a thermometer or temperature gauge to make sure the designated temperature is reached.
- Use the list here to determine the appropriate amount of time to sterilize instruments and other items for different temperatures.
- Do not begin timing until the oven reaches the desired temperature, and do not open the oven door or add or remove any items.
- The times shown here represent the amount of time that items must be kept at the desired temperature to ensure that sterilization is achieved. Keep in mind that the total cycle time--including heating the oven to the correct temperature, sterilization, and cooling--is usually twice as long as the time noted here.
- Because dry heat can dull sharp instruments and needles, these items should not be sterilized at temperatures higher than 160 degrees C.
- Leave items in the oven to cool before removing. When they are cool, remove items using sterile pickups and use or store immediately.
- Store items properly. Proper storage is as important as the sterilization process itself:

UNIT III

Study of morphology and classification of fungi

FUNGI:

Fungus is a member of a large group of eukaryotic organisms that includes microorganisms such as yeasts and molds. Most fungi are **saprophytes**, feeding on dead or decaying material.

.

Yeasts. These are large (5 to 8 μ), single-celled organisms that rarely form filaments. Most yeasts reproduce by the asexual process of budding. Yeast colonies are usually characterized by a smooth surface similar to that of many bacteria.

Classification of Fungi

- Fungi are **classified by their reproductive structures**
- The 4 phyla of fungi are **Basidiomycota, Zygomycota, Ascomycota, & Deuteromycota.**

zygomycota

- Called sporangium fungi or common molds
- Includes molds & blights such as Rhizopus stolonifer (bread mold)
- No septa in hyphae (coenocytic)
- Asexual reproductive structure called sporangium & produces sporangiospores
- Rhizoids anchor the mold, release digestive enzymes, & absorb food
- Asexual reproductive structure called sporangium & produces sporangiospores
- Sexual spore produced by conjugation when (+) hyphae & (-) fuse is called zygospore
- Zygospores can endure harsh environments until conditions improve & new sporangium.

Basidiomycota

- Called club fungi
- Includes mushrooms, toadstools, puffballs, bracket fungi, shelf fungi, stinkhorns, rusts, & smuts
- Some are used as food (mushroom) & others cause crop damage (rusts & smuts)

- Seldom reproduce asexually

Ascomycota

- Called sac fungi
- Includes yeast, cup fungi, truffles, powdery mildew, & morels.
- Some are parasites causing Dutch elm disease & chestnut blight
- Sac Fungi can reproduce both sexually and asexually

Asexual Reproduction in Fungi

- **Fungi reproduce asexually when environmental conditions are favorable**
- Some unicellular fungi reproduce by **mitosis**
- Yeast cells reproduce by **budding** where a part of the cell pinches off to produce more yeast cells.
- A**Sexual Reproduction in Fungi**

- **Fungi reproduce sexually when environmental conditions are unfavorable**
- **No male or female** fungi
- Two mating types — **plus (+) and minus (-)**

Study of morphology of viruses

Virus

- A virus is defined as a very tiny infectious agent which is acellular, obligate intracellular parasite.
- Study of virus is known as virology.

History of virus:

- Experiments on virology began with the discovery of small pox vaccine using milder cow pox viruses by Edward Jenner

- Actual credit of discovery of virus goes to ivanowski. He demonstrated that the agents responsible for causing tobacco mosaic disease were filterable agents, indicating the existence of disease causing agents which are smaller than bacteria.
- Distinct nature of virus was studied by beijerinck, he coined the term virus which means poison (or) living infectious fluid for the substance infecting tobacco plants.
- Stanley, successfully crystallized Tobacco Mosaic Virus (TMV) from tobacco leaves infected with tobacco mosaic disease & found that virus was made of nucleic acid & protein

Characteristics of a virus:

1. They are acellular,
2. They cannot carry on metabolic activities independently, therefore they requires a host for survival. They considered as not living organisms outside the host.
3. It contains a single type of nucleic acid: either DNA or RNA but not both. It has a protein coat (capsid) surrounding the nucleic acid, some also have a lipid envelope around the capsid
4. They can only reproduce inside of a living host cell using its raw materials & enzymes
5. They have a specific host range (i.e only infect certain specific cells)
6. They are ultra-microscopic and can only be visualized under electron microscope.

Morphology of virus:

Size: viruses Are extremely small particles ranging from 20 - 400 nm on average and can only be visualized under electron microscope.

Smallest virus- Bacteriophage f_2

Largest virus- Vaccinia virus

Structure and composition:

- Viruses when exists in extracellular state are called as virions. Virions are metabolically inert and do not carry out replication.
- It consists of nucleic acid i.e either DNA or RNA.
- The DNA or RNA core is surrounded by protein coat called capsid. Capsid helps in the transfer of genetic material between host cells and protects it.
- The Capsid is composed of several protein subunits called capsomers.

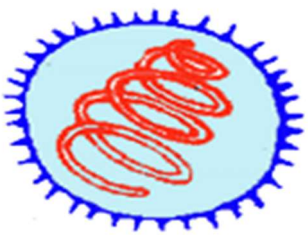
Viral symmetry:

Viral symmetry is of two types based on the arrangement of capsomers in the capsid

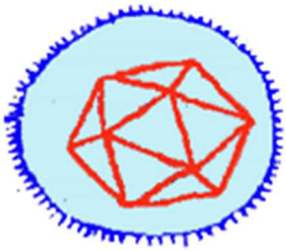
1. Helical symmetry
2. Icosahedral symmetry

Helical symmetry:

In helical symmetry, capsomers are systematically arranged in a helical manner around the core of nucleic acid.



Influenza virus



Herpes virus

Complex symmetry is exhibited by few viruses which do not have clearly defined capsids, but have several other coats around the nucleic acid.

e.g., pox virus

Binal symmetry: In some viruses, like bacteriophages binal symmetry is exhibited where head of the phage exhibits icasohedral symmetry and tail of the phage exhibits helical symmetry.

e.g, T-even bacteriophages (T2, T4, T6)

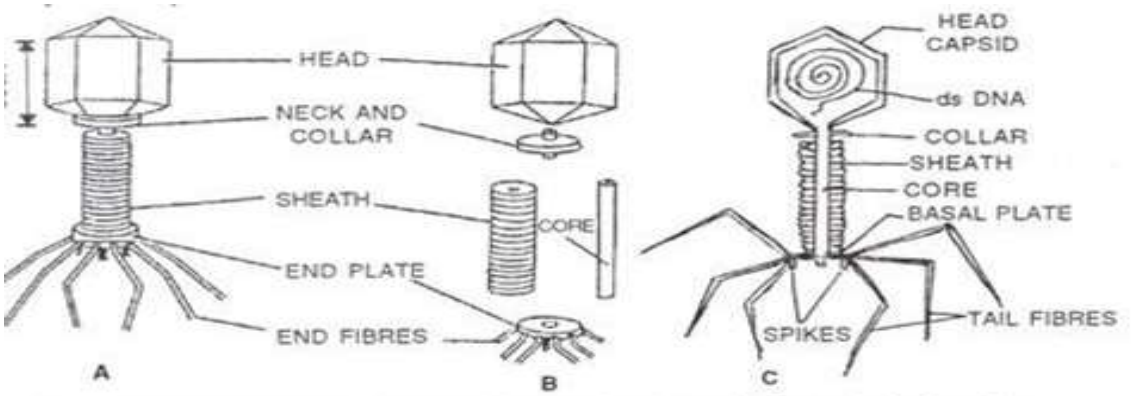


Figure 10.5 A T_4 Bacteriophage. **A.** External Structure ; **B.** Parts ; L.S. of a virion

Evaluation of bacteriostatic and bactericidal actions

Evaluation of bactericidal activity of disinfectants:

1.Phenol coefficient method:

- In phenol co-efficient method, the antimicrobial activity of test disinfectant is compared with the reference standard, phenol.
- The Phenol coefficient of a disinfectant is defined as the ability of a disinfectant dilution to kill a standardized strain of test micro-organisms compared to the dilution of phenol.

Procedure:

- Test organism used in rideal walker test is *Salmonella typhi*
- Dilutions of different concentrations for test disinfectant and phenol are prepared using sterile water
- 5ml from each of these dilutions are transferred into a sterile test tube and inoculated with 0.2ml of 24 hours broth culture of the test micro-organism
- These inoculated test tubes are kept in a water bath at a temperature of 17-18 c
- Then Subculturing is performed at every 2.5, 5, 7.5 and 10 min by transferring 0.2ml sample from inoculated test tubes (i.e., the test tubes containing reaction mixture) into sterile test tubes containing 5ml nutrient broth medium.
- The broth tubes are incubated at 37 c for 48 to 72 hrs and observed for the presence or absence of growth in the form of turbidity and then phenol-co-efficient is calculated.

Determination of Phenol-co-efficient:

- The rideal- walker co-efficient or phenol co-efficient of test disinfectant is calculated using the formula,

$$P.C = \frac{\text{Highest dilution of the test disinfectant killing in 7.5min ,but not in 5min}}{\text{Highest dilution of the phenol killing in 7.5min, but not in 5min}}$$

Disadvantages of the Rideal-Walker test: No organic matter is included; the microorganism *Salmonella typhi* may not be appropriate; the time allowed for disinfection is short; it should be used to evaluate phenolic type disinfectants only.

ii) Chick martin test:

- Test organism used in chick martin test is *Salmonella typhi*
- The test disinfectant dilutions in this method are carried out in 3% feacal matter suspension to simulate the presence or organic matter. Garrod suggested the use yeast suspension instead of feacal matter suspension later.
- 5ml of each dilution is transferred into a sterile test tube and inoculated with 0.2ml of 24 hours broth culture of the test micro-organism.

Sterility testing of products

Sterility testing:

Principle: The test for sterility is based upon the principle that if micro-organisms are placed in a nutritive medium and kept at a favorable temperature, the micro-organisms will grow and their presence can be indicated by turbidity in the originally clear medium.

The probability of detecting viable micro-organisms in the tests for sterility increases with the number present in a given amount of the preparations being examined and varies according to the species of the micro-organisms present.

(*a*) Sterility testing, due to its inherent nature, is intimately associated with a statistical process wherein the portion of a batch is sampled almost randomly* ; and, therefore, the chance of the particular batch (lot) duly passed for actual usage (consumption) solely depends upon the 'sample' having passed the stringent sterility test.

(*b*) Sterility tests should be performed under conditions designed to avoid accidental contamination of the product (under investigation) during the test. Nevertheless, such particular precautions precisely taken for this purpose must not, in any case, adversely affect any microbes that should be revealed in the test ultimately.

Table 1 gives guidance on the minimum number of items recommended being tested in relation to the number of items in the batch (as per I.P)

Culture media: The following media are used for the tests of sterility.

1. Fluid thioglycollate medium: It is used with clear fluid products.
2. Alternative thioglycollate medium: It is used with turbid or viscid products and for devices having tubes with small lumina.
3. Soyabean-casein digest medium.

All the media are adjusted to pH 7.1 ± 0.2 and sterilized by autoclaving at 121 ^{o}c for 20 minutes.

Sterility of media:

Incubate portions of the fluid thioglycollate medium/ alternate thioglycollate medium at 30 to 35^{o} c and soyabean casein digest medium at 20- 25 ^{o} c for not less than 7days and observe the presence of the growth of micro-organisms in sterile media.

UNIT IV

Designing of aseptic area

Building design, Construction and Production Facilities

• Production of sterile products should be carried out in a clean environment with a limit for the environmental quality of microbial and dust particle contamination.

• This limit for contamination is necessary to reduce the product contamination.

Air Supply

• The air supplied to a clean room must be filtered through high efficiency particulate air (HEPA) filters.

• The HEPA filter must be positioned at the inlet of the clean room and the pre- filter may be fitted upstream of the HEPA filters to prolong the life of final filter.

• HEPA filters are used in the construction of vertical and horizontal laminar air flow bench.

• The air filtered from the laminar air flow is claimed to be 99.97% free from the microbial contamination.

• These filters are supported to provide class 100 air and they should be certified every 6 to 12 months.

• Air quality is evaluated using settle plates, microbial air sampler or by particle counters.

Laminar flow equipment

1. Vertical laminar air flow bench
2. Horizontal laminar air flow bench

Air flow pattern

The air flow pattern within the clean room must be carefully regulated to avoid generating particles from the clean room floor, walls and operators.

The general airflow patterns in in clean rooms are,

1. Unidirectional airflow
2. Non-unidirectional airflow
3. Combined airflow

Sources of contamination in an aseptic area and methods of prevention

Sources of contamination in aseptic area

1. Atmosphere
2. Water
3. Raw material
4. Process operators
5. Equipment
6. Building
7. Packaging

Personnel

Personnel who are supervising or performing drug manufacturing or control can be a potential source of microbiological contamination and a vector for other contaminants.

The main reasons for contamination from the personnel include:

- Lack of training
- Direct contact between the operator's hands and starting materials, primary packaging materials and intermediate or bulk product
- Inadequate personnel cleanliness

Materials

The raw materials used for production can be a potential source of contamination.

The main reasons for contamination from the raw materials include:

- Storage and handling mistakes causing mix-ups or selection errors
- Contamination with microorganisms or other chemicals
- Degradation from exposure to excessive environmental conditions such as heat, cold, sunlight, moisture, etc.

Manufacturing Process

There are various opportunities for contamination of raw material, intermediates or packaging materials throughout the manufacturing process.

To minimize the risks of manufacturing contamination

- Manufacture products in a campaign, with the appropriately qualified cleaning processes and checks performed in-between batches to minimize the amount of product changeovers

HVAC System

A poor HVAC system can be a potential source of microbes growth and a transportation mode for dispersing contaminants throughout the manufacturing facility.

The main reasons of contamination due to HVAC issues include:

- Accumulations of organic material in or near HVAC air intakes
- Ineffective filtration of the supply air

Process of Elimination:

- Eliminate the source material
- To remove the contaminant carrier:

    - Reduce human involvement
    - Regulate the use of the equipment

- Use proper tools designed for the product
- Ensure proper cleaning of equipment to prevent any biological contamination
- Regularly check if the cleaning process is effective
- Regularly check equipment for wear and tear to prevent any compromise to its integrity
- Properly design airflow system to prevent airflow contamination

Methods for standardization of vitamins

Microbial assays of vitamins:

Microbial assays of the following *three* water-soluble vitamins would be discussed individually in the sections that follow:

(*a*) Calcium Pantothenate,
(*b*) Niacin (or Niacinamide), and
(*c*) Vitamin B_{12} (or Cyanocobalamin).

Calcium Pantothenate

It refers to one of the B complex vitamins (or vitamin B complex). The various steps involved for the assay are enumerated under sequentially :

(1) Reagents. The various reagents essentially required for the assay of 'calcium pantothenate' are

(*a*) Standardized Stock Solution. Each mL of this stock solution consists of 50 mcg of calcium panthothenate. It may be prepared by carefully dissolving 50 mg of BPCRS* calcium pantothenate in 500 mL of double-distilled water ; 10 mL of 0.2 M acetic acid, 100 mL of 1.6% (w/v) sodium acetate ; and volume made upto 1 L with DW.

(*b*) Standard Solution. The standard solution should contain approximately 0.04 mcg of calcium pantothenate in 1 mL, and is duly prepared by diluting the Standard Stock Solution (*a*).

(2) **Stock Culture of Organism :** The **stock culture of organism** may be prepared dissolving 2 g water-soluble yeast extract in 100 mL DW, 500 mg anhydrous dextrose, 500 mg anhydrous sodium acetate, and 1.5 g agar. The resulting mixture is heated gently so as to dissolve the agar. Now, 10 mL of hot solution is transferred to test tubes and sterilized at 121°C by keeping in an upright position. The **'stab culture'******* is now prepared duly in *three* tubes employing *Lactobacillus plantarum*, incubated at 30 to 37°C for 16 to 24 hours, and stored in a refrigerator ultimately.

(3).Preparation of Inoculum. The cells consequently obtained from the **stock culture,** (*a*) above, organism are duly transferred to a sterile tube containing 10 mL of the culture emdium (*d*). Finally, it is incubated at 30 to 37°C for duration of 16–24 hours.

(4) Methodology. The various steps involved are as stated below:

(*i*) **Standard Solution** (*b*) is added to *five* test tubes in varying amounts *viz.,* 1, 2, 3, 4 and 5 mL **in duplicate.**
(*ii*) To each of the *five* above test tubes plus another *four* similar tubes without any **standard solution** is added 5 mL of culture medium, and the final volume made upto 10 mL with DW.
iii) Now, volumes of **test solution** (*c*) corresponding to either *three* or more of the levels as taken above, are incorporated carefully to similar test tubes, in **duplicate.**

Vitamin B_{12} [or Cynocobalamin]

It is pertinent to state here that the **'basic culture medium'** employed for the assay of **vitamin B_{12}** is found to be extremely complex in nature, and essentially comprises of a large number of varying constituents in the form of a mixture in solution.

Various steps are as follows:

(1) First set of tubes contains solely the measured quantum of a **Standard Cyanocobalamin** Solution**.**

(2) Second set of tubes essentially comprise of the graded volumes of the **'test sample'** (*i.e.,* **unknown).**

(3) All the **'tubes'** (*i.e.,* ***first*** **set** + ***second*** **set**) are carefully inoculated with a small quantity of the culture of ***Lactobacillus leichmanni,*** and subsequently incubated duly.

(4) The precise extent of growth is assayed by measuring the percentage transmittance by the help of a standardized (calibrated) spectrophotometer.

Methods for standardization of amino acids

Microbiological assays of amino acids

Amino acids are essential for the growth of microorganisms. The basis of this assay is to measure the ability of test organism to utilize the substance being assayed under a proper nutritional condition. The organisms require amino acids in micro or nano grams. Amino acids, i.e. arginine, leucine, histidine, isoleucine, methionine, t,hreonine, phenylalanine, tryptophan, valine, glutamic acid, serine, cystine, and tyrosine, are essential for the growth of the organism. The omission of any one of these amino acids from an otherwise complete medium resulted in either very little or no growth. The response (growth of test organism) is proportional to the dose (amount of amino acid) added to medium.

Conventional microbiological assays for amino acids may be carded out in three ways: (1) by plate diffusion, (2) by turbidimetry, and (3) by acidirnetry.

An assay medium which contains all essential growth factors except the amino acid in question is selected. When increasing quantities of the amino acid are added to the medium followed by inoculation with an appropriate assay microorganism, growth or acid production occurs in a linear fashion over a given concentration of the test amino acid. Upon the construction of a standard curve by use of known quantities of the amino acid, one can determine the quantity of that amino acid in an unknown sample by reading from the standard curve.

Materials required for Microbial assay of amino acids:

1. Stock solution.
2. Inoculum media.
3. Assay medium.
4. Standard curve

Turbid metric assay

The stock culture was maintained on a culture medium. To prepare the inoculum, a quantity of cells was transferred from newly prepared stab culture that had been grown over night onto a pre culture. It is grown for 7 to 18 hours at 37°C. After 16 to 18 hours incubation the reaction was stopped by heating at l00 ^{0} c for 10 minutes. Each tube was diluted by the addition of 2 ml of water with hand shaking. The turbidity was measured by Spectrophotometer. The value obtained by the use of distilled water was taken as a control. Standard concentration response curves were prepared each time and the amino acid content in samples was calculated by interpolation.

Amino acids (omit amino acid the one being assayed),

DL-a-alanine b 200 mg, DL-leucine 250 mg, L-arginine—HCI 242 mg, L-lysine—HCI 250 mg, L-asparagine 400 mg, oL-methionine 100 mg, L-aspartic acid 100 mg ,DL-phenylalanine 100 mg, L-cysteine 50 mg, L-proline 100 mg, L-glutamic acid 300 mg, DL-serine 50 mg,Glycine 100 mg, oL-threonine 200 mg, L-histidine—HCI 62 mg, DL-tryptophan 40 mg, oL-isoleucine 250 mg, L-tyrosine 100 mg , DL-valine 250 mg.

According to modified procedure from Guirard and Snell (1981). To assay for alanine with this medium, folinic acid must be added.

General aspects-environmental cleanliness

Clean is a condition of the environment that is free of unwanted matter. Cleaning is the process used to achieve the clean condition. Best viewed as a fundamental environmental management process, cleaning is a systematic, science-based process that puts unwanted matter in its proper place or where it does not cause harm or adverse effects. Understanding the importance and effectiveness of cleaning allows us to fully appreciate its usefulness and the contributions it makes to the quality of life.

Man cannot live and survive amidst waste. A clean environment that includes clean

UNIT V

Types of spoilage, factors affecting the microbial spoilage of pharmaceutical products

Microbial Spoilage include the contamination of Pharmaceutical products with the microbes which lead to spoilage of the product affecting Drug safety and quality, and is not intended for use. Shortly Microbial Spoilage is defined as deterioration of pharmaceutical products by the contaminant microbe. Contamination of pharmaceutical products with microorganisms could make Changes in physico-chemical characteristics as well as the toxicity of pharmaceutical preparations.

Types of spoilage: spoilage is of two types

Microbial: it includes contamination of pharmaceutical products that lead to spoilage of the product.

Non-microbial: these involve chemical reactions caused by foreign material in the pharmaceutical product.

Other spoilage: it is due to insects, rodents, birds and result in change of colour, odour,and chemical nature.

Based on rate of spoilage foods are classified as

- Highly perishable: meat, poultry, milk, eggs, fruits, vegetables.
- Semi-perishable: potatoes, some apple varieties etc.
- Stable or non-perishable: sugar, dry beans etc.

Types of spoilage: spoilage is of two types

Microbial: it includes contamination of pharmaceutical products that lead to spoilage of the product.

Non-microbial: these involve chemical reactions caused by foreign material in the pharmaceutical product.

- chemical spoilage
- enzymatic spoilage
- physical spoilage
- other spoilage

Chemical spoilage-non-enzymatic chemical reaction is involved

eg: presence of lactic acid in dairy products causes souring

Enzymatic spoilage:

It is based on enzymatic reactions occurring In foods and changes in chemical nature and also result in rancidity.

eg: lipases cause hydrolytic rancidity of milk and oils.

Physical spoilage

It occurs due to temperature, light, relative humidity and result in mechanical damage of food components.

eg: oxidation of food occurs due to light and result in change of colour, flavor and chemical nature.

Other spoilage: it is due to insects, rodents, birds and result in change of colour, odour,and chemical nature.

FACTORS AFFECTING MICROBIAL SPOILAGE OF PHARMACEUTICAL PRODUCTS

1. Types and size of contaminant inoculum
2. Nutritional factors
3. Moisture content –water activity
4. Redox potential
5. Storage temperature
6. pH
7. Packaging design
8. Protection of microorganisms within pharmaceutical products

Sources and types of microbial contaminants

SOURCE OF CONTAMINATION OF PHARMACEUTICALS

In manufacture

- raw materials (water and materials with natural origin) microbiological quality
- pharmaceutical industry environment –wet sites, cleaning equipment
- packaging e.g. cardboard ,corks, papers are unsuitable packaging materials
- containers that are frequently re-used
- contamination of disinfectants due to re-used containers
- repackaging of products purchased in bulk into smaller containers
- processing
- storage
- transportation

In use

a. Human sources

- normal flora of the patients – highest risk of contamination in topical products – Staphylococcus, Micrococcus species, diphtheroids, Pseudomonas
- disposable applicators and spoons for topical and oral products
  in hospital -cross contamination between patients during use
- hand washing and creams used by staff –source of cross infection
- improper handling

b. Environmental sources

- small number of airborne contaminants may settle in product
- water-borne contaminants -Pseudomonas

c. Equipment sources

- equipments for pharmaceutical drug administration

- hospital equipments –breath tubes, ventilator, humidifiers and incubators may be the living habitats of opportunistic pathogens colonizing wet sites (Pseudomonas)

Assessment of microbial contamination and spoilage

Assessment of microbial contamination: Microbial contamination may initiate from the raw materials, during product development and even during storage and use. The most frequent reasons of contamination of pharmaceutical products include microbial contamination of the water used in product development, contaminated raw materials, and poor preservative system.
Many microorganisms that were isolated from the tested samples were gram negative bacilli and Aspergillus spp. Pathogens become challenging when they disturb the normal flora hence creating health tribulations. The presence of Klebsiella in the tested samples signals insanitary conditions that may have instigated from personnel working in the pharmaceutical areas.

Types of preservatives

Preservatives are classified on variety of the basis and some of these are as follows

A. CLASSIFICATION BASED ON MECHANISM OF ACTION

1. Antioxidants:

The agent which prevent oxidation of Active pharmaceutical ingradient which otherwise undergo degradation due to oxidation as they are sensitive to oxygen.

Eg. Vitamin E Vitamin C Butylate dhydroxy anisole (BHA). Butylate dhydroxy toluene (BHT).

2. Antimicrobial agents:

The agent which active against gram positive & gram negative micro-organism which causes degradation of pharmaceutical preparation which are active in small inclusion level.

Eg. Benzoates Sodium benzoate Sorbates

3. Chelating agents:

The agents which form the complex with pharmaceutical ingredient and prevent the degradation of pharmaceutical formulation.

Eg. Disodium ethylenediamine tetraacetic acid (EDTA) Polyphosphates Citric acid

B. CLASSIFICATION BASED ON SOURCE

1. Natural Preservatives:- These drugs are obtained by natural sources that are plant, mineral sources, animal etc.

Eg. Neem Oil,Salt (sodium chloride), Lemon, Honey

2. Artificial Preservatives: These preservatives are man made by chemical synthesis active against by various micro-organisms in small concentration.

Eg. Benzoates, Sodium benzoate, Sorbates, propionets, nitrites.

3. Phenolic compounds

- chlorinated and isopropyl derivatives of meta-cresol

4 Organic acids

- acetic acid, lactic acid, citric acid, propionic acid
- benzoic acid and hydroxyl-benzoic acid
- salicylic acid and salts

Factors Affecting the efficacy and availability of Preservatives

- Chemical Structure of the Preservatives
- Temperature

- capacity of Preservative
- Presence of inactivating agents-dirty condition
- changes of Concentration
- Great Inoculums Size

Evaluation of microbial stability of formulations

Evaluation of microbial stability of formulations:

Contamination occurs from two origins:

- during production and filling
- during usage

Common test procedures:

- screening tests
- quantitative tests

Screening tests

- Dip slides
- Plate count methods

Dip slide is used to detect aerobic bacteria in aqueous samples. It is coated with solid culture medium with agar gels.a small quantity of the dye 2,3,5-triphenyl tetrazolium chloride(TTC) is added to detect aerobic bacteria in the sample. Dip the slide in to aqueous samples for 10 seconds. Drain off excess liquid from the slide. Incubate at 35^0C-37^0C for 18 -48 hrs. The slide appearance is compared to calibration charts.

Aerobic bacteria species grow on this medium and can detect by their ability to reduce TTC to a red coloured formazan dye.

Developing bacterial colonies alter the TTC and they appear as red spots.

THIs method is used to determine the total aerobic microbial contamination in aqueous samples.

Animal cell culture

A. Primary cell culture

This is the cell culture obtained straight from the cells of a host tissue. The cells dissociated from the parental tissue are grown on a suitable container and the culture thus obtained is called primary cell culture. Such culture comprises mostly heterogeneous cells and most of the cells divide only for a limited time. However, these cells are much similar to their parents.

Depending on their origin, primary cells grow either as an adherent monolayer or in a suspension.

Adherent cells

These cells are anchorage dependent and propagate as a monolayer. These cells need to be attached to a solid or semi-solid substrate for proliferation. These adhere to the culture vessel with the use of an extracellular matrix which is generally derived from tissues of organs that are immobile and embedded in a network of connective tissue. Fibroblasts and epithelial cells are of such types.

When the bottom of the culture vessel is covered with a continuous layer of cells, usually one cell in thickness, these are known as monolayer cultures. Majority of continuous cell lines grow as monolayers. As being single layers, such cells can be transferred directly to a cover slip to examine under microscope.

Suspension cells

Suspension cells do not attach to the surface of the culture vessels. These cells are also called anchorage independent or non-adherent cells which can be grown floating in the culture medium. Hematopoietic stem cells (derived from blood, spleen and bone marrow) and tumor cells can be grown in suspension. These cells grow much faster which do not require the frequent replacement

of the medium and can be easily maintained. These are of homogeneous types and enzyme treatment is not required for the dissociation of cells; similarly these cultures have short lag period.

Applications of cell culture

Applications of Cell culture is one of the major tools used in cellular and molecular biology, providing excellent model systems for studying the normal physiology and biochemistry of cells (e.g., metabolic studies, aging), the effects of drugs and toxic compounds on the cells, and mutagenesis and carcinogenesis. It is also used in drug screening and development, and large-scale manufacturing of biological compounds (e.g., vaccines, therapeutic proteins). The major advantage of using cell culture for any of these applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells.

A. Vaccines Production

One of the most important uses of cell culture is in research and production of vaccines. The ability to grow large amounts of virus in cell culture eventually led to the creation of the polio vaccine, and cells are still used today on a large scale to produce vaccines for many other diseases, like rabies, chicken pox, hepatitis B, and measles.

B. Virus cultivation and study

Cell culture is widely used for the propagation of viruses as it is convenient, economic, easy to handle compared to other animals. It is easy to observe cytopathic effects and easy to select particular cells on which virus grow as well as to study the infectious cycle. Cell lines are convenient for virus research because cell material is continuously available. Continuous cell lines have been extremely useful in cultivating many viruses previously difficult or impossible to grow.

C. Cellular and molecular biology

Cell culture is one of the major tools used in cellular and molecular biology, providing excellent model systems for studying the normal physiology and biochemistry of cells (e.g., metabolic

studies, aging), the effects of different toxic compounds on the cells, and mutagenesis and carcinogenesis. The major advantage of using cell culture for any of these applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells