PHARMACEUTICAL MICROBIOLOGY

Shaw 650

PHARMACEUTICAL MICROBIOLOGY

B.Pharm, Semester-III

According to the syllabus based on 'Pharmacy Council of India'

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Syllabus

(BP303T) PHARMACEUTICAL MICROBIOLOGY

Module-1 (10 Hours)

- Introduction, history of microbiology, its branches, scope and its importance.
- Introduction to Prokaryotes and Eukaryotes.
- Study of ultra-structure and morphological classification of bacteria, nutritional requirements, raw materials used for culture media and physical parameters for growth,growth curve, isolation and preservation methods for pure cultures, cultivation ofanaerobes, quantitative measurement of bacterial growth (total and viable count).
- Study of different types of phase contrast microscopy, dark field microscopy and electron microscopy.

Module-2 (10 Hours)

- Identification of bacteria using staining techniques (simple, Gram's and acid fast staining) and biochemical tests (IMViC).
- Study of principle, procedure, merits, demerits and applications of physical, chemical gaseous, radiation and mechanical method of sterilization.
- Evaluation of the efficiency of sterilization methods.
- Equipments employed in large-scale sterilization.
- Sterility indicators.

Module-3 (10 Hours)

- Study of morphology, classification, reproduction/replication and cultivation of Fungi and Viruses.
- Classification and mode of action of disinfectants.
- Factors influencing disinfection, antiseptics and their evaluation for bacteriostatic and bactericidal actions.
- Evaluation of bactericidal and Bacteriostatic.
- Sterility testing of products (solids, liquids, ophthalmic and other sterile products) according to IP, BP and USP.

Module-4 **(08 Hours)**

- Designing of aseptic area, laminar flow equipments; study of different sources of contamination in an aseptic area and methods of prevention, clean area classification.
- Principles and methods of different microbiological assay. Methods for standardization of antibiotics, vitamins and amino acids.
- Assessment of a new antibiotic.

Module-5 **(07 Hours)**

- Types of spoilage, factors affecting the microbial spoilage of pharmaceutical products, sources and types of microbial contaminants, assessment of microbial contamination and spoilage.
- Preservation of pharmaceutical products using antimicrobial agents, evaluation of microbial stability of formulations.
- Growth of animal cells in culture, general procedure for cell culture, Primary, established and transformed cell cultures.
- Application of cell cultures in pharmaceutical industry and research.

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CHAPTER 1
Introduction to Microbiology

1.1. MICROBIOLOGY

1.1.1. Introduction

The branch of life-sciences, involving the study of unicellular or cell-cluster microorganisms is termed as **microbiology**, and the specialist is known as **microbiologist**. Thus, microbiology is the specific area, concerned with the study of microbes that are too small and cannot be seen without magnification.

Microbes or microorganisms are microscopic (**mikros** – small + **scopein** – to see), independently living cells that form colonies. Microorganisms include a large and diverse group of microscopic organisms that exist as a single cell of or clusters (**e.g.,** bacteria, archaea, fungi, algae, protozoa, and helminths), and the viruses. Viruses are microscopic organisms but not cellular; while bacteria and archaea are classed as prokaryotes (**pro** - before + **karyon** - nucleus); the fungi, algae, protozoa, and helminths are eukaryotes (**eu** - true or good + **karyon** - nucleus).

Microorganisms are omnipresent or ubiquitous (i.e., present everywhere on earth) and includes humans, animals, plants soil, water, atmosphere, and other living creatures. Due to their diversified activity range, microorganisms affect the human life in many ways, **e.g.,** causing infection and disease in humans, other animals, and plants; production and deposition of minerals; enhancement of soil fertility; and formation of coal. The species of microorganisms beneficial for human and environment are larger in number than the disease-causing species.

The term **microbiology** was introduced by **Louis Pasteur** (a French chemist), who demonstrated that fermentation was caused by the growth of yeasts. He is known as the **Father of Microbiology**. These microorganisms may be seen only by magnifying their images with the microscope.

1.1.2. History of Microbiology

Development of microbiology can be divided into the following eras:

1) Discovery Era,
2) Transition Era,
3) Golden Era,
4) Era of Chemotherapy and Microbial Genetics, and
5) Modern Era.

1.1.2.1. Discovery Era

In **1665**, an English scientist **Robert Hooke** developed the **first microscope** made up of a simple lens with a magnification power of approximately 30 times. He viewed numerous small structures (**e.g.,** eye of a fly, a bee stinger, and the shell of a protozoan), and with this the study of microorganism or microbiology began. **Hooke** also viewed thin slices of cork obtained from the specific type of Oak tree and observed that cork was

made up of very small box-like structures, which named as **cells**. **Hooke** published his work in a book **Micrographie** which included various studies performed by him like his thoughts on chemistry as well as a description of microscope and its uses. In **1665**, the fruiting **structures of moulds** were also illustrated by him. Consequently, for the first time microorganisms were described by him.

Unicellular life was first described just a few years after **Hooke** recorded his observations of the microscopic world. **Antony van Leeuwenhoek** (a Dutch tradesman and scientist) developed microscope and first time studied the microbes. Due to his work and development in the field of microbiology, he was recognised as the **Father of Microbiology**.

Antony van Leeuwenhoek used handcrafted microscopes and firstly observed and described single celled organisms. He named them as **animalcules** (known as microorganisms nowadays). He also observed and recorded other microscopic organisms and structures such as muscle fibres, blood flowing in capillaries (small blood vessels), spermatozoa, and bacteria.

Important Contributions of Antony Van Leeuwenhoek in Microbiology

1) He observed single-celled organisms (**e.g.,** amoeba) and bacteria for the first time.
2) He observed characteristic microbes in vinegar, curd, and human mouth.
3) He discovered spermatozoa and RBCs.
4) He discovered other microscopic structures, **e.g.,** seeds and embryos of plants and some invertebrates.

1.1.2.2. Transition Era

Development of microbiology raised some interesting questions and ultimately led the scientists to question some of the long-held beliefs. At that period, scientists used a theory known as **spontaneous generation** to explain the origins of life. According to the theory of spontaneous generation, lifeless objects give rise to living organisms, i.e., simple life forms arose spontaneously from non-living material.

Although the theory of spontaneous generation was accepted widely; but had some strong opponents. **Francesco Redi** (an Italian scientist) was among the first who argued over this theory. After studying drawing of **Leeuwenhoek**, he concluded that flies had reproductive organs and lay eggs when they land on pieces of exposed meat lay their eggs. These eggs are then hatched to maggots and thus maggots appear instinctively. In the **1670s**, **Redi** performed a series of experiments in which he prevented the entry of flies in jars of meat by covering them with a fine lace. Thus meat would not produce maggots as it was protected; by this the idea of spontaneous generation was temporarily revoked by **Redi**.

Though the theory of spontaneous generation was difficult to abandon but **Redi's** work was recognised extensively. In **1748, John Needham** (a British priest) put forth the idea that in flasks of mutton gravy, microorganisms arise by spontaneous generation. He even boiled several flasks of gravy, sealed the flasks with corks (as **Redi** had sealed his jars) and still the microorganisms developed.

The experiment of **Needham** was disapproved by **Abbe Lazzaro Spallanzani** (an Italian scientist). In **1767**, **Spallanzani** performed an experiment, in which he took vegetable broth and meat, and boiled for a longer duration of time; after that he sealed the neck by

melting the glass. Some flasks were left open to the air, some were closed by using corks, and like **Needham** some were partially boiled as a control experiment. The organisms were swarming in control flask after 2 days, while no organisms were found in the sealed flask. In response to this experiment, **Needham** said that by using excess amount of heat, "vital force" of life has been demolished by **Spallanzani**.

When the spontaneous generation was being debated, a group of scientists was concerned about the transmission of disease. In **1546**, **Girolamo Fracastoro** (an Italian scientist) put forth the concept that "contagion in an infection that passes from one thing to another". He also recognised three forms of passages, *viz.*, contact, lifeless objects, and air.

Table 1.1: Early Observations in Microbiology before the Dawn of Golden Era

Time Period	Investigator	Observations
Fourth Century BC	**Aristotle**	Living things do not need parents, spontaneous generation apparently occurs.
Mid 1500s	**Fracastoro**	"Contagion" passes among individuals, objects, and air.
Mid 1600s	**Kircher**	"Microscopic worms" are present in blood of plague victims.
Mid 1600s	**Francisco Redi**	Fly larvae arise by spontaneous generation.
Late 1600s	**Van Leeuwenhoek**	Microscopic organisms are present in numerous environments.
Early 1700s	**Christian Fabricius**	Fungi cause rust and smut diseases in plants.
Early 1700s	**Joblot**	Existence of various forms of protozoa.
Mid 1700s	**John Needham**	Microorganisms in broth arise by spontaneous generation.
Mid 1700s	**Lazzaro Spallanzani**	Heat destroys microorganisms in broth.
Late 1700s	**Edward Jenner**	Recoverers from cowpox do not contract smallpox.
Mid 1800s	**Ignaz Semmelweis**	Chlorine disinfection prevents disease spread.
Mid 1800s	**John Snow**	Water is involved in disease transmission.

The concept of disease transmission got little tenability and microorganisms were considered as contagion material. In 1600s, **Athanasius Kircher** (a German scientist) paid attention to the disease transmission when he observed blood sample of plague patients and found "microscopic worms". In 1700s, **Christian Fabricius** proposed that rust and smut diseases in plants may be due to fungi; but his work did not got recognition.

In **1798**, **Edward Jenner** discovered immunisation for smallpox though he was unable to explain the etiology of smallpox. He accorded honours for his great work and gift to mankind which was his vaccine for smallpox (characterised by production of skin lesions called **pox** caused by Variola, belonging to the category of pox viruses). Thus the modern era of vaccines and vaccination began in **1798** with the discovery of a vaccine against smallpox by **Edward Jenner**.

In **1847**, **Ignaz Semmelweis** (Hungarian physician) observed the transfer of blood poisoning agent from autopsies in the mortuary to the maternity patients, via physicians. **Semmelweis** showed that hand washing in chlorine water could stop the spread of disease. In **1854**, **John Snow** (a British physician) identified the source of cholera to the municipal water supply of London and he concluded that this disease can be prevented by

evading the polluted source of water. Both **Semmelweis** and **Snow** concluded to the fact that a poison or unseen object in the environment was responsible for the disease, but the exact cause was still unknown.

In **1867**, **Joseph Lister** developed a system of antiseptic surgery to prevent microorganisms from entering wounds. In **1878**, **Lister** gave the specific cause of milk souring after studying the lactic acid fermentation of milk. He also developed a method of isolation of a pure culture of a bacterium, *Bacterium lactis*. **Lister** introduced the principle of sterile surgery in the medical field and was designated as the **Father of Antiseptic Surgery**.

Table 1.1 enlists the observations made before the dawn of golden era.

1.1.2.3. Golden Era

The Golden Era of Microbiology is the time period of 60 years during which the science of microbiology blossomed. The period started with the work of **Louis Pasteur** in **1857** and continued up to the 20th century till the beginning of World War I. In this period, various branches of microbiology were laid for the maturing process that has led to modern microbiology.

In **1848**, **Louis Pasteur** reported the role of microorganisms in fermentation for the first time. He gained prominence for the discovery of two different types of crystals of tartaric acid (a four-carbon containing organic compound). In **1857**, **Pasteur** resolved the mystery of sour wines. He explained the role of yeasts in fermentation of fruits and grains resulting in the production of alcohol. He also observed that bacteria causes spoilage of wine. He performed Swan-Neck Flask experiment and strictly disproved the theory of spontaneous generation.

Louis Pasteur proposed the **germ theory of disease** and discovered the anaerobic bacteria (existence of bacteria in the absence of free oxygen). He discovered **pasteurisation technique** which involves mild heating to kill microorganisms in broth. He developed anti-rabies and anthrax (*Bacillus anthracis*) vaccines, gave methods to control the pebrine disease in silkworm, and isolated the causative agent of cholera (*Vibrio cholerae*) and rabies (Lyssa) virus.

While **Pasture** failed to relate a specific organism to specific disease, his contribution encouraged others to investigate the nature of microorganism, and to establish their relation with diseases.

Ferdinand Cohn (a German botanist) discovered multiplication of bacteria by dividing into two cells. He also viewed that some bacteria develop a highly resistant structure, known as endospore in the cell.

Table 1.2: Louis Pasteur – Notable Contributions

1857	Lactic acid fermentation is due to a microorganism.
1860	Yeasts are involved in alcoholic fermentation.
1861	Disproved the theory of spontaneous generation.
1861	Introduction of the terms aerobic and anaerobic for yeasts. Production of more alcohol in the absence of oxygen during sugar fermentation (**The Pasteur Effect**).
1862	Proposed germ theory of disease.
1867	Devised the process of destroying bacteria known as pasteurisation.
1881	Development of anthrax vaccine. Resolved Pebrine problem of silkworms.
1885	Development of a special vaccine for rabies (the Pasteur treatment).

Cohn gave the entire lifecycle of *Bacillus* bacteria (vegetative cell → endospore → vegetative cell). He used cotton plugs for closing the flasks and tubes to prevent the contamination of sterile culture media. In **1855**, **Cohn** studied *Beggiatoa mirabilis* (a filamentous sulphur-oxidising bacterium) and also identified the minute granules of sulphur present in the cell that are produced from the oxidation of H_2S.

Robert Koch proved the germ theory of disease undisputedly. His major work was on anthrax (a fatal disease of blood of sheep and cattle). He injected mice with the blood of diseased animals (sheep and cattle), performed autopsies, and observed that the same symptoms appeared regularly. He isolated some rod-shaped bacilli from the blood of a mouse by placing the bacilli in the sterile aqueous humour from an ox's eye. Similar symptoms of anthrax develop in ox within hours. Koch performed autopsies of the animal and found their blood swarming with bacilli and re-isolated the bacilli in sterile aqueous humour. The method adopted by him is known as **Koch's postulates** which are as follows:

1) The suspected microorganism must be present in the diseased organism but absent in the healthy organism.
2) The microorganism must be isolated in pure culture (one free of all other types of microbes) on a nutrient medium.
3) If the isolated microorganism is inoculated into a healthy host, the same disease must result.
4) Similar microorganism (causing agent) must be re-isolated from the experimentally infected host.

Table 1.3: Robert Koch-Notable Contributions

1876	Demonstrated that anthrax is caused by *Bacillius anthracis*.
1877	Methods for staining bacteria, photographing and preparing permanent visual records on slides.
1881	Developed solid culture media and the methods for studying bacteria in pure cultures.
1882	Isolated the bacterium, *Mycobacterium tuberculosis*, that causes tuberculosis.
1882	Agar as a support medium for solid culture in Koch's lab by **Hesse**.
1883	Isolated *Vibrio cholerae*, that cause cholera.
1883	Verification of the germ theory of disease by relating a specific organism to the specific disease.
1883	Koch put forth his postulates, known as **Koch's postulates**.

In **1880**, **Koch** observed small masses of bacteria on a slice of potato, which he named **colonies**. Colonies have millions of just one kind of bacteria.

Koch's proof of the germ theory was proposed in **1876**. Within two years, **Pasteur** verified the proof and moved a step ahead. **Pasteur** observed that bacteria were sensitive to temperature because chickens did not get infected with anthrax at normal body temperature of 42°C, but get infected if animals were cooled down to 37°C.

In **1880**, the most remarkable discovery of **Pasteur** came in light when he inoculated some chickens but no signs of chicken cholera were observed. He had been working on the methods to weaken bacteria using heat, different growth media, passages among animals, and many more things which he could think. Lastly, he developed two cultures with reduced ability to produce disease, suspended the bacteria in a mildly acidic medium, and left the culture undisturbed for a long period of time. When chickens were inoculated with this culture and followed by a dose of lethal cholera bacilli, the animals did not become infected. This principle is the basis for the use of many vaccines for immunisation. In **1881**, **Pasteur** developed anthrax vaccine by using this principle and protected sheep against the disease.

Koch isolated causative agents of tuberculosis, *Tubercle bacillus*. *Typhoid bacillus* and *Diphtheria bacillus* were isolated in **1884**, by colleagues of **Koch**, **George Gafky** and **Friederich Loeffler**, respectively. After a few years, **Emil von Behring** produced diphtheria antitoxin by injecting a toxin into animals. Antitoxins are antibody preparation or a blood product. **Emil von Behring** received the first Nobel Prize in Physiology and Medicine for the successful use of this antitoxin in the treatment of diphtheria.

In **1885**, **Pasteur** successfully immunised young **Joseph Meister** against the dreaded disease, rabies. **Pasteur** never saw the agent of rabies, yet he produced it in the animal's brains and injected in the boy with bits of the tissue. Thus, he successfully applied the principles to resolve the practical problem.

Other Pioneers of Microbiology

1) Anaerobic organism, ***Tetanus bacillus*** was effectively cultivated by a Japanese scientist, **Shibasaburo Kitasato** and **Koch**.
2) In **1884**, **Elie Metchnikoff** (an associate of Pasteur) published an account of phagocytosis, a defensive process in which the body's white blood cells engulf and destroy microorganisms.
3) **Metchnikoff** gave the term **phagocytosis** (eating of cells) and explained it as a defensive process in which the body's White Blood Cells (WBCs) engulf and destroy microorganisms. Thus, he explained the basic theory on which the science of immunology is established that the body is protected from infection by leukocytes that engulf bacteria and other invading organisms (cellular immunity). In **1908,** Nobel Prize was awarded to him. Metchnikoff's notable contribution was on the *Bacillus bulgaricus* therapy and his underlying concept of health.
4) **Charles Nicolle** (a scientist of a Pasteur Institute) proved that typhus fever was transmitted by lice.
5) **Albert Calmette** (a scientist of a Pasteur Institute) developed a harmless strain of *Tubercle bacillus* used for immunisation.
6) **Jules Bordet** (a Belgian bacteriologist) isolated the bacillus of pertussis (whooping cough) and developed the complement fixation test, a procedure once widely used in the diagnosis of disease.
7) In **1898**, **Ronald Ross** (an English physician) proved that mosquitoes were the vital link in malaria transmission. For this discovery, he was awarded Nobel Prize in **1902**.
8) **David Bruce** (a British scientist) isolated the cause of undulant fever.
9) **Bruce** also said that tsetse flies transmit sleeping sickness. **Almroth Wright** (a British scientist) described opsonins, the chemical substances that promote phagocytosis in the body.
10) In **1897**, **Masaki Ogata** (a Japanese physician) reported that rat fleas transmit bubonic plague. One year later, **Kiyoshi Shiga** isolated the bacterium causing bacterial dysentery, an intestinal disease; the organism was later named *Shigella*.
11) **Daniel E. Salmon** and **Theobald Smith**, (American microbiologists) were among the first who used heat killed bacteria for immunisations. **Salmon** later studied swine plague and gave his name to *Salmonella*, an agent causing typhoid fever. **Smith** discovered that Texas fever (a disease of cattle) was transmitted by ticks.

12) **Howard Taylor Rickkets** (a pathologist from University of Chicago) discovered the agent of Rocky Mountain spotted fever in the human bloodstream and demonstrated its transmission through ticks.

13) **Winogradsky** and **Beijerinck** examined the role of non-infectious microorganisms in the soil and reported the importance of microorganisms in nitrogen, sulphur, and carbon cycling. They also discovered the process of nitrogen fixation by symbiotic or free living soil bacteria.

1.1.2.4. Era of Chemotherapy and Microbial Genetics

In **1910**, **Paul Ehrlich** along with **Sakahiro Hata** discovered the drug, **Salvarsan**, an arsenobenzol compound used for the treatment of syphilis caused by *Treponema pallidum*. Thus, **Ehrlich** laid the foundation of the era of chemotherapy. Chemotherapy involves selective inhibition or killing of pathogens without causing damage to the host.

In **1935**, **Gerhard Domagk** of Germany discovered first sulpha drug in the form of **Prontosil** (a red dye used for staining leather). He reported that it was active against pathogens, *Streptococci* and *Staphylococci*. For this discovery **Domagk** got a noble prize in **1939**.

In **1929**, **Sir Alexander Fleming** (a Scottish physician and bacteriologist) discovered **penicillin** from the mould *Penicillium notatum*. Penicillin was the **first antibiotic** and is also known as wonder drug. Penicillin is a microbial product that can kill susceptible microorganisms and inhibit their growth. At Oxford University in England procedures for **industrial production of penicillin** was established by **Sir Howard W. Florey** and **Ernst B. Chain** in **1941**. For the discovery and production of penicillin, Noble Prize was awarded to **Fleming**, **Florey**, and **Chain** in **1945**.

In **1944** another antibiotic (Streptomycin) was obtained from *Streptomyces griseus* (an actinomycetes) by **S.A Waksman** and **Albert schatz**. In **1952**, **Waksman** received Nobel Prize for his notable contribution and for the discovery of streptomycin used in the treatment of tuberculosis (bacterial disease caused by *Mycobacterium tuberculosis*).

In **1947**, **Dr. Paul R. Burkholder** isolated **chloramphenicol** (chloromycetin) from *Streptomyces venezuelae*. In **1948**, **Dr. B.M. Dugger** identified **aureomycin** from *Streptomyces aureofaciens*. **Tetramycin** was discovered by **Finlay**, **Hobby**, and his collaborators in **1950** from *Streptomyces rimosus*.

Antibiotic production remains to be the important area of industrial research. Now, there are over 8000 antibiotics known, out of which only a few are being used as chemotherapeutic agents.

In **1943**, a series of experiments were performed by **Salvador Luria** (an Italian microbiologist) and **Max Dulbriick** (a German physicist) with bacteria and viruses. They addressed basic questions regarding the nature of mutations, spontaneous or induced by using bacterium *Escherichia coli*. They also concluded that sometimes bacteria may develop resistance to viral infection due to spontaneous mutation.

George Beadle and **Edward Tatum** carried out their experiments on fungus *Neurospora* and showed that one gene codes for one enzyme, i.e., one-gene one enzyme hypothesis. **Oswald Avery**, **Colin Mcleod**, and **Maclyn McCarty**, worked on the bacterium *Streptococcus pneumoniae*, and concluded that Deoxyribonucleic Acid (DNA) is the genetic material in cells.

In **1953**, **Alfred Hershey** (an American biochemist) and geneticist **Martha Chase**, studied bacterial viruses and gave indisputable evidence that DNA is the substance of genetic material. In **1958**, **Joshua Lederberg** received Nobel Prize in Physiology or Medicine for his discoveries concerning genetic recombination and organisation of genetic material in bacteria.

1.1.2.5. Modern Era

By the 1970s, research on bacterial physiology, biochemistry, and genetics had advanced to such an extent that it was possible to manipulate the genetic material of living organism very easily. Discovery of restriction enzymes had made possible the introduction of DNA from foreign sources into bacteria and control its replication.

It led to the development of biotechnology. The **genetic code** was deciphered by **Prof Har Gobind Khorana**, along with **Nirenberg** and co-workers. In **1968**, they were awarded with Nobel Prize for their work.

In **2005**, **Barry J. Marshall** and **Robin Warren** won Nobel Prize for showing that painful ulcers in the stomach and intestine result due to infection of *Helicobacter pylori* (*Campylobacter pylori*) and not due to stress.

Every field of biology was influenced by the development of sequencing techniques of nucleic acid simultaneously. Sequencing technology helped microbiologists to establish phylogenetic (evolutionary) relationships among prokaryotes, which led to evolutionary new concepts in the field of biological classification. In this era, various branches of microbiology like medical microbiology, microbial ecology, industrial microbiology, etc., has been developed.

The long span of 450 years of microbiology brought amazing insight into the biology of microbes, and also brought with it new challenges which affects society both positively and negatively. Various microbial diseases like AIDS, Bird's flu, and SARS seem to appear without a trace and have challenged the basic understanding of microbial diseases.

Simultaneously, new discoveries and inventions have helped for better understanding of the cell's physiology. Microbial ecology has given the evidences of microbial importance in the environment. Biofilms are discovered as the dominant form of organisation of microbial communities. Microorganisms have more positive roles other than causing infectious diseases. Many microorganisms are known for their essential and important beneficial roles that can provide humanity with an even better and more healthful existence.

1.1.3. Branches of Microbiology

Microbiology is the largest and most complex branch of biological sciences as it deals with many diverse biological disciplines. It has played a major role in the progression of human health and welfare.

Along with the study of the natural history of microbes, it also deals with every aspect of microbe-human and environmental interaction including ecology, genetics, metabolism, infection, disease, chemotherapy, immunology, genetic engineering, industry, and agriculture. Microbiology can be broadly categorised into **basic** and **applied disciplines** as given **table 1.4:**

Table 1.4: Basic and Applied Disciplines in Microbiology

Discipline	Nature of Study
1) **Basic Disciplines** i) **Algology or Phycology**	It is the study of algae (simple aquatic organisms ranging from single-celled forms too large seaweeds).
ii) **Bacteriology**	It is the study of bacteria (smallest, simplest, single-celled prokaryotic microorganisms) and archaeabacteria (prokaryotic microorganisms which constitute an ancient group intermediate between the bacteria and eukaryotes).
iii) **Mycology**	It is the study of fungi, microscopic eukaryotic forms (moulds and yeasts), higher forms (mushrooms, toadstools and puffballs), and slime moulds.
iv) **Protozoology**	It is the study of protozoans (animal like and mostly single-celled, eukaryotic organisms).
v) **Virology**	It is the study of viruses (infectious agents containing either DNA or RNA that require living cells for their replication/or reproduction) and viral diseases.
vi) **Parasitology**	It is the study of parasitism and parasites that include pathogenic protozoa, helminth worms, and some insects.
vii) **Microbial Ecology**	It is the study of interrelationships between microbes and environment.
viii) **Microbial Morphology**	It is the study of detailed structures of microorganisms.
ix) **Microbial Systematics**	It is the study of classification, naming, and identification of microorganisms and constructions of the phylogenetic tree of life.
x) **Microbial Physiology**	It is the study of metabolism of microbes at the cellular and molecular levels.
xi) **Microbial Biochemistry**	It is the study of discovery of microbial enzymes and the chemical reactions carried out by them.
xii) **Molecular Microbiology**	It is the study of genome (i.e., genomics) of microorganisms and construction of phylogenetic tree-based on rRNA.
xiii) **Microbial Genetics**	It is the study of heredity and variation in varieties.
xiv) **Molecular Biology**	It is the study of advanced study of genetic material (DNA, RNA) and protein synthesis.
2) **Applied Disciplines**	
i) **Immunology**	It is the study of immune system that protects against infections and attempts to understand phenomena responsible for both acquired and innate immunity, in addition to the study of antibody-antigen reactions in laboratory.
ii) **Agricultural Microbiology**	It is the study of relationships of microbes and crops with an emphasis on control of plant diseases and improvement of yields.
iii) **Food Microbiology**	It is the study of interaction of microorganisms and food in relation to food bioprocessing, food spoilage, food-borne diseases and their prevention.
iv) **Agricultural Microbiology**	It is the study of relationships of microbes and crops with an emphasis on control of plant diseases and improvement of yields.
v) **Food Microbiology**	It is the study of interaction of microorganisms and food in relation to food bioprocessing, food spoilage, food-borne diseases and their prevention.
vi) **Dairy Microbiology**	It is the study of production and maintenance of quality control of dairy products.

vii) **Industrial Microbiology**	It is the study of industrial uses of microbes in the production of alcoholic beverages, vitamins, amino acids, enzymes, antibiotics and other drugs.
viii) **Marine Microbiology**	It is the study of microorganisms and their activity concerning human and animal health in fresh, estuarine, and marine waters.
ix) **Air Microbiology**	It is the study of role of aerospora in contamination and spoilage of food and dissemination of plant and animal diseases through air.
x) **Exomicrobiology**	It is the study of exploration for microbial life in outer space.
xi) **Diagnostic Microbiology**	It is the study of fundamental principles and techniques involved in the study of pathogenic organisms as well as their application in the diagnosis of infectious diseases.
xii) **Epidemiology and Public Health Microbiology**	It is the study of control and spread of diseases in communities.
xiii) **Biotechnology**	It is the study of scientific manipulation of living organisms, especially at the molecular and genetic level to produce useful products.

1.1.4. Scope of Microbiology

The study of microbiology involves the following:

1) **Production of Antibiotics:** Microorganisms are directly used in pharmaceutical industry for the production of antibiotics by isolating antibiotic-producing microorganisms from nature (**e.g.,** water or soil).

2) **Production of Enzymes, Vaccines, Bio-Surfactants, Alcoholic and Other Pharmaceutical Products:** Pharmaceutical microbiology includes detection and assay of pharmaceutical and chemical products of microbial activity.

3) **Diagnosis of Diseases and Treatment:** Various infectious microorganisms are detected and recognised by using different tests (**e.g.,** Widal test, Elisa, etc.). For the selection of appropriate treatment for microbial infections, antimicrobial susceptibility testing is performed.

4) **Treatment of Industrial Waste Material:** Waste organic matter, salts, spent media, untreated-water, etc., are produced after most of the industrial processes. For the treatment and decomposition of such industrial and organic wastes many microbial species (**e.g.,** protozoans, fungi, actinomycetes, etc.) are used.

5) **Plant Growth Promotion:** Certain species of microbes (**e.g.,** *Rhizobium*, *Rhodospirillum*, *Azatobacter*, *Agrobacterium rhizogenes*, *Pseudomonas*, *Acetobacter*) are present in soil to participate in soil fertility, herbicidal resistance, insect resistance, enhancement of quality of plant products, and protein/oil content alteration.

6) **Sterile Product Preparation:** Microbiology is involved in the preparation of sterile room, aseptic techniques, and detection of microbes by sampling and sterility testing of different sterile preparations.

7) **Sterilisation:** This process involves killing of microbes by different means to make the pharmaceutical product free from microorganisms.

8) **Steroid Biotransformation:** With the help of microbiological transformations of naturally occurring steroids, some important steroids can be produced. Steroids are physiologically active compounds with complex molecular structure, **e.g.,** testosterone, ergosterol, progesterone, cholesterol, etc.

9) **Identification of Microorganisms:** Microbial species that can be used for the production of enzymes, antibiotics, and other active pharmaceutical compounds are identified and isolated on the basis of their morphological, biochemical, cultural, microscopic characteristics, and genetic features.

10) **Testing of Pharmaceuticals**
   i) In pharmaceutical raw materials and finished products, the *Salmonella* species, *Pseudomonas aeruginosa, Staphylococcus aureus*, and *Escherichia coli* are detected by performing several tests described in the British Pharmacopoeia (BP), European Pharmacopoeia (EP), United States Pharmacopoeia (USP), and Indian Pharmacopoeia (IP),
   ii) Endotoxin testing,
   iii) Microbiological assays of antibiotics,
   iv) Tests for support of the sterility assurance system,
   v) Evaluation of disinfectants, and
   vi) Antimicrobial preservation efficacy testing.

1.1.5. Importance of Microbiology

Microorganisms are used in almost every aspect of life due to their ubiquitous nature, i.e., they are found in soil, mud, water, air, animals, plants, food products, dead wood, clothes, etc.

Applications of microbiology in pharmaceuticals are as follows:

1) **Microbes in Food and Dairy Industries:** Food microbiology is the study of those microbes which supply food due to their higher protein value (**e.g.,** yeast). It is also the study of those microbes which grow using our food supply as a source of nutrient. When microbes attack the food products, they increase in number, utilise nutrients, produce enzymatic changes, and destroy the flavours by breakdown of products that ultimately results in the deterioration of food. Microbes used in food industries are discussed below:
   i) **Moulds:** Many microorganisms, especially moulds (**e.g.,** *Mucor*, *Rhizopus*, *Botrytis*, *Aspergillus*, *Penicillium*, etc.) deteriorate the food. Thus, in the manufacturing of certain food and other related substances some specific species of moulds are used. **For example,** mould ripened cheese (blue, Roquefort, camembert, etc.), production of oriental foods (soy sauce, miso, sonti, etc.). Other than these, some microorganisms are involved in the preparation of enzymes, **e.g.,** amylase in bread-making and citric acid used in soft drinks. Some moulds are harmful, **e.g.,** *Aspergillus flavus*, and some moulds produce mycotoxins (toxic metabolites), **e.g.,** *Aspergillus parasiticus*.

   ii) **Yeasts:** These are unicellular eukaryotes, classified under fungi, and generally or ovoid or spherical in shape. They are mostly non-filamentous, and reproduce by fission or budding. They may be both harmful, as well useful for food, and in food industry.

   During the food manufacturing where fermentation is required (**e.g.,** bread, beer, wines, vinegar and surface ripened cheese), yeast is used. Yeasts are grown to produce enzyme involved in the fermentation process. The growth and enzyme of yeast is also undesirable as they spoil fruits, juices, molasses, syrups, beer, wine, and other foods. **Examples** of yeast used in food industry are *Saccharomyces*, *Schizosaccharomyces*, *Candida*, *Kluyveromyces*, *Zygosaccharomyces*, *Pichia*, *Hansenula*, *Debaromyces*, *Hanseniaspora*, etc.

   iii) **Bacteria:** Like fungi, bacteria can also be harmful and beneficial for food and food industry. Some pigmented bacteria produce colour changes on the food surface, form a film over liquid food, etc. This results in sedimentation and produce undesirable cloudiness.

Bacteria of genera ***Acetobacter***, oxidise ethyl alcohol to acetic acid. These pathogens are called **aeromonas** (a facultative anaerobe) which are pathogenic to humans and other animals (fish, frogs, and other mammals). **Alcaligenes** (as the name indicates) produce an alkaline reaction in the medium for growth, causes ropiness in milk, and results in slimy growth on cottage cheese.

Few **examples** of beneficial bacteria are:

a) ***Bacillus coagulan*:** A proteolytic species used for curdling of milk.
b) ***Bacillus purimilus*:** Recommended test organism in sterility testing.
c) ***B. stearothermophilus*:** Used for testing procedures of steam sterilisation.
d) ***B. subtilis* var. niger (ATCC 9372):** Recommended for ethylene oxide sterilisation testing.
e) ***Microbacterium lacticum*:** Used in production of vitamins.
f) ***Micrococcus luteus* and *M. rosens*:** Used in meat-curing brines.

2) **Microbes in the Production of Industrial Products:** Microorganisms are actively involved in the production of amino acids, alcohol, organic acids, enzymes, vitamins, and antibiotics. In these products, few are used by the bacteria itself for their growth, therefore known as **primary metabolites** or **primary microbial products** (amino acids, enzymes, and vitamins) and some are not essential for the growth of the cells, therefore, named as **secondary metabolites** or **secondary microbial products** (organic acids, alcohol, and antibiotics).

Following are the **extracellular microbial enzymes** which are available in market in crude form:

i) **Amylases:** These are used in the preparation of starch hydrolysates used in various product formation, **e.g.,** beer, vinegar, etc.
ii) **Penicillin Acylases:** These are used in the production of semi-synthetic penicillin.
iii) **Lipases and Proteases:** These are used as supplements to detergents, but proteases are also used in leather and food industry.
iv) **Pectinases:** These are used in clarifying fruit juice.
v) **Asparaginases:** These enzyme of microbial origin are used in the treatment of leukaemia and lymphomas by acting upon amino acid asparagine present in cancer cells in high concentration.

3) **Microbes in Genetic Engineering and Biotechnology:** Genetic engineering is a molecular technique used for manipulating genetic information, and biotechnology is the use of genetically engineered microbes in industrial process. By using the genetically engineered microorganisms, many proteins of pharmaceutical importance can be produced. Diagnostic kits and vaccines depend on the use of improved strains of microorganisms.

By cloning **Human Growth Hormones** in bacteria, sufficient amount of it can be produced required in clinical trials. In animal cells, interferons are produced during viral infections. These interferons are used for testing interleukins involved in the stimulation of T-lymphocytes. These are used in the production of bacterial, viral, or protozoan antigens for treating typhoid, cholera, dysentery, and other infections in humans caused by microorganisms.

By using the principles of genetic engineering, viral vaccines with cloned polypeptide antigens can be produced for treating foot and mouth disease, and hepatitis B. Lactic acid and acetic acid are obtained as by-products of many microorganisms. Lactic acid is used in textile industry and to preserve food in tanning animal hide, while acetic acid is the main component of vinegar.

Many antibiotics such as β-lactam (penicillin and cephalosporin) are produced by microorganisms to be used in the treatment of various diseased conditions; **for example**, *Gonoccocal*, *Treponemal*, and *Streptococcal* infection are treated with penicillin which is also effective against Gram-positive bacteria; nosocomial Gram-negative sepsis is treated with cephalosporin.

4) **Microbes in Environmental Microbiology:** Microbes are omnipresent, i.e., they are found in water, soil, deep inside the earth (deep sea vents), and air. Recycling of biological elements (**e.g.,** oxygen, carbon, nitrogen, sulphur, and phosphorus) is also performed by microbes.

i) **Microbes in Biogeochemical Cycles:** A cell contains 70% of its weight as oxygen, and in atmosphere above 23% oxygen is present that remains available to all microbes. Oxygen is also found in mineral deposits as salts of carbonate, silicate, aluminate, and oxide. About 80% of oxygen cannot be used by microorganisms; however, cyanobacteria, heterotrophs, and chemolithotrophs use it. Water remains available for photosynthesis as it is the product of aerobic processes. The affect of microbes on some important biogeochemical cycles is discussed below:

a) **Carbon Cycle:** In atmosphere, carbon remains in the form of CO_2 gas produced abundantly by the burning of biological preparations and fossil fuel (petroleum, coal, etc.) and microbial decomposition. From the total carbon present on the earth, about 20% is found as an organic compound, and 1% is found in fossil fuel.

CO_2 gets converted into organic carbon by photosynthesis and chemosynthesis performed by the microorganisms. Methanogenic archaebacteria produces methane (CH_4) anaerobically from CO_2 and H_2. Fungi and bacteria present in soil uses organic matter present in soil. By using their enzymatic machinery, microbes release soluble products that are used by plants and microbes itself. Microbes decompose the dead material of animals and plants.

b) **Nitrogen Cycle:** There are four main parts of nitrogen cycle and microbes (especially bacteria) play a role in all of them. Bacteria (**e.g.,** cyanobacteria) help in **nitrogen fixation**, either as free-living bodies or through symbiotic relationships with animals and plants (such as legumes). Bacteria break down the plant and animal proteins into ammonia through the process of decay; this process is termed **ammonification**. The next step in nitrogen cycle is **nitrification**, which is carried out by two nitrifying bacteria (nitrosomonas and nitrobacter); the former converts ammonia to nitrite, while the latter converts nitrite to nitrates. The final step is **denitrification**, which is carried out by denitrifying bacteria (anammox or ammonia oxidizing bacteria); in this step, nitrates are broken down into nitrogen to replenish the atmosphere.

ii) **Microbes in Pollution Microbiology:** Bacteriophages (bacterial viruses) control the bacterial population by performing lysis of susceptible group. Bacteria also perform decomposition of waste. In aquatic environment, many microorganisms are found performing **eutrophication** [**e.g.**, protozoa, algae and other smaller, non-motile forms of microorganisms (including fungi, and the bacteria, and certain viruses)]. On adapting special procedures, these microorganisms can be detected in the aquatic environment.

Viruses that infect all the biological grouping have a bearing on water pollution problems. Unrestricted host development can be prevented by plants and algal viruses. By seeding with specific viruses (LPP and AS viruses), excessive growth of blue-green algae can be controlled.

Mineralisation of organic pollutants and utilisation of dissolved oxygen results due to biological sewage treatment and self-purification. Complex microbial associations which dominate the ecology of treatment plants are involved in self-purification. Some microorganisms decompose organic molecules present in industrial effluents, while some compounds resist the biological attack.

5) **Microbes and Medical Microbiology:** Microbes cause various infections to animals and human but they are also helpful in creating "disease-free world" by treating many mental and physical deformities of people. Vaccination reduces the chances of many epidemic diseases, **e.g.,** diphtheria, whooping cough, poliomyelitis, and smallpox, but is not effective or feasible for many organisms vaccination. It is especially important for diseases transmitted by respiratory droplets.

Viral infection in a living cell produces viral proteins (glycoproteins) that have a broad spectrum antiviral action and are known as **interferons**. Other than the anti-viral action, interferon has many biological effects also and inhibits parasitic infections caused by rickettsiae, protozoa, bacteria, and chlamydia.

6) **Microbes and Agriculture:** Soil microbes (bacteria and fungi) are essential for decomposing organic matter and recycling old plant material. Some soil bacteria and fungi form relationships with plant roots that provide important nutrients like nitrogen or phosphorus. Some of their vital functions are described below

   i) **Soil Microbes Break Down Organic Matter:** Microbes play an important role in the decomposition of organic matter.

   ii) **Soil Microbes Help to Recycle Nutrients:** Soil microbes play a crucial role in returning nutrients to their mineral forms, which plants can take up again. This process is known as **mineralisation**.

   iii) **Soil Microbes Create Humus:** When the soil microbes have broken down all they can, the residue is called **humus** (a dark brown jelly-like substance that can remain unchanged in the soil for ages). Humus helps the soil retain moisture, and encourages the formation of soil structure.

   iv) **Soil Microbes Create Soil Structure:** Some soil microbes secrete polysaccharides, gums and glycoproteins, which glue soil minerals together, forming the basis for soil structure. Fungal hyphae and plant roots further bind soil aggregates together. Soil structure is essential to good plant growth.

   v) **Soil Microbes Fix Nitrogen:** Agriculture depends heavily on the ability of certain microbes (mainly bacteria) to convert atmospheric nitrogen (N_2) to ammonia (NH_3). Some live freely in the soil, while others live in association with plant roots (e.g., *Rhizobium* bacteria in the roots of legumes). The process of conversion is known as **nitrogen fixation**.

   vi) **Soil Microbes Promote Plant Growth:** Some soil microbes produce a variety of substances that promote plant growth, including auxins, gibberellins, and antibiotics.

   vii) **Soil Microbes Control Pests and Diseases:** The best known **example** of the use of soil microbes in pest control is the commercial production of the soil, *Bacillus thuringiensis* to control caterpillar pests of crops. Some strains of *B. thuringiensis* are used to control beetles and flies as well. Several strains of the fungal genus *Trichoderma* have been developed as bio-control agents against fungal diseases of plants (mainly root diseases). Various other genera of fungi are used for the control of insect pests.

1.2. SUMMARY

The details given in the chapter can be summarised as follows:

1) The branch of life-sciences, involving the study of unicellular or cell-cluster microorganisms is termed as **microbiology**, and the specialist is known as **microbiologist**.

2) The development of microbiology can be divided into the following eras:
   i) Discovery Era,
   ii) Transition Era,
   iii) Golden Era,
   iv) Era of Chemotherapy and Microbial Genetics, and
   v) Modern Era.

3) In **1665**, an English scientist **Robert Hooke** developed **first microscopes** made up of the simple lens with a magnification power of approximately 30 times.

4) **Hooke** published his work in a book **Micrographie** which included various studies performed by him like his thoughts on chemistry as well as a description of microscope and its uses.

5) **Antony van Leeuwenhoek** (1632-1723) was a Dutch tradesman and scientist who developed microscope and first time studied the microbes. Due to his work and development in the field of microbiology, he was recognised as the **Father of Microbiology**.

6) **Antony van Leeuwenhoek** firstly described single celled organisms and named them as **animalcules**, known as microorganisms nowadays.

7) **Louis Pasteur** proposed the **germ theory of disease** and discovered the anaerobic bacteria (existence of bacteria in the absence of free oxygen).

8) **Louis Pasteur** discovered **pasteurisation technique** which involves mild heating to kill microorganisms in broth. He developed anti-rabies and anthrax (*Bacillus anthracis*) vaccines.

9) **Applications of Microbiology**
   i) Production of antibiotics,
   ii) Production of enzymes, vaccines, bio-surfactants, alcoholic and other pharmaceutical products,
   iii) Diagnosis of diseases and treatment,
   iv) Treatment of industrial waste material,
   v) Plant growth promotion,
   vi) Sterile product preparation,
   vii) Sterilisation,
   viii) Steroid biotransformation,
   ix) Identification of microorganisms, and
   x) Testing of pharmaceuticals.

10) Viral infection in a living cell produces viral proteins (glycoproteins) that have a broad spectrum antiviral action and are known as **interferons**.

1.3. EXERCISE

1.3.1. True or False

1) Compost is the store house of major (NPK, i.e., nitrogen, phosphorus, and potassium) and micro animal nutrients.
2) Antony van Leeuwenhoek firstly described multicellular organisms and named them as animalcules, known as microorganisms nowadays.
3) Robert Hooke proposed the germ theory of disease and discovered the anaerobic bacteria (existence of bacteria in the absence of free oxygen).
4) The term microbiology was introduced by Louis Pasteur.
5) Louis Pasteur developed anti-rabies and anthrax (*Bacillus anthracis*) vaccines.

1.3.2. Fill in the Blanks

6) Viral infection in a living cell produces viral proteins (glycoproteins) that have a broad spectrum antiviral action and are known as_____.
7) Theory of _____ was proposed in transition era.
8) Hooke published his work in a book _____ which included various studies performed by him.
9) Lister introduced the principle of sterile surgery in the medical field and was designated as the father of_____.
10) Louis Pasteur proposed the _____ and discovered the anaerobic bacteria.

Answers:

1) False 2) False 3) False 4) True 5) True
6) Interferons 7) Spontaneous generation 8) Micrographie
9) Antiseptic surgery 10) Germ theory of disease

1.3.3. Very Short Answer Type Questions

1) Enlist various ranches of microbiology.
2) What is Pasteur Effect?
3) What are interferons?
4) Give any four examples of beneficial bacteria.
5) Write a note on contribution of Louis Pasteur in the development of microbiology.
6) What is theory of spontaneous generation?

1.3.4. Short Answer Type Questions

1) Write a short note on scope of microbiology.
2) Write a note on Koch's postulates.
3) Give a short note on golden era of microbiology.
4) Write a note on contribution of Robert Koch.

1.3.5. Long Answer Type Questions

1) Write a detail note on history of microbiology.
2) Give a descriptive note on various branches of microbiology.
3) Write a note on scope and importance of microbiology.

CHAPTER 2

Cell

2.1. CELL

2.1.1. Introduction

All organisms are built of cells, thus cell is the fundamental unit of life. All animal tissues including human tissues are made up of cells.

All organisms can be broadly categorised into **prokaryotes** and **eukaryotes** (**pro** means primitive; **eu** means true; and **karyote** means nut indicating the cellular nucleus). First time in **1937**, **Edward Chatton** introduced these terms. The major difference between prokaryotes and eukaryotes is that the former does not have a nucleus while the latter possess a distinct one. **Examples** of prokaryotes are bacteria and cyanobacteria; and that of eukaryotes are fungi, protozoa, plants, animals, and humans.

2.1.2. Prokaryotes

Examination of a prokaryotic cell reveals various component structures. Some of the structures are naturally common to almost all prokaryotes, but some structures are present in certain species. Structures found external to the bacterial cell wall aid in protection, attachment, motility, or conjugation. **Figure 2.1** shows a typical prokaryotic cell.

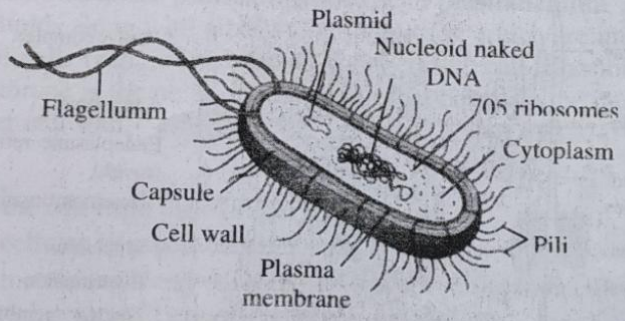


Figure 2.1: Typical Prokaryotic Cell

The prime **features** of a prokaryotic cell are:

1) The cell is enclosed within a **cell wall**, made up of peptidoglycan (a mixture of carbohydrates, and amino acids). The cell wall prevents the cell from bursting.

2) Inner to the cell wall, **plasma membrane** is present which controls the movement of materials in and out of the cell. Some substances are pumped in and out of the cell via active transport.

3) The inner area of cell is occupied by cytoplasm, containing enzymes (required for cellular chemical reactions) and the genetic material.

4) The region in the cytoplasm having chromosome is known as **nucleoid**. The **DNA** is not bound by a nuclear envelope and is naked, i.e., not associated with any protein. Some bacteria contain **plasmids** (small circular DNA) which replicate independently and passes from one cell to another.

5) **Ribosomes** are the site of protein synthesis and are present in all prokaryotic cells. Cells actively involved in producing protein have large number of ribosomes. In prokaryotic cells **70S ribosomes** are present that are smaller than 80S ribosomes found in eukaryotic cells.
6) Some prokaryotic cells possess **flagellum** (singular flagella) projecting out from the cell wall, and enabling cell movement.
7) The cell wall of some bacterial cells has **pili** (singular pilus), through which they can connect with other bacterial cells. This helps in exchange of genetic material.

The volume of prokaryotic cells is less than that of the complex cells, since they lack nucleus. They undergo division via **binary fission**. With their growth, the DNA replicates and separates into two different cytoplasmic areas, which further divides into two. This division mechanism is slightly different from mitosis undergone by eukaryotic cells.

2.1.3. Eukaryotes

Eukaryotic cells are about 10 times the size of a typical prokaryotic cell, and can be as much as 1000 times greater in volume. The major difference between prokaryote and eukaryote is that the latter contains membrane-bound compartments in which specific metabolic activities take place. Most important difference is the presence of a cell nucleus (a membrane-delineated compartment that houses the eukaryotic cell's DNA). Eukaryotic cells are often found in multicellular organisms.

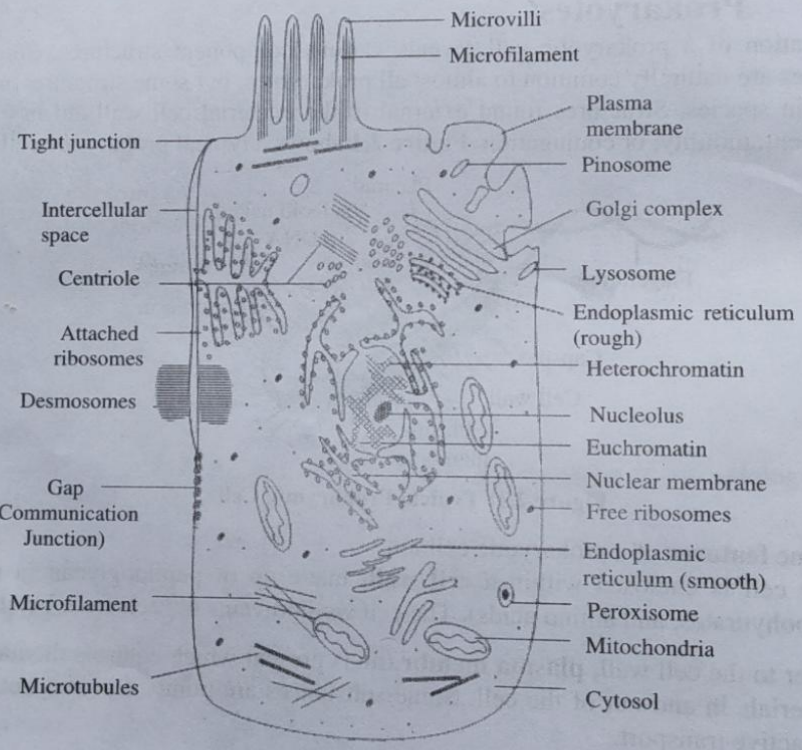


Figure 2.2: Typical Eukaryotic Cell

A eukaryotic cell comprises of the following basic components (**figure 2.2**):
1) Cell wall,
2) Cell membrane (or plasma membrane),
3) Cytoplasm, and
4) Nucleus.

2.1.3.1. Cell Wall

Cell wall is the characteristic feature of plant cells (it is absent in animal cells). Most eukaryotic plant cells have cell wall; although they are much simpler than that of prokaryotic cells.

Structure

Cell wall is the non-living outermost covering of plant cells. In most of the plants, cell wall is primarily composed of **cellulose**; though it may also be made up of other chemical compounds like **pectin** and **lignin**. Cell wall is composed of fine threads of fibres known as **microfibrils**.

Functions

1) It **protects** the delicate inner structures of the cell.
2) As it is rigid, it **provides shape** to the cell.
3) It facilitates the **movement of substances** and **other chemicals** in and out of the cell, thus cell wall is a permeable membrane.
4) Sometimes, it is modified to function as a **food reserve**, **e.g.,** hemicellulose in some seeds.
5) It **reduces water loss** and protects the cell from infection by forming a coating of waxy cutin known as **cuticle**.
6) It has **plasmodesmata**, which are cytoplasmic strands connecting one cell to the other.
7) **Middle lamella** (composed of calcium pectinate) joins the walls of two adjacent cells firmly.

2.1.3.2. Cell Membrane

Cell membrane is a selectively permeable biological membrane, allowing only certain substances to pass through. It acts as a barrier between the inner and outer surface of the cell. It is also known as the **plasma membrane** or **plasmalemma**. It comprises mainly of proteins and lipids along with other living molecules, which are important for the normal functioning of cells (**e.g.,** in ion channel conductance, cell adhesion, and cell signalling). The cell membrane is the point of attachment that connects the inner cytoskeleton of the cell to its outer cell wall.

Properties

1) It guards the cell from the external environment.
2) It allows cellular transport between cells.
3) The transmembrane receptors (binding the signalling molecules) present on it help in signal transduction and provide selective receptivity.
4) It helps in recognising a cell.
5) It has binding sites for the constituents of cytoskeleton present in the external environment.
6) It divides the cell into smaller sections having different structure and functions.
7) It offers a stable site for the catalysis and binding of enzymes.
8) It fuses with other membranes of cell organelle through special points of attachment.
9) It regulates transfer of certain specific molecules through the cell membrane, **e.g.,** gap junctions.
10) It enables motility of the cellular organelles.

Structure

To explain the nature and functions of cell membrane, various **models** have been proposed in past, as mentioned below:

1) Danielli and Davson model/Lamellar theory,

2) Unit membrane model,
3) Robertson's model,
4) Lipid or phospholipids bilayer model, and
5) Fluid mosaic model.

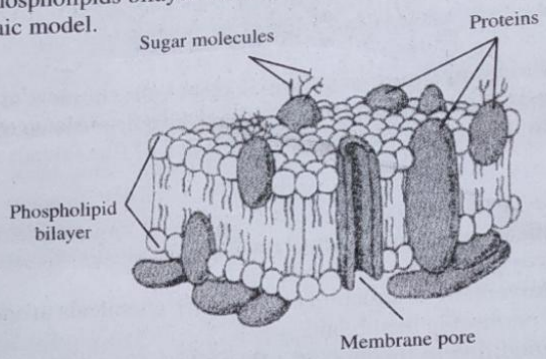


Figure 2.3: Structure of the Cell Membrane

However out of the above given models, the **Fluid Mosaic Model** is the most accepted one (**figure 2.3**), given by **S.J. Singer** (University of California) and **G.L. Nicholson** (Salk Institute) in the early 1970s. According to this model, proteins do not form a continuous layer extending from one side of the membrane to the other; rather they are present in the form of globules, dotted randomly in a mosaic pattern.

Some globules of the protein penetrate into the membrane to deeper extents; some of them remain attached to the membrane surface; while some extend through it and adhere to the other side. Due to this, membrane shows less rigid nature as compared to the original one.

Experiments on the viscosity of membrane show that it has a fluid consistency. This fluidity allows movement of lipid and protein molecules within it. Due to the fluidity and mosaic arrangement of the protein molecules, this model is known as the **Fluid Mosaic Model**.

Chemical Composition

1) **Phospholipids:** These are the major components of the cell membrane. These are fat (lipid) molecules with a charged phosphate group at one end (**figure 2.3**). Phosphate group attached to the lipid bilayers are hydrophilic in nature, whereas lipid molecules are hydrophobic in nature. The lipid 'tails' of the molecules face each other creating a lipid bilayer.

2) **Proteins:** The lipid bilayer is embedded with proteins of various shapes and sizes. **Three groups** of membrane proteins can be identified as:
   i) **Integral Proteins:** These proteins extend into or through the lipid bilayer.
   ii) **Transmembrane Proteins:** These proteins span the entire lipid bilayer and protrude into both the cytosol and the ECF.
   iii) **Peripheral Proteins:** These proteins associate more loosely with the polar heads of membrane lipids or with integral proteins at the inner or outer surface of the membrane.

According to their distribution, **membrane proteins** are of **two types**:

i) **Extrinsic Proteins:** These peripheral proteins occur on outer and inner surface of the membrane outside the lipid bilayer.

ii) **Intrinsic Proteins:** These proteins are intra-membranous, and are embedded in the lipid bilayer.

3) **Carbohydrates:** These molecules are found attached to the proteins and lipids forming **glycoproteins** and **glycolipids**, respectively. These glycoproteins (**glycocalyx** in most of animals) form a part of the protective coat of carbohydrate that surrounds most animal cells, and are responsible for cellular differentiation and identification (**e.g.,** difference in blood type). **Examples** of two major glycolipids are glycerol-based and sphingolipid-based glycolipids.

Functions

1) **Protection of Cells:** The receptors present on the cell membrane allow chemical messages to pass between the cells and systems, which provides protection to cells.

2) **Providing Cytoskeleton and Mechanical Support:** It provides a specific shape to the cell. It provides mechanical support and maintains the membrane potential. Attachment proteins present on cell membrane bind the extracellular and intracellular components.

3) **Separation from Surroundings:** In an animal cell, it separates the internal environment from the external. Other organisms, including fungi, plants, and bacteria, consist of an additional layer known as cell wall, which forms the outermost protective layer.

4) **Permeability:** It is a semi-permeable membrane. The membrane in this state is called **selectively permeable**, as it allows the passage of solvent and several selected solutes, while holding back the others.

5) **Tissue Formation:** Groups of similar cells attach to the extracellular matrix and form tissues.

6) **Particle Transportation:** Various mechanisms, like ion channel, carrier proteins, and ion pumps are involved in particle transportation. Chemical exchange of solute between extracellular medium and cytosol is mediated by transport of channel and carrier proteins.

7) **Contribution to Enzyme Activity:** It controls the activity of enzymes which further participate in metabolism and immune system.

8) **Control of Neurohormonal Flow:** Receptor proteins on cell membrane mediate the flow of neurohormonal and other chemical information from extracellular medium to the cytosol.

2.1.3.3. Cytoplasm

Cell consists of a cytoplasm enclosed within the cell membrane. The cytoplasm includes various cellular organelles and therefore these organelles are also referred to as cytoplasmic organelles. The cell performs most of the metabolic activities (**e.g.,** glycolysis, and processes like cell division) in cytoplasm. Basically, cytoplasm is the fluid in which cell organelles (mitochondria, Golgi body, nucleus, etc.) remain suspended.

Functions

1) Various vital biochemical reactions, important for the maintenance of life, occur in the cytoplasm.

2) It provides the site for cell growth and expansion.

3) It provides space for organelles to remain suspended in the medium.

4) It also helps in the movement of several elements within the cell.
5) It has enzymes, which help in breaking the large macromolecules into smaller ones so that other organelles can use them easily. **For example,** in mitochondria, glucose cannot be used directly until it is broken down into pyruvate by the enzymes that act as a catalyst in the process of glycolysis, or in the synthesis of amino acid, sugar and fatty acid.
6) Other important functions performed by the cytoplasm involve protein synthesis, cytokinesis, anaerobic glycolysis, cell reproduction, etc.

For the study point of view, cytoplasm is **divided** into **three parts** namely the cytosol, cytoplasmic organelles, and cell inclusions:

1) **Cytosol:** It refers to the **fluid present inside the cells**. This fluid bathes the cytoplasmic organelles. Important **characteristic features** of cytosol include:
   i) Majority of the cell metabolism takes place within the cytosol.
   ii) Cytosolic proteins perform a significant function in signal transduction and glycolytic pathways.
   iii) Cytosolic proteins also act as ribosomes and intracellular receptors.

2) **Cytoplasmic Organelles:** Cell consists of specialised organelles (*viz.* Golgi bodies, mitochondria, endoplasmic reticulum, lysosomes, etc.) having some specific characteristics that help in the maintenance of life. They are present within the cytoplasm and include:
   i) **Mitochondria:** Mitochondria (singular mitochondrion) is a **double membranous** cell organelle found in most of the eukaryotic cells. The diameter of mitochondria ranges from 0.5-10μm. Mitochondria are also known as the **powerhouse of cells** because they generate most of the cell's supply of Adenosine Triphosphate (ATP), which is used as a source of chemical energy. Hence mitochondria are the **chief source of energy** in the cell.

Structure

a) **Outer Membrane:** This membrane contains **transport proteins**, and is helpful in transporting ions and atoms across the organelle.

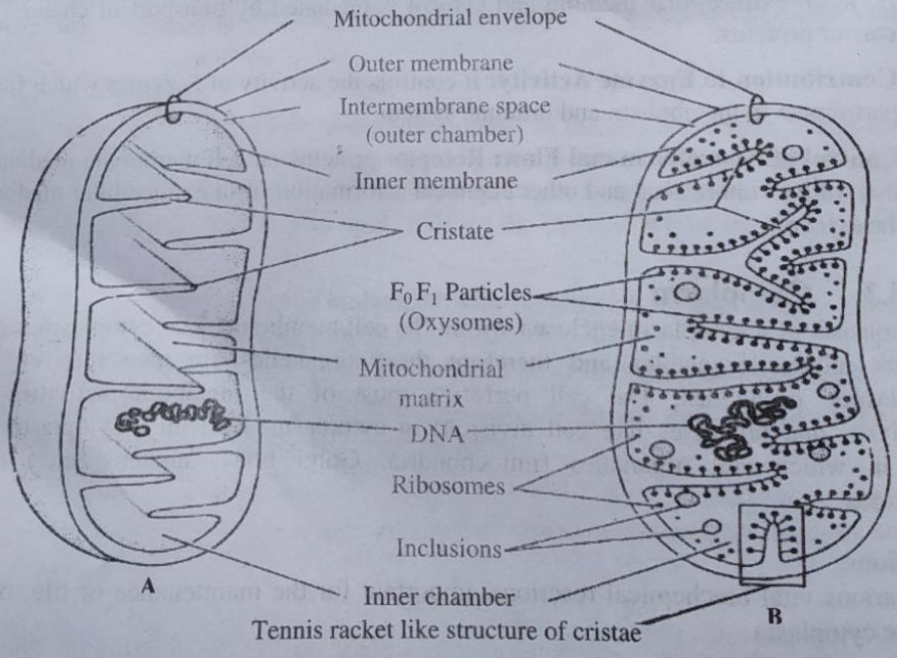


Figure 2.4: (A) Enlarged Diagram of Mitochondrion showing Membranes and Cristae (B) Internal Structure of Mitochondrion

b) **Inner Membrane:** This membrane is involved in the formation of **cristae [figure 2.4 (A)]**. Cristae are finger-like projections in the matrix, bearing small particles called **F1 particles** or **oxysomes [figure 2.4 (B)]**. Enzymes of respiratory chain are present in cristae.

c) **Mitochondrial Matrix:** Matrix of the mitochondria is formed by the fluid present within it. This fluid contains enzymes involved in Kreb's cycle, to oxidise fatty acids and pyruvic acid.

Functions

a) It mainly performs aerobic respiration to create energy for the functioning of cell.
b) It regulates plasma membrane potential and cellular anabolism and catabolism.
c) It actively participates in apoptosis.
d) It is involved in synthesis of steroids.
e) It is involved in reaction of haem synthesis.
f) It performs cellular signaling and regulates cellular proliferation.
g) It performs detoxification in liver cells. Mitochondria in liver cells have enzymes that allow them to detoxify ammonia (a waste product of protein metabolism).

ii) **Golgi Bodies:** The Golgi body comprises of folded and compressed membranous sacs piled upon each other. All cells contain Golgi bodies, however it is larger in those cells that facilitate the synthesis and export of proteins to the Golgi complex via endoplasmic reticulum. These proteins are then 'enclosed' into membrane-bound vesicles called **secretory granules**, which are stored, and when required, moved to the plasma membrane, by which the proteins are exported.

Structure

The Golgi body (**figure 2.5**) consists of membrane-bound stacks (usually between four and eight) called **cisternae** (singular cisterna). Each cisterna consists of flattened membrane disks that carry Golgi enzymes. The cisternae stack comprises of the following **four functional portions**:

a) *Cis*-Golgi network, b) Medial-Golgi,
c) *Trans*-Golgi, and d) Trans-Golgi network.

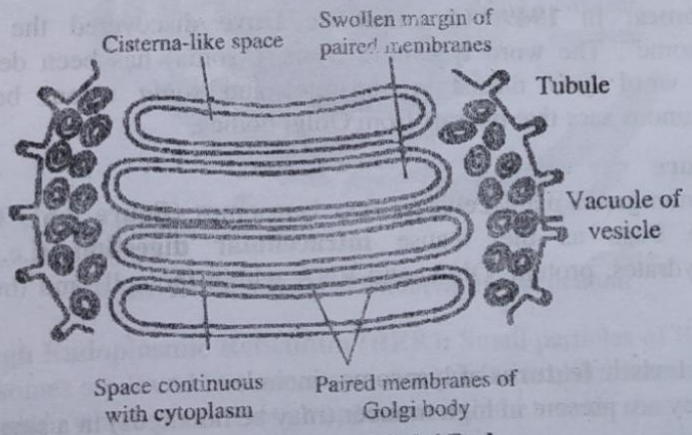


Figure 2.5: Structure of Golgi Body

The formation of new cisternae takes place on *Cis*-Golgi network. The ***cis*-** and ***trans*-Golgi** networks are considered to be specialised cisternae through which the Golgi body moves in and out.

Functions

Golgi body is involved in the synthesis of different number of macromolecules.

Golgi bodies perform the following **functions**:

a) **Modifying, Sorting, and Packaging:** It plays an important role in sorting, modifying, and packaging of substances for use within the cell or for cell secretion.

b) **Modification of Proteins:** Proteins delivered by the rough endoplasmic reticulum are modified by the Golgi body. It also takes part in lysosome formation and in the transport of lipids to the adjoining areas of the cell.

c) **Transportation of Substances:** Enzymes present in the cisternae bind to the carbohydrate and phosphate groups by the process of glycosylation and phosphorylation, respectively, in order to modify the substrate by the transport of substances, **e.g.,** nucleotide sugars from the cytosol into the cell organelle. Proteins are also marked with the molecules' signal sequences that determine their final destination, **e.g.,** Golgi bodies add a mannose-6-phosphate label to proteins meant for lysosomes.

d) **Sulphation of Molecules:** It performs sulphation of certain molecules (**e.g.,** GAGs of proteoglycans as well as on the core protein). Sulphation occurs with the help of sulphotranferases when the molecule passes through lumen. PAPs (3'-Phosphoadenosine-5'-phosphosulphate) functions as a donor of sulphur atom. The level of sulphation is very important to give signalling abilities and overall negative charge to the proteoglycans.

e) **Phosphorylation of Molecules:** Phosphorylation of various biochemical molecules occur during transportation of ATP molecules into the lumen, in the presence of kinases enzyme (like casein kinases). **For example**, apolipoprotein gets phosphorylated to form VLDL (Very Low Density Lipoprotein), one of the blood serum constituents. It is assumed that these molecules get phosphorylated so as to help in sorting of secretion of these molecules in the blood serum.

f) **Formation of Spindle:** It is involved in the formation of meiotic and mitotic spindles.

g) **Excretion of Water:** Golgi bodies help in excreting excess amount of water.

iii) **Lysosomes:** In **1949**, **Christian de Duve** discovered the cell organelle "Lysosome". The word **lysosome** (lysis + soma) has been derived from the Greek word ***lysis*** means to separate; and ***soma*** means body. They are membranous sacs that bud off from Golgi bodies.

Structure

Some non-green plant cells possess lysosomes (**figure 2.6**). They are called **suicide bags** as they cause **intracellular digestion** (i.e., destruct the carbohydrates, protein, DNA, and RNA within the cell) and thus, **destroy the cell**.

Characteristic features of lysosomes include:

a) They are present in high number (may be hundreds) in a single cell.

b) They possess nearly 40 different enzymes.

c) Lysosomal enzymes act upon the substances that enter into lysosome.

d) They are referred to as **suicidal bags** since the enzymes present in them can digest the whole cell, in case of damage or death of the cell.

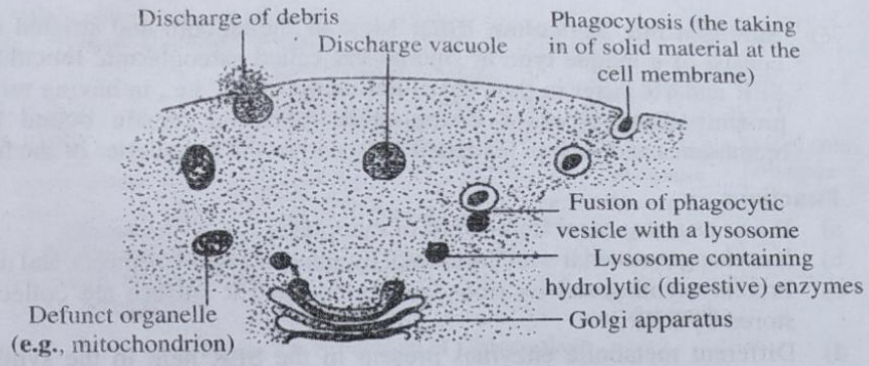


Figure 2.6: Structure of Lysosomes

Functions

a) They function as the **cell's digestive system**; the substances are engulfed by the cell via the process of endocytosis, **e.g.,** proteins, foreign particles, and other substances are digested by the lysosomes.
b) They have the ability to engulf and thus destruct the complete cell in case of diseases.
c) They are known to **trigger cell division**.

iv) **Endoplasmic Reticulum:** The endoplasmic reticulum is a complex channel system enclosed within the membrane in the form of three dimensional networks that consists of vesicles, flattened sacs, and branched tubules.

Structure

The lamina and the nuclear envelope of endoplasmic reticulum together extend up to the surface of the cell. On the basis of structure, there are three types of endoplasmic reticulum (**figure 2.7**):

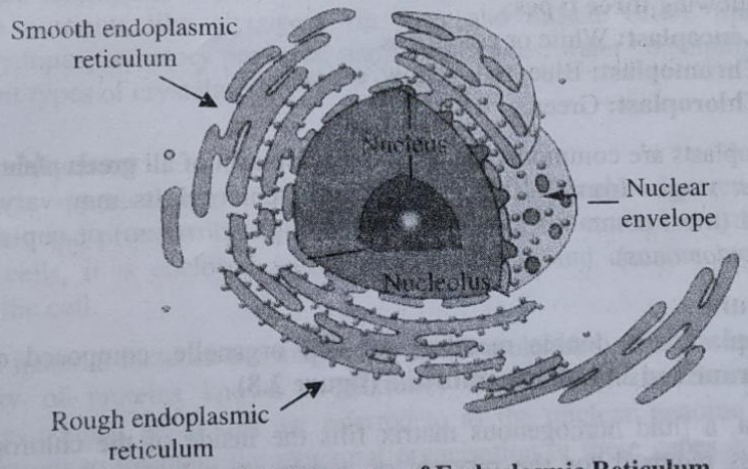


Figure 2.7: Structure of Endoplasmic Reticulum

a) **Rough Endoplasmic Reticulum (RER):** Small particles of RNA known as ribosomes are found in close association with the membrane of endoplasmic reticulum which gives a rough appearance to the membrane.

b) **Smooth Endoplasmic Reticulum (SER):** The membranes of SER do not contain ribosomes and help in the metabolism of carbohydrates and lipids. They are involved in the synthesis of steroid hormones in adrenals and testes.

c) **Sarcoplasmic Reticulum (SR):** Most of the smooth and striated muscles consist of a unique type of smooth ER called sarcoplasmic reticulum. The SER and SR differ in their structural composition, i.e., in having mixture of proteins. Both types of endoplasmic reticulum remain bound to their membranes and remain restricted only within the boundaries of the lumens.

Functions

a) It acts as a structural framework of cytoplasm.
b) It exchanges material with the cytosol by active transport, osmosis, and diffusion.
c) Proteins synthesised by ribosomes bound to ER surface are collected and stored by RER.
d) Different metabolic enzymes present in the SER help in the synthesis of phospholipids, cholesterol, and triglycerides.
e) SER found in the liver helps in the synthesis of glycogen and lipoproteins. It is also involved in the lysis of macromolecules.

v) **Vacuoles:** Vacuoles are sac-like structures filled with fluid and are bounded by a single membrane, known as **tonoplast** (only in a plant cell). Mineral salts, sugar, amino acids, wastes, and sometimes pigments are present in vacuoles and are together referred to as **cell sap**. In plant cells, vacuoles are larger in size and are placed centrally.

Functions

a) They **support** the cell and help in its **growth**.
b) They act as **storehouse for pigments**, which impart colours to the flower, fruit, buds, and leaves.
c) They also possess **hydrolytic enzymes**.
d) They act as sites for **temporary storage of food and waste**.

vi) **Plastids:** Plastids are self-reproducing double membranous organelles of plants and algae. Plastids can either be coloured or can be colourless and are mainly of the following **three types**:

a) **Leucoplast:** White or colourless,
b) **Chromoplast:** Blue, red, yellow, etc. and
c) **Chloroplast:** Green.

Chloroplasts are commonly found in the cytoplasm of all green plant cells. Their number ranges from 1-1008. The shape of chloroplasts may vary from disc-shaped (most common) to ribbon-shaped (alga *Spirogyra*) or cup-shaped (alga *Chlamydomonas*).

Structure

Chloroplast is a double membranous cell organelle, composed of an **outer membrane** and an **inner membrane** (**figure 2.8**).

Stroma, a fluid homogenous matrix fills the inside of the chloroplast. It has enzymes required for the process of carbon dioxide fixation (dark reaction) during photosynthesis.

Grana (singular granum) are found scattered within stroma. Each granum includes a membrane bound disc-like vesicles known as **thylakoids**, arranged like a pile of coins.

Intergranal lamella connects one granum to the other. Two layers of thylakoids form the **thylakoid membrane**, comprising of all the energy-generating system, including chlorophyll, electron transport chain, and ATP synthetase.

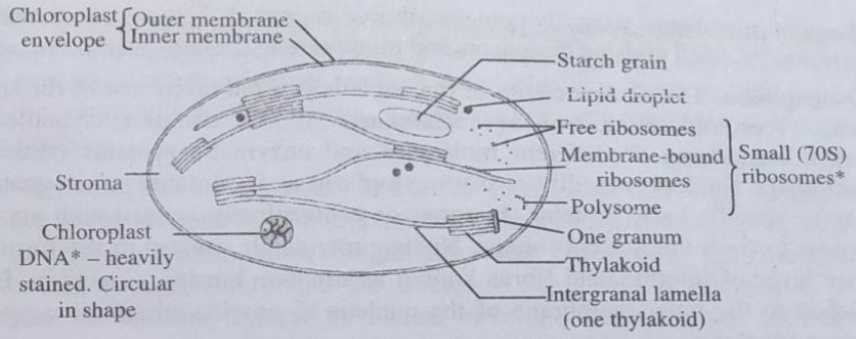


Figure 2.8: Structure of Chloroplast

Functions

a) It provides the **site for photosynthesis**.
b) It helps in **synthesis of fatty acids**. Carbohydrates are converted into fatty acids with the help of ATP and NADPH in the stroma.
c) It **reduces nitrite** to ammonia.

vii) **Peroxisomes:** Peroxisomes occur in cells of green leaves in the higher plants. Nucleoid, a central core of crystalline material is made up of urate oxidase crystals. The nucleoid is usually spherical or ovoid and is nearly of the same size as that of the mitochondria and lysosomes. Peroxisomes closely resemble ER.

Functions

a) They perform **substrate level oxidation** and form hydrogen peroxide.
b) In plant cells, they take part in **photorespiration**.
c) They bring about **metabolism of fat** in plants.

3) **Cell Inclusions:** Certain bodies are present within a cell but are unable to perform their own metabolic processes. These structures also lack membranes binding them. They are referred to as cell inclusions since they are present within the cell, and include nutrients like glycogens (in liver and muscle cells), lipids (fat cells), carbohydrates, secretory products, pigment granules, water-containing vacuoles, and different types of crystals.

2.1.3.4. Nucleus

Nucleus is a spherical organelle that consists of a double layered membrane of phospholipids and proteins. Nucleus is also known as the **control centre** of the cell. In eukaryotic cells, it is enclosed within the membrane and consists mostly of genetic material of the cell.

The genetic material includes the long linear DNA molecules, which interwine around the huge variety of proteins known as **histones** for forming the chromosomes. These chromosomes contain genes that are referred to as the **nuclear genome** of the cell. The nucleus functions to maintain the structural organisation of these genes and regulates gene expression for controlling the cellular activities. Thus, it is called as the **cell's control centre**.

Structure

Nucleus has **five components (figure 2.9):**

1) **Nuclear Envelope:** A large number of small circular or octagonal nuclear pores are present at specific points on the nuclear envelope. These nuclear pores function as a channel for the regulated movement of macromolecules from nucleus to cytoplasm and *vice-versa*. The proteins enclosing the pores combine with various other granular and fibrillar substances and form a complicated structure, known as **nuclear pore complex**

The outer membrane generally contains ribosomes over it. The nuclear envelope in the early stages of cell division disappears and re-appears in the later stages.

2) **Nucleoplasm:** The nuclear cavity of the nucleus is filled up by one of the specialised forms of protoplasm, known as nucleoplasm. It is a complex crystallo-colloidal system comprising of different molecules and enzymatic proteins (structural and functional). Nucleoplasm differs from cytoplasm as it contains an adequate amount of some specific basic proteins (**histones** or **protamines**) coupled with nucleic acids in order to form the nucleoproteins. Nucleoproteins are present in the form of a thin dense layer of intermediate fibres known as **nuclear lamina** or **cortex**. It remains attached to the inner membrane of the nucleus to provide structural support to the nuclear envelope.

3) **Chromatin Material:** The chromatin material, saturated with **Deoxyribonucleic Acid** (DNA) transmits the coded structural and functional information between the cell and organism, respectively. Notably, all the body cells of organisms and individuals that belong to the same species contain an equal amount of DNA, i.e., the amount of DNA present in an individual or organism indicates its specificity.

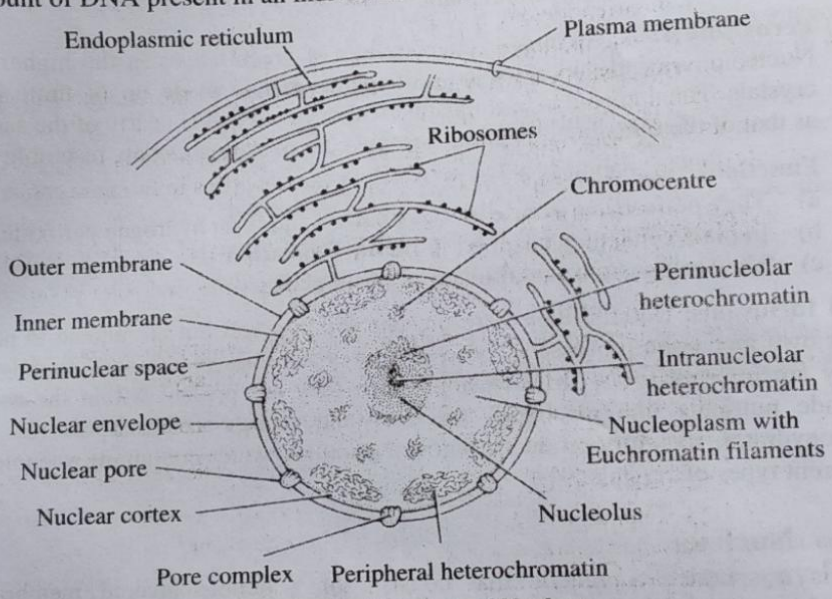


Figure 2.9: Structure of Nucleus

All the chromatin fibres condense together by the process of **coiling** and **supercoiling** to form visible thick and separate threads known as **chromosomes**. In an eukaryotic nucleus, the chromatin material consists of a complex of protein and DNA that packages the chromosome. Depending on the DNA requirement, the chromatin structure in different stages of cell cycle varies significantly.

Interphase Chromatin: During the interphase (the period of cell cycle where the cell is not dividing), **two types** of chromatin can be distinguished:

i) **Euchromatin:** It includes the active DNA, **e.g.,** expressed as protein.

ii) **Heterochromatin:** It includes the inactive DNA. During the chromosomal stages, it particularly helps in structural development. It can further be divided into **two types**:
   a) Constitutive heterochromatin, and
   b) Facultative heterochromatin.

4) **Nucleolus:** The nucleus of the cell has a nucleolus, also known as **nucleoli**. It is a non-membranous structure containing proteins and nucleic acids. The ribosomal RNA gets transcribed inside the nucleoli. Nucleolus is a dense and spheroidal mass located eccentrically in the nucleus. It is the largest and most important component of the nucleus.

   Generally, a nucleus contains only a single nucleolus, but there may be two or more. They are not membrane-bound structures but are usually surrounded by an irregular layer of heterochromatin, known as **perinucleolar chromatin**. The heterochromatin strands can even penetrate into the nucleolus mass and is referred to as **intra-nucleolar chromatin**. Similar to nuclear envelope, the nucleolus also disappears during the early stages of cell division. However, in the later stages of the cell division, it re-appears.

5) **Chromosome:** The word chromosome has been derived from the Greek word ***chroma*** means colour and ***soma*** means body, as it has the strong ability to be stained with vital and supravital dyes. Chromosome can be defined as DNA-bound proteins (histone proteins) that carry genetic information from one generation to other.

Functions

1) Most of the physiological activities of cell are controlled by the nucleus via specified enzymatic proteins formed in the cell.
2) Along with its direct involvement in reproduction, it also functions to encode all the genetic information that carries the hereditary characteristics in living organisms.

2.1.4. Difference between Prokaryotes and Eukaryotes

Table 2.1 enlists the distinguishing features between prokaryotes and eukaryotes:

Table 2.1: Difference between Prokaryotic and Eukaryotic Cells

Basis	Prokaryotes	Eukaryotes
Typical Organisms	Bacteria and archaea	Protists, fungi, plants and animals.
Typical Size	~1-10µm	~10-100µm (sperm cells, apart from the tail, are smaller).
Types of Nucleus	Nucleoid region; no real nucleus	Real nucleus with double membrane.
DNA	Circular (usually)	Linear molecules (chromosomes) with histone proteins.
RNA-/Protein-Synthesis	Coupled in cytoplasm	RNA-synthesis inside the nucleus; protein synthesis in cytoplasm.
Ribosomes	50S + 30S	60S + 40S
Cytoplasmatic Structure	Very few structures	Highly structured by endomembranes and a cytoskeleton.
Cell Movement	Flagella made of flagellin	Flagella and cilia containing microtubules; lamellipodia and filopodia containing actin.
Mitochondria	None	One to several thousand (though some lack mitochondria).
Chloroplasts	None	In algae and plants.
Organisation	Usually single cells	Single cells, colonies, higher multicellular organisms with specialised cells.
Cell Division	Binary fission (simple division)	Mitosis (fission or budding) and meiosis.

2.2. SUMMARY

The details given in chapter can be summarised as follows:

1) All organisms are built of cells thus cell is the **fundamental unit of life**.
2) All organisms can be broadly categorised into **prokaryotes** (**e.g.,** bacteria and cyanobacteria) and **eukaryotes** (**e.g.,** fungi, protozoa, plants, animals, and humans).
3) The major **difference** between prokaryote and eukaryote is that the latter contains membrane-bound compartments in which specific metabolic activities take place.
4) **Cell wall** is the characteristic feature of plant cells (it is absent in animal cells), forming a non-living outermost covering of plant cells. In most of the plants, cell wall is primarily composed of **cellulose**; though it may also be made up of other chemical compounds like **pectin** and **lignin**.
5) **Cell membrane** is a selectively permeable biological membrane, allowing only certain substances to pass through. It acts as a barrier between the inner and outer surface of the cell. It is also known as the **plasma membrane** or **plasmalemma**.
6) According to **Fluid Mosaic Model** cell membrane is chemically composed of:
   i) Phospholipids, ii) Proteins, and iii) Carbohydrates.
7) Cell membrane encloses **cytoplasm** which includes various cellular organelles and therefore these organelles are also referred to as cytoplasmic organelles.
8) In cytoplasm specialised organelles (**e.g.,** Golgi bodies, mitochondria, endoplasmic reticulum, lysosomes, etc.) are found that help in the maintenance of life.
9) Most of the eukaryotic cells contain a **double membranous** cell organelle called **mitochondria** (singular mitochondrion) with a diameter ranging from 0.5-10micrometers (μm). Mitochondria are also known as the **powerhouse of cells** because they generate most of the cell's supply of Adenosine Triphosphate (ATP).
10) The **Golgi body** comprises of folded and compressed membranous sacs piled upon each other.
11) **Lysosomes** are called as **suicide bags** as they cause **intracellular digestion** (i.e., destruct the carbohydrates, protein, DNA, and RNA within the cell).
12) **Endoplasmic reticulum** is a complex channel system enclosed within the membrane in the form of three dimensional networks that consists of vesicles, flattened sacs, and branched tubules. There are three types of endoplasmic reticulum:
   i) Rough endoplasmic reticulum (RER) ii) Smooth endoplasmic reticulum (SER)
   iii) Sarcoplasmic reticulum (SR)
13) **Vacuoles** are sac-like structures filled with fluid and are bounded by a single membrane, known as **tonoplast** (only in a plant cell).
14) **Plastids** are self-reproducing double membranous organelles of plants and algae. Plastids can either be coloured or can be colourless and are mainly of the following three types:
   i) Leucoplast (white or colourless), ii) Chloroplast, and
   iii) Chromoplast (blue, red, yellow, etc.).
15) **Cell inclusions** are present within a cell but are unable to perform their own metabolic processes.
16) **Nucleus** is a spherical organelle that consists of a double layered membrane of phospholipids and proteins. Nucleus is also known as the "**control centre**" of the cell. Nucleus consists of following structures:
   i) Nuclear envelope, ii) Nucleoplasm, iii) Chromatin material
   iv) Nucleolus (or nucleoli), and v) Chromosome.
17) **Chromosomes** are defined as DNA-bound proteins (histone proteins) that carry genetic information from one generation to other.

2.3.

2.3.1.
1) Tissues a
2) Vacuole known a
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4) Plastids
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2.3. EXERCISE

2.3.1. True or False

1) Tissues are the fundamental unit of life.
2) Vacuoles are sac-like structures filled with fluid and are bounded by a single membrane, known as tonoplast (only in a plant cell).
3) Cell inclusions are present within a cell but are unable to perform their own metabolic processes.
4) Plastids are self-reproducing single membranous organelles of plants and algae.
5) Nucleus is also known as the "power house" of the cell.
6) Cristae are finger like projections in the matrix, bearing small particles called F1 particles or oxysomes.
7) Plasmodesmata are cytoplasmic strands that connect one cell to the other.

2.3.2. Fill in the Blanks

8) Lysosomes are called as _____.
9) Middle lamella composed of _____ that joins the walls of two adjacent cells firmly.
10) Outer membrane of mitochondria contains _____, and is helpful in transporting ions and atoms across the organelle.
11) Mitochondria are known as _____ of the cell.
12) The Golgi apparatus consists of membrane-bound stacks called _____.
13) Each granum includes a membrane bound disc-like vesicles known as_____, arranged like a pile of coins.

Answers:

1) False	2) True	3) True	4) False	5) False
6) True	7) True	8) Suicide bags	9) Calcium pectinate	
10) Transport proteins		11) Powerhouse	12) Cisternae	13) Thylakoids

2.3.3. Very Short Answer Type Questions

1) Enlist double membranous and single membranous cellular structures.
2) Draw a clean diagram of mitochondria with naming.
3) Lysosomes are also known as suicide bags. Why?
4) Give examples of prokaryotic and eukaryotic cells.
5) Write a note on chromatid.

2.3.4. Short Answer Type Questions

1) Give difference between prokaryotes and eukaryotes.
2) Write note on following cellular structures:
   i) Mitochondria
   ii) Nucleus
3) Write a note on plastids.
4) Write a short note on structure and chemical composition of cell membrane.
5) Give a detail note on plastids.

2.3.5. Long Answer Type Questions

1) Write a detail note on plasma membrane.
2) Give a descriptive note on eukaryotic cell.

CHAPTER 3

Study of Bacteria

3.1. BACTERIA

3.1.1. Introduction

The word **bacteria** (plural) evolved from the Latin word ***bacterium*** (singular), which means **rod** or **staff**. On the earth, these are most **abundant prokaryotes**. They are omnipresent and majority of them perform positive and vital role in nature; but few are harmful and causes diseases and infections in animals and plants. Bacteria are most adaptive microorganisms that modulate according to their living conditions very rapidly as compared to any other group of organisms.

Bacteria are a group of prokaryotic, unicellular microorganisms having wide range of shapes (from spheres to rods and spirals) and are of few micrometres in length. They are ubiquitous microorganisms, which can grow in almost every habitat on earth such as soil, acidic hot springs, radioactive waste, water, and deep in the sea. Bacteria are actively engaged in the recycling process on the earth. Recycling involves several steps depending on these organisms, **e.g.,** fixation of nitrogen from the atmosphere and putrefaction. Still, many of bacteria are not identified and only about half of the phyla of bacteria have species that cannot be grown in the laboratory. **Bacteriology** is the branch of microbiology that deals with the **study of bacteria**.

3.1.2. Ultrastructure of Bacteria

Despite of being the most primitive and simplest microorganism, bacteria have well-developed cell structures which possess many unique biological properties. Bacteria differ from archae bacteria and other eukaryotic microorganisms in many ways. Due to their simplicity, bacteria had been studied thoroughly on the genetic level, revealing many biochemical and genetic principles that have been further used in various studies.

Cell-envelope or the outer layer of bacteria consists of two components (**figure 3.1**):

1) A rigid cell wall, and
2) Underlying cytoplasmic or plasma membrane.

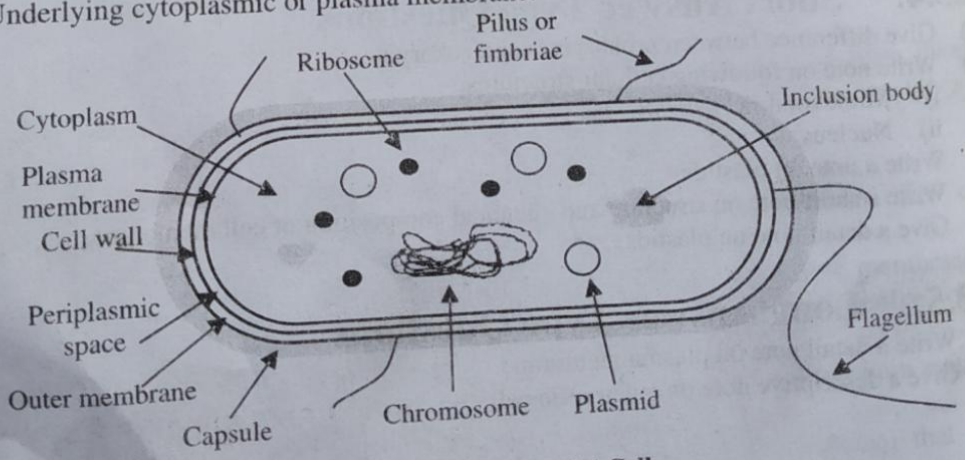


Figure 3.1: Bacterial Cell

Protoplasm of the cell includes cytoplasm, cytoplasmic inclusions (mesosomes, ribosomes, inclusion granules, and vacuoles) and single circular **chromosome** of DNA (**figure 3.1**). The protoplasm is enclosed by a cell-envelop. Along with these essential components, some bacteria have additional structures, **e.g.,** flagella, capsule, and fimbriae.

On examining the bacterial cell, many cell components are observed; few of them are naturally common to almost all prokaryotes and some are present in only certain species. In these structures, few lie externally to the cell wall, and few lie internally. Structures present on the external surface of the bacterial cell wall perform attachment, motility, conjugation, or protection.

3.1.2.1. Capsules and Slime Layers

Extracellular polymers are secreted outside the bacterial cell walls; these polymers are composed of polysaccharides and proteins. Capsules are relatively **impermeable structures**, as compared to cell wall. Capsule **cannot be stained with dyes** (such as India ink), and protect bacteria from phagocytosis and desiccation. Attachment of bacteria to other cells or inanimate surfaces forms bio films through slime layers.

Gelatinous covering present around the cell wall is known as **slime layer**. In bacteria it can be removed easily. It is a diffuse, unorganised layer of extracellular material surrounding the bacteria cells. The major constituents of slime layer are **exopolysaccharides**, **glycoproteins**, and **glycolipids**.

Slime layer (different to slime layer) is a highly organised glycoprotein layer surrounding the bacterial cells.

Functions of Capsule

1) It **protects** the cells against temporary drying by preserving water molecules.
2) IT blocks **attachment** of bacteriophages.
3) It **prevents engulfment** of bacterial cells by WBCs. This **inhibition of engulfment** promotes invasive or infective ability of bacteria known as virulence.
4) It helps in **attachment of bacterial cells** to the surfaces, **e.g.,** *Streptococcus mutans* causing dental caries, by adhering to the smooth surfaces of teeth through its water-insoluble capsular glucan.
5) It provides **stability** to bacterial suspensions. It has an electrical charge due to the presence sugar-uronic acids, which prevent cells from aggregating and settling out since cell surfaces with same charges tend to repel each other.

Functions of Slime Layer

1) It protects the bacterial cells from external environment like antibiotics and desiccation.
2) It allows bacteria to adhere to smooth surfaces just like prosthetic medical devices and catheters.
3) It allows bacterial colonies to sustain under chemical sterilisation (**e.g.,** chlorine, iodine, and other chemicals), except autoclaving or flushing with boiling water used for decontamination.

3.1.2.2. Flagella

Flagella (singular flagellum) form the organ of locomotion in a number of bacilli and spirilla in addition to a few species of cocci. In prokaryotes, flagella represent long whip-like, filamentous, helical appendages that protrude through the plasma membrane and the cell wall. They measure 0.1-0.2µm in thickness and may range up to 15-20µm in length. They are significantly thin as compared to the flagella or cilia of eukaryotes.

Flagella are extremely thin structures, therefore cannot be seen using a bright-field microscope. Special staining techniques are used to see them. Detailed structure of flagellum can be studied by using an electron microscope.

Location of flagella on bacterial cell depends on the bacterial species; therefore it may vary with different species. Flagella may either be positioned at one end or both the ends of the bacterial cell (polar), or may be located along the side of bacterium (lateral).

Four types of flagellar arrangements are seen in bacteria (**figure 3.2**):

1) **Monotrichous:** Only a single flagellum protrudes from either of the ends or poles of bacterial cell.
2) **Lophotrichous:** Numerous flagella protrude from a single pole of the bacterial cell.
3) **Amphitrichous:** Either a single flagellum or a bunch of flagella protrude from both the poles of the bacterial cell.
4) **Peritrichous:** Flagella protrude from all over the bacterial cell.

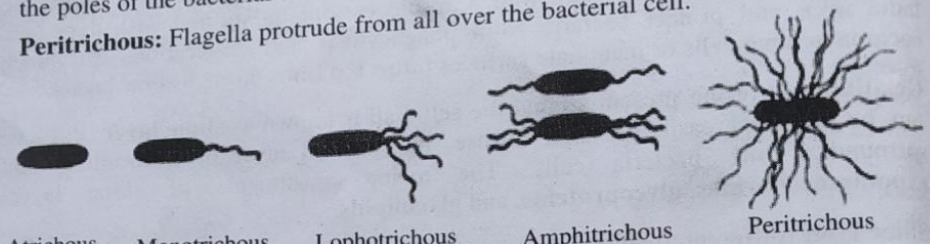


Figure 3.2: Types of Flagellar Arrangement on Bacterial Cell

Atrichous is a condition in which there is absence of flagella. The arrangement of flagella is also useful in classifying the species taxonomically.

Flagellum is remarkably thickened in some prokaryotic genera. An extension of the cytoplasmic membrane surrounds such flagella and they are known as **sheathed flagella**, **e.g.,** the predaceous *Bdellovibrio* characteristically shows the presence of a polar-sheathed flagellum. Few bacteria (**e.g.,** *Vibrio*) show the presence of polar-sheathed flagellum in addition to numerous peritrichously arranged flagella that are unsheathed, under specific growth conditions. **Mixed flagellation** is the term used for such type of flagellar arrangement.

According to the transmission electron microscope, the **bacterial flagellum** has been shown to be made up of **three parts** (**figure 3.3**), which are as follows:

1) **Basal Body:** It is the most complex part of flagellum. It shows different configuration in both gram-negative and gram-positive bacteria. The basal body of gram-negative bacteria shows the presence of four rings, namely the **M (membrane) ring**, the **S (super membrane) ring**, the **P (peptidoglycan) ring**, and the **L (lipopolysaccharide) ring**, all attached to a rod that forms the central axis.

   In a gram-negative bacterium, basal body are arranged as follows:
   i) **Inner membrane**, i.e., plasma membrane embeds the M-ring.
   ii) **Periplasmic space** between the inner membrane and the outer membrane houses the S-ring.
   iii) **Peptidoglycan layer** of the cell wall shows attachment with the P-ring.
   iv) **Lipopolysaccharide layer** of the outer membrane of the cell wall shows attachment with the L-ring.

The P- and L-rings form a channel for the rod to pass through the outer cell wall membrane. In case of gram-positive bacteria, the outer set of rings (P- and L-rings) is absent. M-ring is embedded in the inner membrane, and S-ring is attached to the inside of the thick peptidoglycan layer. The chemical composition of basal body is unknown.

2) **Hook:** The basal body is connected to the main filament or the shaft by the hook. The hook in a gram-negative flagellum is shorter than that in a gram-positive flagellum. In hook, the protein sub-units are (monomers) arranged in a helical fashion.

3) **Main Filament or Shaft:** Protein sub-units (monomers) arranged in a helical fashion forms the main filament or shaft just as the hook. The filament protein is recognised as **flagellin**. Instead of the base, the flagellum of the bacteria shows growth at its tip. The hollow centre of the flagellum forms the path for the passage of the flagellin monomers synthesised inside the bacterial cell. From within the cell, the flagellin monomers are carried and added to the end (situated away from the point of attachment of the filament).

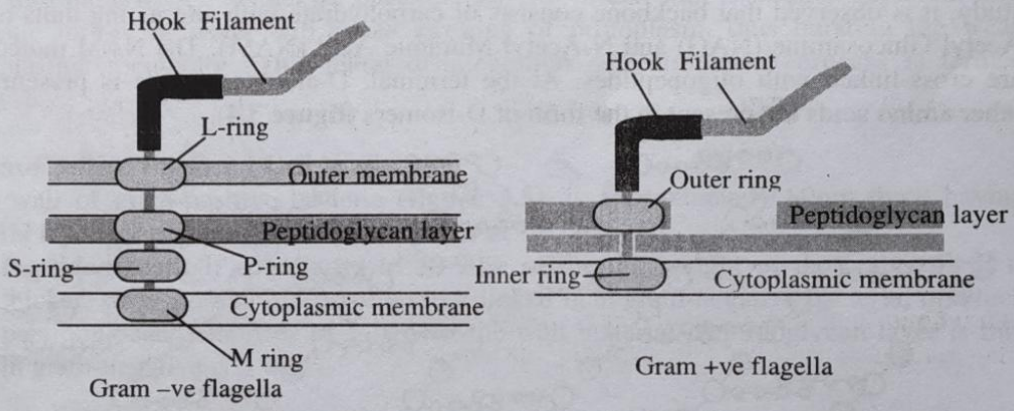


Figure 3.3: Structure of Bacterial Flagellum

3.1.2.3. Pili (Fimbriae)

Duguid *et al.* in 1955 proposed the term **fimbriae** (singular fimbria) and **Brinton** in 1959 proposed the term **pili** (singular pilus). Gram-negative rods (*Salmonella typhi*, typhoid fever; *Shigella dysenteriae*, bacillary dysentery), cocci (*Neisseria gonorrhoeae*, gonorrhoea), and gram-positive bacilli (*Corynebacterium renale*) have fimbriae or pili.

Fimbriae are thin, short, filament-like, non-flagellar appendages which project peritrichously from the cell surface. Each cell possesses around 100-500 of fimbriae, and they are 0.5-20µm in length and 3-25nm in diameter. They are made up of protein sub-units, i.e., the pilin are arranged helically forming a hollow filament.

Fimbriae or pili are categorised into **three types**:

1) **Common Pili:** These are responsible for cell adhesion.
2) **Sex Pili or F (Fertility) Pili:** These are of distinct type which facilitates bacterial mating. They are exclusively formed on cells that contribute DNA during the process of bacterial mating. F pilus regulates the cell-to-cell contact required during mating. F pili are assumed to play a part in the process of conjugation possibly by standing as conjugal tubes required for the transfer of DNA from the donor to the recipient.
3) **Col I (Colicin) Pili:** These are another type of pili which show a strong inclination of sticking to each other and causing agglutination, i.e., clumping together.

Pili also act as receptor sites for some bacteriophage (viruses that replicate within bacterial cells). The bacteriophage attaches to the pili and subsequently transfers their genetic information to the bacterial cell.

3.1.2.4. Cell Wall

Bacterial cell wall is also responsible for the **structural integrity of the cell**. Cell wall is a porous membrane and is not considered as a barrier for permeability of small substrates. Peptidoglycan is present in all bacterial cell wall (with a few exceptions, **e.g.,** intracellular parasites such as *Mycoplasma*), but all cell walls do not have the same structure.

Cell wall of bacteria differs from other organisms due to the presence of **peptidoglycan** (poly-*N*-acetyl glucosamine and *N*-acetylmuramic acid). This peptidoglycan layer is present immediately outside the cell membrane.

The main **function of peptidoglycan** is to provide rigidity to the bacterial cell wall and to determine the shape of bacterial cell.

Chemical Composition

The bacterial cell wall is a unique **biopolymer** of D- and L-amino acids. In a molecular study, it is observed that backbone consists of carbohydrate with alternating units of N-Acetyl Glucosamine (NAG) and N-Acetyl Muramic Acid (NAM). The NAM molecules are cross-linked with oligopeptides. At the terminal, D-alanine peptide is present and other amino acids are present in the form of D-isomers (**figure 3.4**).

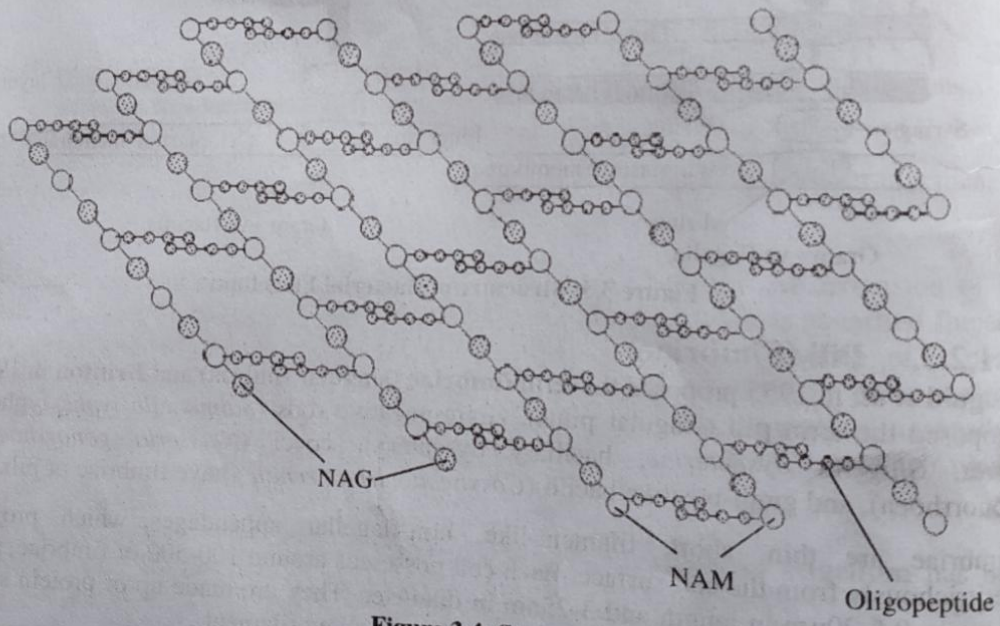


Figure 3.4: Structure of Peptidoglycan

This is the only biological molecule that contains D-amino acids and it is the target of numerous antibacterial antibiotics. There is a small space present between the cell wall and cell membrane (made up of pepidoglycan) of gram-positive bacterium. Up to 40 layers of polymer pepidoglycan are present that provide enormous mechanical strength to the cell wall. Few other polymers, **e.g.,** teichoic and teichuronic acids, are also present in the cell wall of gram-positive bacteria, which act as surface antigens.

Difference between the Cell Wall of Gram-Positive and Gram-Negative Bacteria

On the basis of the ability to retain a crystal violet-iodine dye complex after treating with acetone or alcohol, bacteria may be divided into **two major groups**, i.e., **gram-positive** and **gram-negative** bacteria. This reaction is known as **Gram reaction** named after **Christian Gram** in 1884, who developed the staining protocol.

There are differences in cell walls of gram-positive and gram-negative bacteria (**table 3.1**). Gram-positive bacteria cell wall has a simpler chemical nature than that of gram-negative bacteria.

Table 3.1: Comparison of Cell Walls of Gram-Positive and Gram-Negative Bacteria

Characteristics	Gram-Positive	Gram-Negative
1) Thickness	Thicker	Thinner
2) Peptidoglycan	Thick layer (16-80nm)	Thin layer (2nm)
3) Teichoic Acid	Present	Absent
4) Variety of Amino Acids	Few	Several
5) Aromatic and Sulphur Containing Amino Acids	Absent or scant	Present
6) Lipids	Absent or scant	Present
7) Porin Proteins	Absent	Present
8) Periplasmic Region	Absent	Present

Cell wall integrity is essential for bacterial survival. If cell wall is weak or ruptured, then osmotic inflow of water will cause swelling of protoplasm, thus bursting the weak cytoplasmic membrane. This lethal disintegration and dissolution process is termed analysis.

Gram-Positive Bacterial Cell Wall

Cell wall of gram-positive bacteria (**figure 3.5**), is approximately 80nm thick having several layers of peptidoglycan. It comprises of:

1) **Peptidoglycan:** It contributes to 50-90% of the dry weight of the cell wall. It is thicker, stronger, and extensively cross-linked than gram-negative bacteria, in which peptidoglycan comprises of 5-10% of the wall material. Peptidoglycan layer is thin in gram-negative cell walls.

2) **Teichoic Acids:** Cell wall of gram-positive bacteria has teichoic acids containing alcohol (either glycerol or ribitol) and phosphate. There are two types of teichoic acids; first is **wall teichoic acid** which is covalently bonded to peptidoglycan, and the second one is **membrane teichoic acid** (lipoteichoic acid) that covalently links to membrane glycolipid and is concentrated in mesosomes. Wall teichoic acids are absent in some gram-positive species, whereas membrane teichoic acids are present in all.

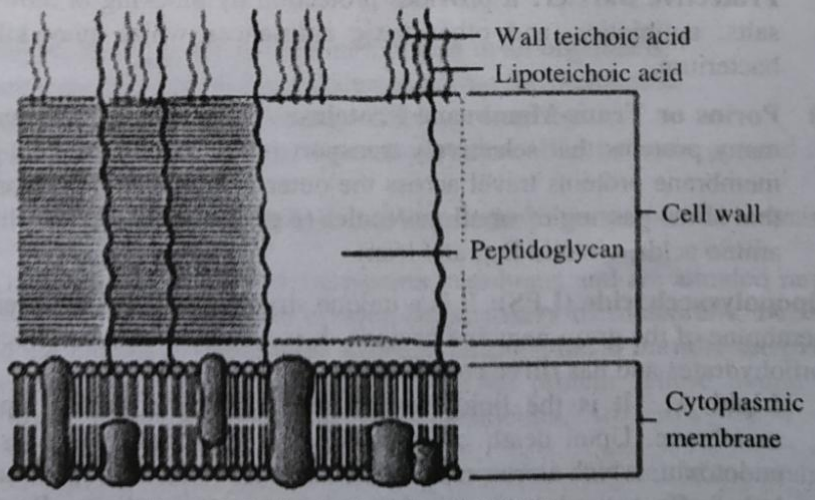


Figure 3.5: Gram-Positive Cell Wall

Gram-Negative Bacterial Cell Wall

Structurally, the gram-negative cell wall (**figure 3.6**) is different from gram-positive cell wall. It is made up of:

1) **Peptidoglycan Layer:** It constitutes approximately 5-10% of the dry weight of the wall of gram-negative bacteria and consists of a single unit. In the outer membrane and plasma membrane, it is linked with lipoproteins. It is present in periplasmic space (a gel like fluid between the outer membrane and plasma membrane) containing high concentration of degradative enzymes and transport proteins. The periplasmic space is around 20-40% of the cell volume.

2) **Lipoprotein:** Lipoprotein/murein lipoproteins are linked (both covalently and non-covalently) to the peptidoglycan through protein portion and to the outer membrane through lipid. It **functions** to stabilise the outer membrane and hold it to the peptidoglycan layer.

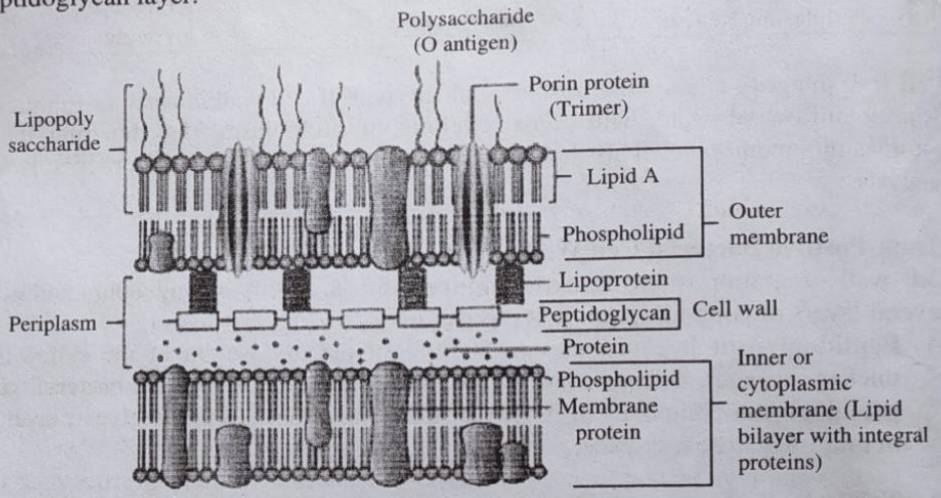


Figure 3.6: Gram-Negative Cell Wall

3) **Outer Membrane:** It is present externally to the peptidoglycan and is attached with lipoproteins. It is a bilayered structure having inner leaflet composed of phospholipid, while outer leaflet composed of lipopolysaccharide (LPS) molecules.

Functions

i) **Protective Barrier:** It provides protection by blocking or slowing the entry of salts, antibiotics, and other toxic substances which may kill or injure the bacterium.

ii) **Porins or Trans-Membrane Proteins:** Along with LPS, outer membrane has many proteins that selectively transport nutrients into the cell. Porins or trans-membrane proteins travel across the outer membrane, making trimeric channels that allow passing of small molecules (**e.g.,** nucleotides, disaccharides, peptides, amino acids, vitamin B_{12}, and iron).

4) **Lipopolysaccharide (LPS):** It is a unique structural component present in the outer membrane of the gram-negative bacteria. It is a large complex molecule of lipids and carbohydrates and has **three components**:

i) **Lipid A:** It is the lipid part of LPS embedded in the top layer of outer membrane. Upon death of gram-negative bacteria, lipid A is released as an endotoxin, which is responsible for the endotoxic activities, i.e., pyrogenicity, lethal effect, tissue necrosis, anti-complementary activity, B-cell mitogenicity, immunoadjuvant property, and antitumor activity.

ii) **Core Polysaccharide:** It is linked to lipid A and a terminal series of repeated units having unusual sugars that maintains stability.

iii) **O-Polysaccharide:** It is composed of sugar molecules. It extends toward outside with respect to the core polysaccharide. O-polysaccharides work as antigens and are differentiating factor for species of gram-negative bacteria. In gram-positive cells, its role is similar to that of teichoic acid. A major surface antigen on polysaccharide of the bacterial cell is O antigen. Bacteria lacking the O antigen form rough colonies, while bacteria with LPS having O antigen forms smooth colonies in bacteriological media.

3.1.2.5. Plasma (Cytoplasmic/Cell) Membrane

Immediately below the cell wall, plasma membrane is present which is also known as cytoplasmic membrane, inner membrane, or cell membrane. In prokaryotes, plasma membrane thickness is about 7.5nm (0.0075μm).

Composition

1) **Phospholipids:** These constitute 40% of the plasma membrane and are arranged in two parallel layers, thus known as **phospholipid bilayer**. Its each molecule has **two components**:
   i) **Polar Head:** It comprises of phosphate group and glycerol. It is hydrophilic (water-loving), therefore is soluble in water.
   ii) **Non-Polar Tails:** Fatty acids being the main component makes them hydrophobic (water-fearing), therefore are insoluble in water.

   On both surfaces of the bilayer, polar heads are present. Inside the bilayer, non-polar tails are found. Bacterial membranes lack sterols and high molecular weight lipids, except the wall-less prokaryotic mycoplasma (they contain membrane sterols). Sterols and high molecular weight lipids are commonly present in eukaryotic cells.

   After studying the **phospholipids** present in **bacteria** (eubacteria) and in **archae bacteria**, it is concluded that in bacteria, the phospholipids are phosphoglycerides having straight-chain fatty acids ester-linked to glycerol; whereas in archaebacteria, lipids are poly isoprenoid branched-chain lipids having branched long-chain alcohols (phytanols) which are ether-linked to glycerol.

   Functions
   i) These are building blocks for cell membranes in all organisms.
   ii) These are transducer of biological signals across the membrane.
   iii) These are efficient storage of energy, like triglycerides.
   iv) These play an important role in the transportation of fat between gut and liver in mammalian digestion.
   v) These are the source of acetylcholine (a neurotransmitter present in mammals).

2) **Proteins:** These constitute 60% of the plasma membrane and are situated randomly in lipid bilayer. Some proteins are present on periphery of membrane, but loosely attached and are known as **peripheral proteins** or **peripheral membrane proteins**, which constitute about 20-30% of total membrane protein. These bound to the hydrophilic layer of membrane through charge interaction.

   About 70-80% of membrane proteins are present within the membrane matrix and constitute **integral proteins** or **trans-membrane proteins** or **membrane-integral proteins**. They are present throughout the bilayers and are bound to fatty acid chains

by hydrophobic amino acid residues. Few proteins act as channels for substances to enter and exit the cell. These peripheral proteins normally bound to the hydrophilic layer of membrane by charge interaction.

Functions of Plasma Membrane

1) It is actively involved in the regulation of the flow of materials inside and outside the cell. It acts as a differentially permeable barrier, that permit smaller molecules (like water, oxygen, carbon dioxide, and simple sugars) to pass. Larger molecules cannot move freely across the membrane. The bacterial cell membrane excretes out molecular waste products outside the cell.
2) It acts as an osmotic barrier that maintains intracellular osmotic pressure for the cell.
3) It provides site for many important enzymatic reactions, **e.g.,** in respiratory metabolism and during synthesis of capsule and cell wall components. Its impermeability to protons (hydrogen ions) results to the site for generation of the proton-motive force, that drives ATP production, some nutrient transport systems, and flagellar motility.
4) Any damage to membrane through physical or chemical agents results in cell death.
5) It also participates in replication of DNA attached to the membrane. Newly formed DNA molecules are physically separated through newly formed individual cell membrane.

3.1.2.6. Cytoplasm and Cytoplasmic Inclusions

Structure enclosed by the cell membrane is known as cytoplasm, which is aqueous, semi-transparent, elastic, and thick. Cytoplasm consists of 80% water and remaining 20% include proteins (enzymes), carbohydrates, lipids, inorganic ions, and many low-molecular weight compounds. In the cytoplasm, most of the vital chemical reactions (metabolism) of the cells take place.

In prokaryotic cytoplasm, the major structures are nucleoid, ribosomes, and various reserve deposits (called inclusion bodies). Most of the membrane-bound structures, **e.g.,** endoplasmic reticulum, mitochondria, Golgi apparatus, lysosomes, and membrane-bound vacuoles are not present in the prokaryotic cytoplasm.

Nucleoid

Prokaryotic cells lack the presence of a well-developed nucleus or a nucleus with a distinct membrane or a mitotic apparatus or a nucleolus. Yet, their nuclear structure is encompassed in the form of a double stranded DNA (dsDNA) present close to the centre of the cell.

This nuclear structure is labelled as a **nucleoid/nucleoplasm region/chromatin body/nuclear equivalent/bacterial chromosome**, as it is not a distinct nucleus. The nucleoid can be visualised by a light microscope using **Feulgen staining**, which is specifically used to visualise DNA. It gives the impression of a light area with a delicate fibrillar structure under an electron microscope.

The genetic information of a bacterial cell is contained in the nucleoid which carries all the information essential for the structure and function of bacterial cell. It is present in the form of a continuous, long thread of single double-stranded DNA, arranged in a circular manner.

Ribosomes

Ribosomes are very small structures, present in the cytoplasm of a bacterial cell, ranging in number from 15,000-20,000. They are responsible for the characteristic granular

appearance of the cytoplasm. The process of translation (protein synthesis) is carried out by ribosomes. In this process, the message in nucleotides is carried by the messenger RNA (mRNA) from the genome to the ribosome, where proteins are formed by the linking of amino acids through peptide bonds.

Approximately, 40% of the dry weight of a bacterial cell is accounted for the ribosomes. Ribosomes are comprised of ribosomal Ribonucleic Acid (rRNA) and proteins. Some bacterial ribosomes are suspended freely within the cytoplasm, and the remaining ribosomes are linked to the inner surface of the cytoplasmic membrane, and are engaged in protein synthesis (to be ultimately transported out of the cell).

Inclusion Bodies

In the cytoplasm of prokaryotic cells, some reserve deposits, known as **inclusions** are present. Cells store certain nutrients present in large amounts to utilise them in deficient conditions. It has been proved that macromolecules in inclusions to prevent the increase in osmotic pressure that would result if the molecules were dispersed in the cytoplasm.

Some inclusions are commonly present in wide variety of bacteria, while some are limited to a small number of species, thus aiding in their identification. Given below are the different inclusion bodies:

1) **Metachromatic Granules:** The name of these large inclusions has been derived from the fact that at times they stain red with certain blue dyes (**e.g.,** methylene blue). Collectively they are termed **volutin**, which is a reserve of inorganic phosphate (polyphosphate) used for ATP synthesis. It is formed by cells grown in phosphate-rich environments. Algae, fungi, protozoa, and bacteria contain metachromatic granules, which are characteristic of *Corynebacterium diphtheriae* (diphtheria causing agent), thus are diagnostically significant.
2) **Polysaccharide Granules:** These inclusions contain glycogen and starch. The glycogen granules appear reddish brown and starch granules appear blue when bacterial cells are stained with iodine, thus aiding the identification of these inclusions.
3) **Lipid Inclusions:** These inclusions are present in various species of *Mycobacterium*, *Bacillus*, *Azotobacter*, *Spirillum*, etc. A common lipid-storage material (unique to bacteria) is poly-β-hydroxybutyric acid (a polymer). The presence of these inclusions is identified by staining the bacterial cells with fat-soluble dyes, **e.g.,** Sudan dyes
4) **Sulphur Granules:** The "sulphur bacteria" (genus *Thiobacillus*) obtain energy from the oxidation of sulphur and sulphur-containing compounds. These bacteria deposit sulphur granules in the bacterial cell to act as a source of energy.
5) **Carboxysomes:** These inclusions contain ribulose 1,5-diphosphate carboxylase enzyme. Bacteria obtaining carbon from carbon dioxide utilise this enzyme for carbon dioxide fixation during photosynthesis. Nitrifying bacteria, cyanobacteria, and thiobacilli contain these inclusions.
6) **Gas Vacuoles:** These hollow cavities are present in some aquatic prokaryotes, including cyanobacteria, anoxygenic photosynthetic bacteria, and halobacteria. Each vacuole has rows of several individual gas vesicles (hollow cylinders covered with protein). Gas vacuoles maintain buoyancy so that the bacterial cells remain in the water at a depth where they can receive optimum amounts of oxygen, light, and nutrients.
7) **Magnetosomes:** These inclusions of iron oxide (Fe_3O_4) are formed by some gram-negative bacteria (*Aquaspirillum magnetotacticum*) and act like magnets. Bacteria

with the help of these inclusions move downward to a suitable attachment site. Magnetosomes decompose hydrogen peroxide formed in the bacterial cells in the presence of oxygen. Researchers have guessed that magnetosomes provide protection to the cells by avoiding accumulation of hydrogen peroxide. Various culture methods are under development by industrial microbiologists to obtain large quantities of magnetite from bacteria, so that they can be further used for producing magnetic tapes for sound and data recording.

3.1.2.7. Bacterial Spores (Endospores)

There are some bacteria (**e.g.,** *Bacillus anthracis*, *Bacillus subtilis*, *Clostridium tetani*, *Clostridium botulinum*, *Coxiella burnetii*, *Sporosarcina*, *Thermoactinomycetes*, *Streptomyces*, *Actinopolyspora* species, etc.) which produce **endospores** (spores within the cell) and **exospores** (spores outside the cell).

Each cell produces a single thick-walled and highly refractile endospore. Each bacterial spore produces a single vegetative cell on germination. Thus, **bacterial sporulation is a preservation method** and not a reproduction method. Spores remain unaffected by desiccation, staining, disinfecting chemicals, radiation, and heat. They are viable for centuries, therefore helping the bacteria to survive long even under unfavourable conditions. Moist heat sterilisation, i.e., autoclaving at 121°C for 20 minutes destroy the spores of all medically important bacteria.

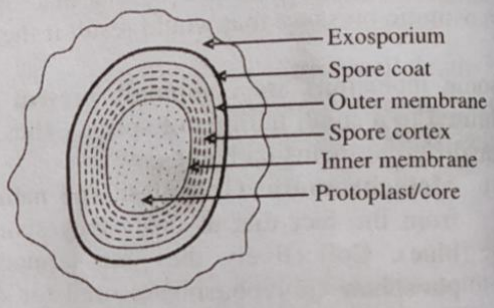


Figure 3.7: Structure of a Spore

Endospores have a **core** or **envelope** or **protoplast**. DNA and protein synthesis components (like ribosome, t-RNA, and enzymes) are present in the protoplast. The **spore envelope** is comprised of the **inner membrane**, **cortex**, **outer membrane**, and **spore coat**. Some bacterial species have outer layer developed into **exosporium**, bearing ridges and folds (**figure 3.7**).

The inner and the outer membrane have a multi-laminated cortex (containing peptidoglycan) in between. The spore coat containing keratin-like protein is present outside the outer membrane, and is not affected by chemical treatment, thus making the spore resistant to chemicals.

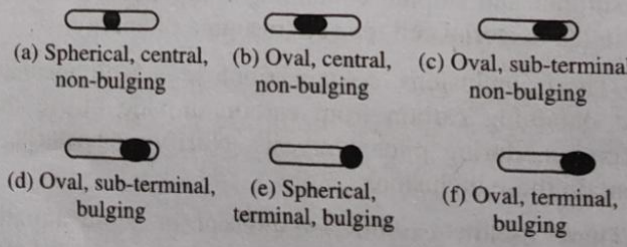


Figure 3.8: Types of Spores

The spores may be round, oval, or elongated (**figure 3.8**). They may occupy a terminal, sub-terminal, or central position within the cell. They may be narrower than the width of the bacilli and bulging.

3.1.3. Morphological Classification of Bacteria

On the basis of morphology, bacteria are classified into the following **three forms**:

1) **Spherical:** These are **coccus** bacteria which are spherical in shape. The word **coccus** originated from the Greek word ***kokkos*** meaning berry. They are the smallest form of bacteria and are almost spherical in shape and appear in many different patterns depending on the planes in which they can be divided:
   i) **Micrococcus (Greek *micros* - small; *kokkos* - berry):** These are present as a single cell, **e.g.,** *Micrococcus*. These bacteria are mostly non-flagellated, gram-positive, and facultative saprophytes or parasites.
   ii) **Diplococci:** These are present in the pair of two, **e.g.,** *Diplococcus pneumonia*. A single coccus can be divided into two cocci by one plane but remain attached in pair.
   iii) **Streptococci (Greek *streptos* - twisted):** These are present in chains, **e.g.,** *Streptococcus lactis*. Although, cocci divides into one plane but remain arranged in the form of chains.
   iv) **Staphylococci (Greek *staphyle* - bunch of grapes):** These are present in groups, **e.g.,** *Staphylococcus aureus*.
   v) **Tetrads (Tetracoccus):** These are present in groups of four and can be divided into two planes.
   vi) **Sarcinae:** These are present in cube-like groups of eight and can be divided into three planes, **e.g.**, *Sarcina*.

   In **figure 3.9**, structures of different forms of cocci are shown:

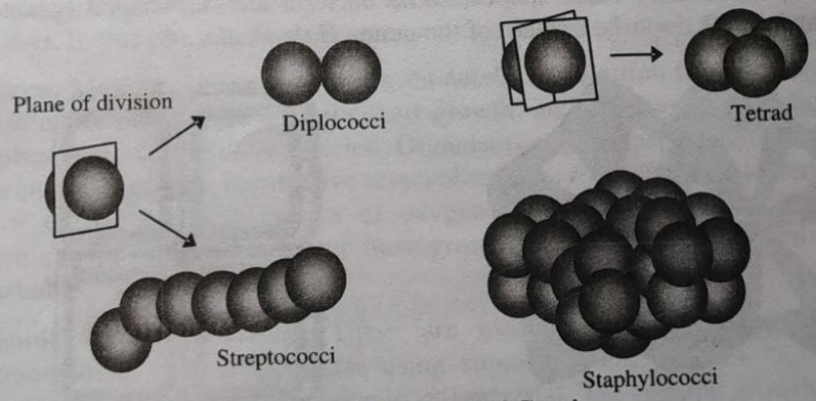


Figure 3.9: Forms of Cocci

2) **Cylindrical or Rod-Shaped:** These bacteria are rod-shaped and are also known as **bacillus** (meaning rod-shaped, singular bacilli). In number, the rod-shaped bacteria are more than the spherical bacteria as their ratio of the surface area to volume is greater. These are of the following types:
   i) **Bacillus:** These are single rod-shaped bacteria.
   ii) **Diplobacillus:** These bacteria occur in pair.
   iii) **Streptobacillus:** These are grouped in the form of chains, **e.g.,** *Bacillus anthracis*.

   In **figure 3.10**, different forms of bacilli are shown:

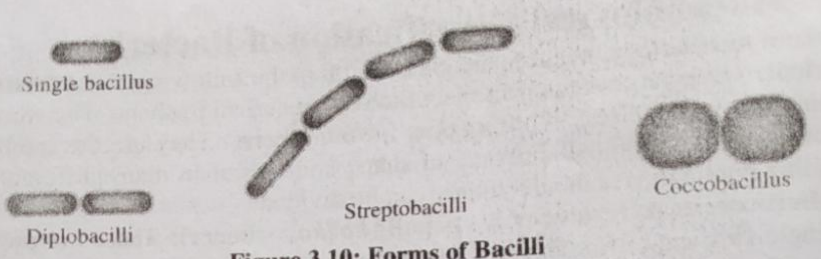


Figure 3.10: Forms of Bacilli

3) **Spiral:** Shape of these bacteria is never straight and one or more twists are found in them. These are sub-divided into:
   i) **Vibrio or Comma:** These bacteria are comma [,] shaped, **e.g.,** *Vibrio cholera.*
   ii) **Spirilla:** The word **spirilla** originated from the Greek word ***spira*** meaning coil. Shape of these bacteria is coiled or curved rod-like. These bacteria have rigid body having a helical shape (like a corkscrew), **e.g.,** *Spirillum ruprem.*
   iii) **Spirochetes:** These bacteria use an axial filament found on the external flexible sheath for their movement, **e.g.,** *Treponema palladium.*

In **figure 3.11**, different forms of spiral bacteria are shown:

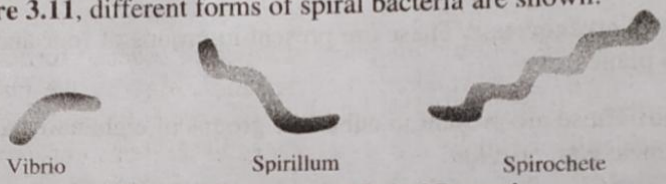


Figure 3.11: Forms of Spiral Bacteria

Other than these three basic shapes, some bacteria are star-shaped (genus *Stella*), square, and flat (halophilic archae) of the genus *Haloarcula*.

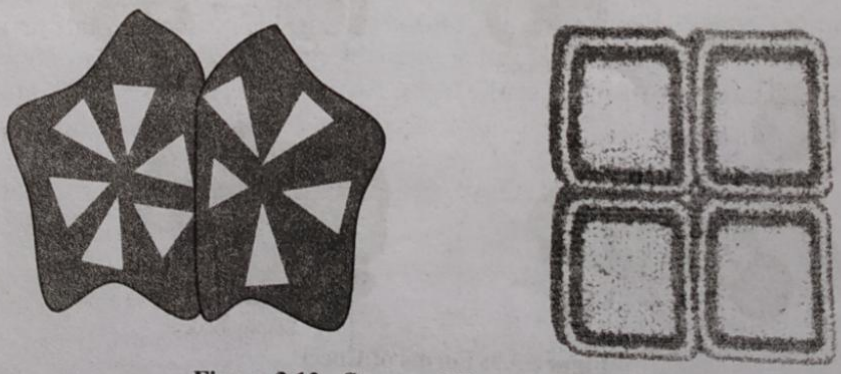


Figure 3.12: Star- and Square-Shaped Bacteria

3.1.4. Nutritional Requirements of Bacteria

Bacteria differ widely in their nutritional requirements. Some bacteria have the ability to synthesise their nutritional requirements from the simplest available elements, while others are artificially provided with organic compounds essential for growth. Given below are the different growth requirements provided to bacteria via different types of culture media:

1) **Water:** It is the major nutrient, forming about 80-90% of the total weight of cells. **Hydrogen** and **oxygen** can be metabolically obtained from water, while other required elements are **carbon**, **nitrogen**, **phosphorus**, and **sulphur**. These 6 elements make about 95% of the dry cell weight. Apart from these elements, potassium, magnesium, calcium, iron, manganese, cobalt, copper, molybdenum, and

zinc are also essentially required. These metallic elements are supplied in the form of their corresponding soluble salts (chlorides or sulphates, especially). **Manganese**, **cobalt**, **copper**, **nickel**, **molybdenum**, and **zinc** are the **micronutrients** or **trace elements** required for bacterial growth. Most of these trace elements are present as impurities in tap water; therefore, the culture media should be prepared with tap water (and not with distilled water). Some biological groups have specific mineral requirements, **e.g.,** diatoms and certain algae require silicate; photosynthetic bacteria, cyanobacteria, and certain marine bacteria require sodium.

2) **Carbon:** Bacteria obtaining energy from oxidised inorganic compounds and photosynthetic organisms utilise CO_2 (oxidised form of carbon) as the major source of cellular carbon. All other organisms derive carbon from organic nutrients. Some bacteria of *Pseudomonas* group utilise as a source of carbon and energy any one out of 90 different organic compounds; methane-oxidising bacteria utilise two organic substrates (i.e., methane and methanol); while some cellulose-decomposing bacteria utilise only cellulose. Microorganisms depending on organic carbon sources also require trace amounts of CO_2 as a nutrient to be utilised in some biosynthetic reactions.

   The organisms which depend on an inorganic diet, using CO_2 or carbonates as a source of carbon for their survival, are **autotrophs** (*autos* means self and *trophe* means nourishing).

3) **Nitrogen and Sulphur:** These elements are used in reduced form, **e.g.,** nitrogen in amino acids or sulphur in sulphydryl compounds. Most of the photosynthetic and non-photosynthetic bacteria and fungi assimilate nitrogen and sulphur as **nitrates** and **sulphates** (oxidised inorganic state). Organic nutrients containing nitrogen and sulphur in reduced organic combination, **e.g.,** amino acids or peptones can also be used for fulfilling their requirement. Some bacteria utilise the most abundant natural nitrogen source (N_2) in the process of nitrogen assimilation, known as **nitrogen fixation**. In this process, N_2 undergoes a preliminary reduction to ammonia.

4) **Oxygen:** Microorganisms depending on aerobic respiration for their energetic needs utilise molecular oxygen (O_2) for their growth; and are known as **obligate** or **strict aerobes**, **e.g.,** *Clostridium* species. Organisms which can survive with or without molecular oxygen are **facultative anaerobes**, **e.g.,** *E. coli*. Obligate aerobes growing below 0.2atm partial pressures of oxygen are **microaerophilic**. Microorganisms which cannot utilise oxygen for their growth but tolerate it well are **aerotolerant anaerobes**.

5) **Organic Growth Factors:** These are essential organic compounds which a microorganism cannot synthesise using simple organic sources, but requires as a precursor or constituent of its organic cell material. The organic growth factors are of the following **types**:
   i) **Amino Acids:** Required as constituents of proteins.
   ii) **Purines and Pyrimidines:** Required as constituents of nucleic acids.
   iii) **Vitamins:** Forming parts of the prosthetic groups or active centres of certain enzymes.

3.1.5. Growth of Bacteria

Bacteria growing in different ecological niches have varied patterns of growth as described below:

1) **Exponential Growth:** When a bacterial culture is growing in unlimited medium, and is kept below a given concentration by dilution at suitable intervals, the culture grows continuously as well as exponentially. On a semi-logarithmic paper, a straight

line graph is obtained between the number of cells per volume (or any other property per volume) against time. The growth curve is simplified by the given formula:

$$N_t = N_0 \cdot 2^{t/\tau}$$

Where,

N_0 = Cell number at zero time
N_t = Cell number at any given time t and
τ = Doubling time of the culture
(Every τ minute, the cell doubles in number)

If a graph is plotted for other cell properties, parallel lines appear because the other properties of the cell also show same growth pattern as the cell number (i.e., double) as the time increases in square (**figure 3.13**).

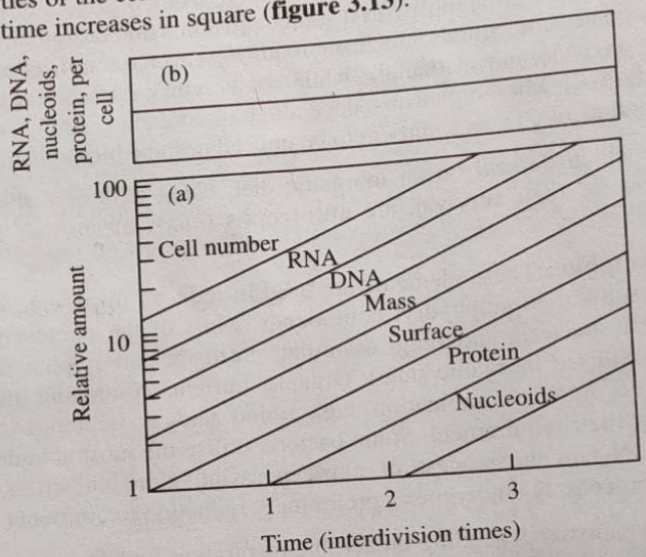


Figure 3.13: Balanced Growth of a Bacterial Culture. Determinations of the cell number, DNA, RNA, protein, or any property of cells in balanced growth give straight, parallel lines when plotted on semi-logarithmic graph paper [panel (a)]. As the rates of increase of all cell properties are the same at all times, the average cell composition is constant. This is plotted in the upper graph where the cell composition is constant [panel (b)].

Average properties of the culture are constant with time. The intensive properties of the culture (average cell size, the RNA per cell, and the DNA per cell) remain constant and invariant with time, whereas the extensive properties (the amount and the number) increases with time.

The culture can be kept growing exponentially for many hours to ensure that it is in a constant state of exponential growth. It is usual to have cells growing exponentially for up to 15 hours before performing an experiment.

2) **Balanced Growth:** Balanced culture is evident from an exponentially growing culture. According to most of the bacteriologists, the bacterial growth is generally presented as a curve in which an overgrown culture is inoculated into a fresh medium. There is a lag phase, before cell number begins to increase and then a period of time when the number increases; this period is known as **early-**, **middle-**, and **late-log[2] phases**.

On entering stationary phase, the growth of the culture stops, followed by a final death phase. However, this pattern of growth does not depict balanced growth. Balanced growth occurs only in middle phase of the classic growth cycle, where the cells in culture grow exponentially with constant properties. This is the main property of balanced growth.

In balanced exponential growth, the properties of the cells are independent of the age of the culture. Any results acquired do not depend on the time when samples were withdrawn from a culture.

3.1.6. Growth Curve

Bacterial growth is defined as **orderly growth of all cellular constituents in a bacterial cell**. During the growth, cell divides and results in an increase in cell number. The most common method of bacterial division is binary fission in which one cell divides to form two daughter cells of equal size.

A definite growth pattern is observed, during the inoculation of bacterium into a suitable culture medium. On counting the bacteria of these cultures at definite time-interval and plotting a graph in relation to time, a growth curve is obtained known as **normal growth curve** of bacteria (**figure 3.14**):

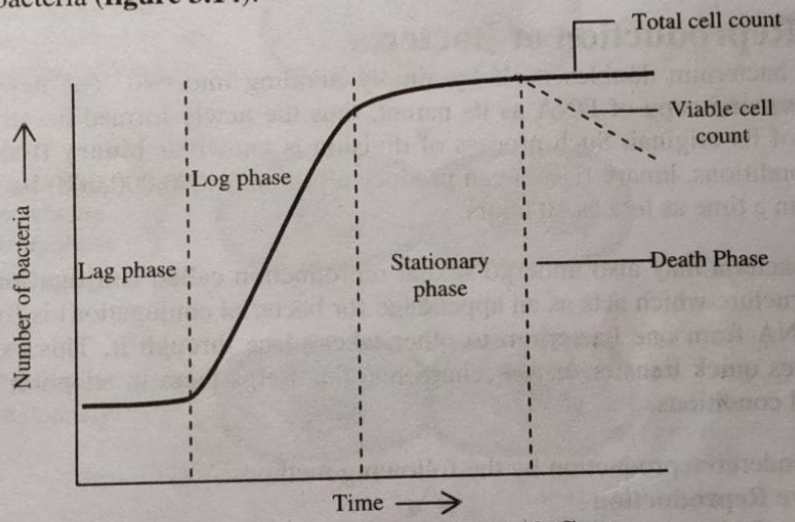


Figure 3.14: Bacterial Growth Curve

The bacterial growth curve consists of the following **four phases**:

1) **Lag Phase:** Generally, multiplication of bacteria does not begin immediately after the culture medium has been inoculated. Lag phase represents the **period between inoculation of the culture medium and initiation of multiplication**. In the lag phase, the microbe adapts itself to the new environment; during this time the enzymes which are essential and the intermediate metabolites are built up in quantities required for multiplication. During this phase, the cells increase its size, but their numbers remain almost same. The lag phase is controlled by factors such as species, temperature, nature of culture medium, etc.

2) **Log (Logarithmic) or Exponential Phase:** Though, the rate of synthesis of the material of the new cell is constant, the culture itself acts as a catalyst therefore an exponential increase is seen in the new cell mass. This process continues until either:
   i) The medium is exhausted of the nutrients (one or more), or
   ii) Accumulation of toxic metabolic products takes place that inhibits their growth.

Generally, oxygen becomes the limiting factor for aerobic organisms. Concentration exceeding approximately 1×107/mL (in case of bacteria), demands higher concentration of oxygen into the culture medium either by means of agitation or by bubbling in the air. If oxygen is not in high concentration into the medium, a decrease is seen in the rate of bacterial growth. On reaching a concentration of 4×109/mL to 5×109/mL, the rate at which oxygen is diffused is not enough to suffice the demand even in a medium that is aerated, and growth shows progressive slowing.

3) **Stationary Phase:** The nutrients get exhausted and toxic products are accumulated after log phase, leading to a more or less complete termination in the growth of bacterial colony. The number of progeny cells formed is just enough to replace the number of cells died. Since a balance exists between the number of cells dying and the cells that are newly formed, the number of viable cells remains stationary.

4) **Death Phase or Phase of Decline:** After a period of stationary phase, the bacterial population decreases due to the death of cells. The decline phase starts due to exhaustion of nutrients, and accumulation of toxic products and autolytic enzymes. There is a decline in viable count and not in total count. With autolytic bacteria, even the total count shows a phase of decline. A small number of cells that have survived may persist for some months or even years. Sometimes, it is seen that after a large number of cells have died, there is a severe decline in the death rate.

3.1.7. Reproduction of Bacteria

Generally, a bacterium doubles itself by simply dividing into two. The newly formed bacterium has same copy of DNA as its parent, thus the newly formed bacterium is the cloned form of its original. Such process of division is known as **binary fission**. Under favourable conditions, binary fission can produce a billion (1,000,000,000) bacteria from a single one, in a time as less as 10 hours.

Sometimes, bacteria may also undergo sexual reproduction called **conjugation**. Pilus (a thread-like structure which acts as an appendage for bacterial conjugation) is formed, and transfer of DNA from one bacterium to other takes place through it. This exchange of DNA facilitates quick transfer of new characters that helps them in adaption of diverse environmental conditions.

Bacteria can undergo reproduction by the following methods:

1) **Vegetative Reproduction**
   i) Binary fission,
   ii) Budding, and
   iii) Fragmentation.
2) **Asexual Reproduction:** Endospore formation.
3) **Sexual Reproduction**
   i) Transformation,
   ii) Transduction, and
   iii) Conjugation.

In **asexual reproduction**, a single organism gives rise to the offspring which inherit the genes exclusively from the parent organism. Fusion of gametes does not take place during asexual reproduction, and negligible change in chromosomal number occurs. Single celled micro organisms (like the archaebacteria, eubacteria, and protists) reproduce mainly by means of asexual reproduction. Asexual reproduction is also evident amongst many plants and fungi.

Sexual reproduction in bacteria is not characterised by meiosis, gamete formation, or zygote formation; rather portion of the genetic material (DNA) is transferred to a recipient cell from a donor cell. This process is termed **parasexuality** or **genetic recombination**.

3.1.7.1. Binary Fission

Binary fission is the most common type of reproduction occurring under favourable conditions (**figure 3.15**). Binary fission involves division of bacterial cell into two similar daughter cells, during which the bacterial chromosomes get attached to the cell membrane and replicates. With the enlargement of cell, the daughter chromosomes separate. A cross-wall builds up between the separating daughter chromosomes, thus dividing the cell into two daughter cells. The daughter cells mature within 20-30 minutes; and under favourable conditions some of them divide within 20-30 minutes.

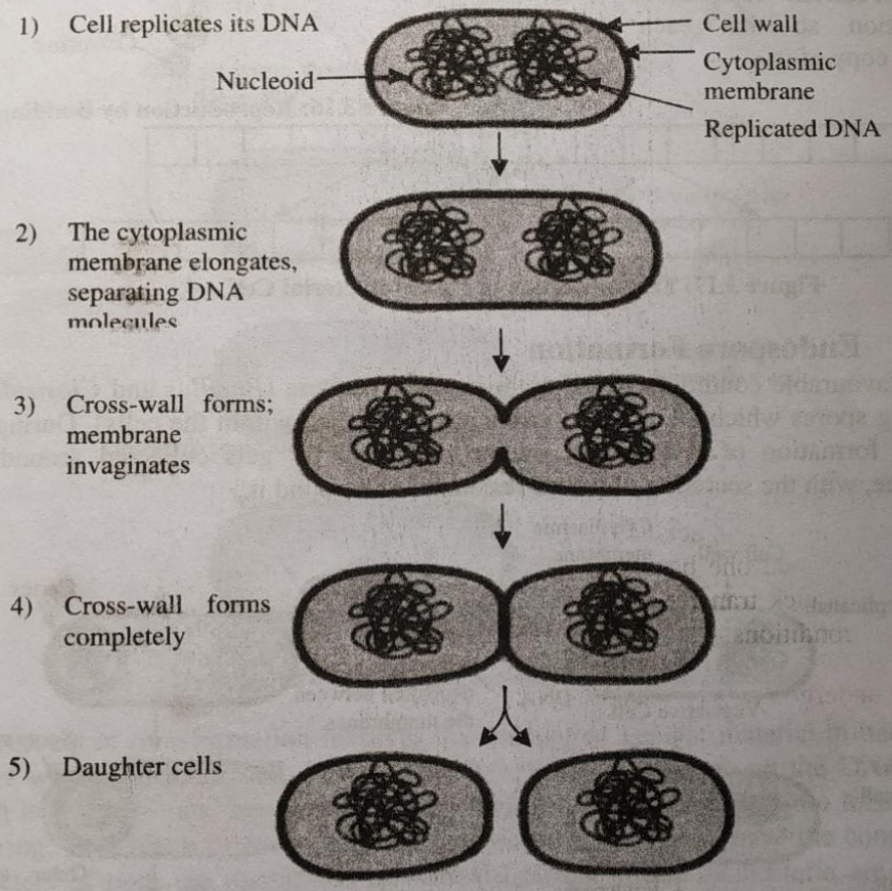


Figure 3.15: Cell under Binary Fission Forming Daughter Cells

3.1.7.2. Budding

Budding is a form of vegetative reproduction in which some reproductive anatomical point in the parent organism gives rise to a new organism (**figure 3.16**). Budding is a characteristic feature of few unicellular organisms (**e.g.,** certain bacteria, yeasts, and protozoans), and few gram-positive bacteria (**e.g.,** Planctomycetes and Cyanobacteria).

Budding is facilitated by the formation of a small protuberance called **bud**, which develops at one end of the cell. Followed by replication of genome (where one copy of the genome gets into the bud), the bud then increases in size, and develops into a daughter cell, and lastly separates from the parent cell, forming an off-spring.

3.1.7.3. Fragmentation

Fragmentation is a vegetative means of reproduction in which a fragment of the parent gives rise to a new organism (**figure 3.17**). All the fragments develop into a fully mature and grown organism. Many organisms like animals (**e.g.,** some annelid worms, turbellarians and sea stars), fungi, and plants reproduce by means of fragmentation.

In case of unfavourable conditions, compartments are formed within the bacterial protoplasm followed by fragmentation, resulting in minute bodies known as **gonidia**. When conditions favour, each gonidium develops into a new bacterium. The bacterial genome should undergo recurrent replications prior to fragmentation so that each fragment acquires a copy of it.

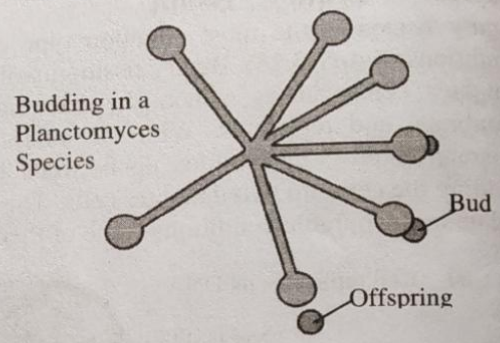


Figure 3.16: Reproduction by Budding

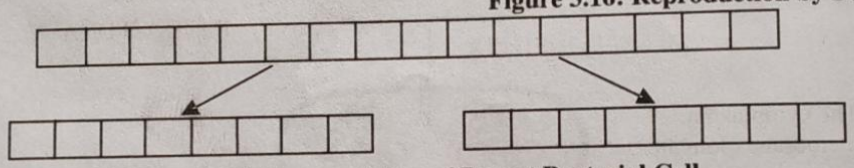


Figure 3.17: Fragmentation of Parent Bacterial Cell

3.1.7.4. Endospore Formation

During unfavourable conditions some gram-positive bacteria (*Bacillus* and *Clostridium*) form resting spores which are actually **endospores** (formed within the cells). During the process of formation of endospores, the protoplast partly gets collected around the chromosome, with the secretion of a hard resistant wall around it.

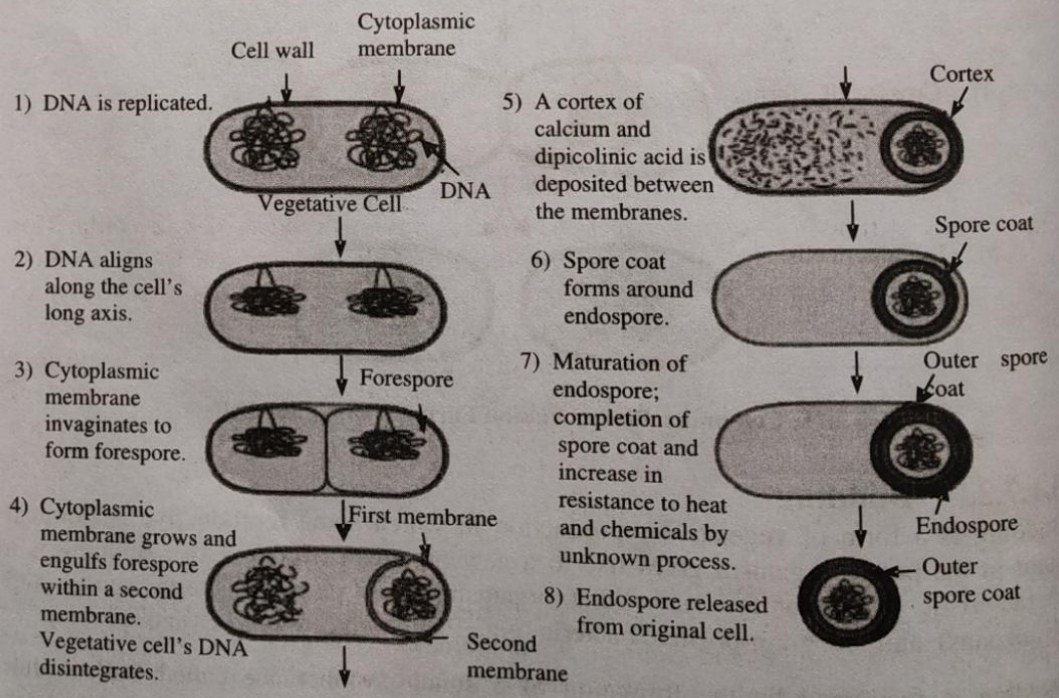


Figure 3.18: Reproduction in Bacteria by Endospores

The remaining bacterial cell undergoes degeneration. Endospores are resistant to chemicals as well as extreme physical conditions. When conditions favour, the wall of the spores rupture and new bacteria arise from the protoplasmic mass. **Figure 3.18** represents the entire process of endospore formation.

3.1.7.5. Transformation

Griffith (1928) was the first who recorded the phenomenon of transformation. The transforming principle was recognised as DNA in the sequential events of transformation of bacteria by **Avery, MacLeod,** and **McCarty (1944)**. **Figure 3.19** represents the entire process of transformation:

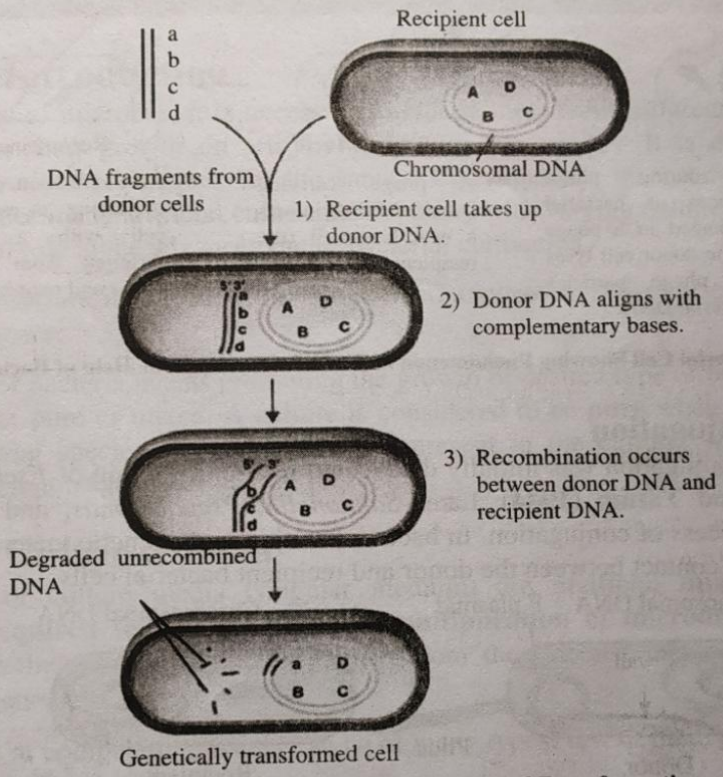


Figure 3.19: Bacterial Cell Showing Phenomenon of Transformation

The process of transformation involves the transfer of genetic material in bacteria. This occurs when the donor cell disintegrates, a recipient cell takes up the DNA molecule which is liberated, and hence some characteristics of the donor cell are inherited by its offspring. As a result of **recombination**, the resulting offspring have the combination of characters of both the parent strains (when different strains of bacteria are found in a mixed state either in nature or in culture). As a result of this process, one kind of bacterium transforms into another type. This is facilitated by the transfer of DNA from a capsulated bacterium into a non-capsulated bacterium, and easily can be seen in genus Diplococcus (**e.g.,** *Diplococcus pneumonia*).

3.1.7.6. Transduction

Transduction process was first observed by **Lederberg** and **Zinder's (1952)** during an experiment using U-tube and *Salmonella typhimurium*. In this, bacterial viruses or bacteriophages are responsible for the transfer of genetic material from one lysogenic and lytic phages to the other. Thus, the genotype of host acquires a new combination. This process occurs in many bacterial species, **e.g.,** *Salmonella*, *Escherichia*, *Micrococcus*, and *Stigella*. **Figure 3.20** represents the entire process of transduction:

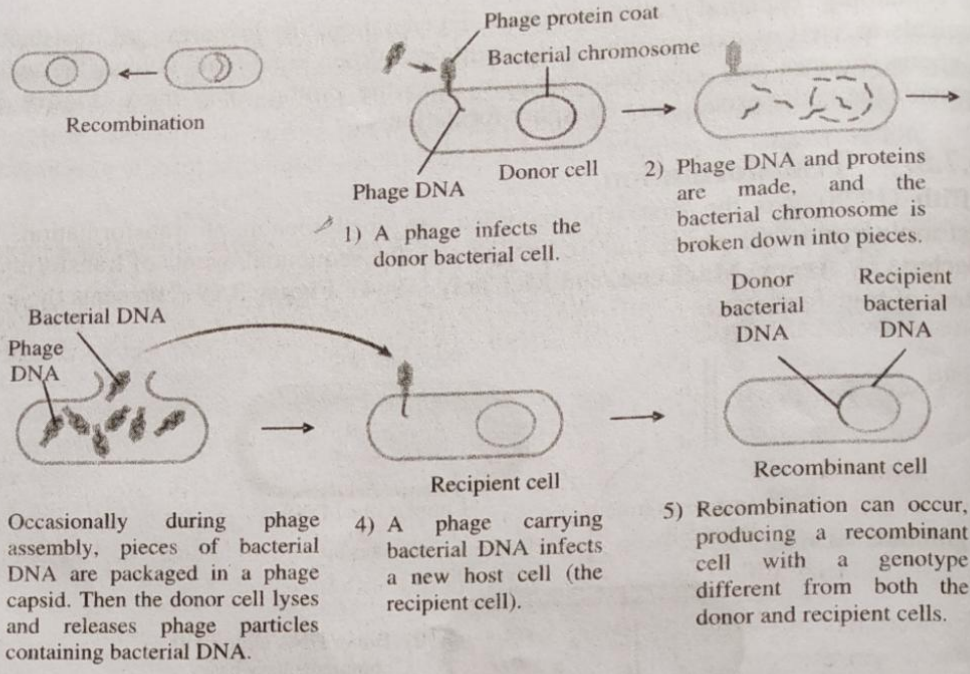


Figure 3.20: Bacterial Cell Showing Phenomenon of Transduction with the Help of Bacteriophage

3.1.7.7. Conjugation

The process of conjugation was initially discovered in a single strain of *Escherichia coli* by **Lederberg** and **Tatum** (**1944**). Later *Salmonella*, *Pseudomonas*, and *Vibrio* also confirmed the process of conjugation. In bacterial conjugation, genetic material (DNA) is transferred by the contact between the donor and recipient bacterial cells.

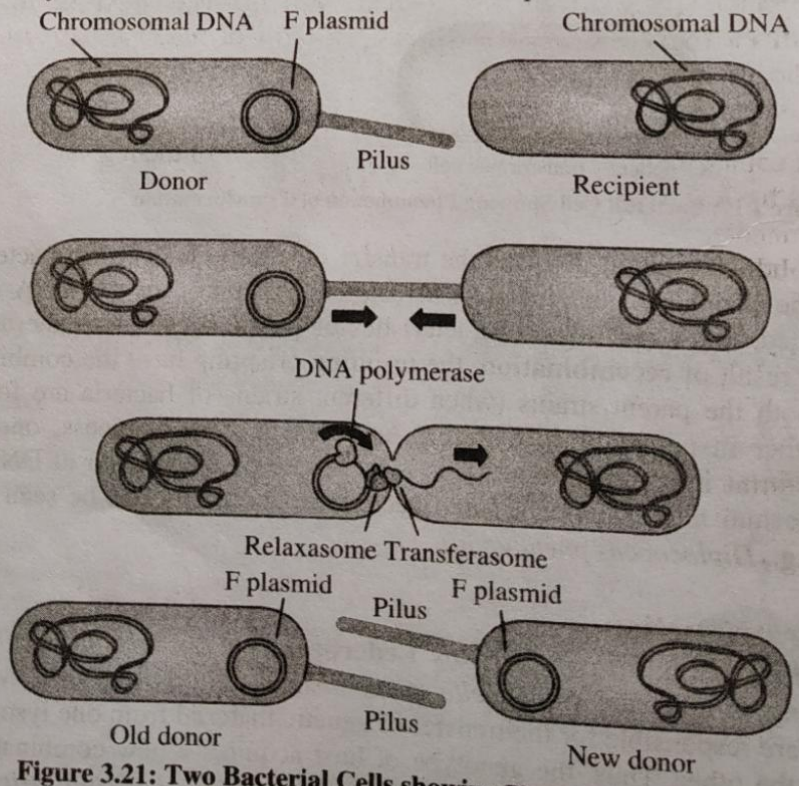


Figure 3.21: Two Bacterial Cells showing Phenomenon of Conjugation

One bacterial strain acts as the donor (male) while the other bacterial strain acts as the recipient (female). A component of the circular DNA (F+ strain) is a **sex factor** or **fertility factor** (F factor), possessed by the donor cells. The recipient cell is denoted as F^- strain, since it does not possess the fertility factor. Conjugation occurs between the cells of F+ and F^- strains, resulting in the formation of F+ bacterial cells in the progeny. **Figure 3.21** represents the entire process of conjugation.

3.2. CULTIVATION AND ISOLATION OF BACTERIA

3.2.1. Introduction

For the study of microbes, it is necessary to isolate them from different natural sources and facilitate their growth on artificial media in laboratories. It is only possible by employing various techniques for the same. Consequently, the needs as well as the development of many artificial culture media, along with varying culture techniques have played a vital role in the advancement of bacterial cultivation.

Microbiologists are using bacterial, fungal, actinomycetes, and viral culture media for various purposes.

Cultivation of bacteria means promoting the growth of desired type of bacteria. A culture may be either pure or mixed. A culture is considered to be **pure** when only a particular single bacterial species (microorganism) is present in the culture; whereas in **mixed culture** different bacterial strains are present.

3.2.2. Culture Media

Nutritional or culture media (singular medium) are **artificial mixture of various nutrients required for the growth and multiplication of microorganisms**. This is required for the growth of microorganism from the infected material to identify the causative agents.

On the basis of composition, media can be of many types; few of them given below:

1) **Based on Physical State**
   - i) Liquid media,
   - ii) Semisolid media, and
   - iii) Solid media.

2) **Based on Presence of Molecular Oxygen and Reducing Substances in Media**
   - i) Aerobic media, and
   - ii) Anaerobic media.

3) **Based on Nutritional Factors**
   - i) Simple media,
   - ii) Complex media,
   - iii) Synthetic media, and
   - iv) Special media:
     - a) Enriched media,
     - b) Enrichment media,
     - c) Selective media,
     - d) Differential media,
     - e) Indicator media,
     - f) Transport media, and
     - g) Sugar media.

3.2.2.1. Raw Materials Used

The raw materials used in culture media are discussed below:

1) **Water:** It is used as a solvent for preparing most of the culture media. Water to be used for the preparation of culture media should be free from substances inhibiting bacterial growth. De-ionised or distilled water are mainly used in the preparation of culture media.

2) **Electrolytes:** These provide basic elements to the cell that are essential to carry fundamental metabolic process, **e.g.,** transport of food molecule, cellular respiration, etc.

3) **Peptones:** These are the water-soluble product obtained from protein hydrolysis. Proteins in meat, milk, and soya bean undergo hydrolysis in the presence of acids or enzymes (pepsin, trypsin, and papain). Peptones are heat stable. Peptones obtained from animal protein are the source of nitrogen, while those obtained from plant (soyabean) protein are the source of carbohydrates in the medium.

   An **ideal peptone** should have the following **characteristics**:
   i) A neutral pH,
   ii) Light in colour,
   iii) Hygroscopic, and
   iv) Large amount of tryptophan for indole production.

   Different forms of peptone are used for different purposes. **For example**, proteose peptone is used in media for toxin production; and tryptone is used for providing amino acids to the media.

4) **Meat Extracts:** These mainly contain degraded protein products; however, inorganic salts, carbohydrates, and growth factors are also present.

5) **Yeast Extracts:** These act as stimulants for bacterial growth in the culture media [especially in Thiosulphate Citrate Bile Salt Sucrose (TCBS) medium].

6) **Mineral Salts:** Bacteria require trace elements, which are obtained from some salts (**e.g.,** sulphur from sulphates and phosphorus from phosphates). In some culture media, traces of magnesium, potassium, iron, calcium, and other elements are also present. All these elements are essential for enzymatic activity. Sodium chloride is also an essential constituent of many media as it actively involves in maintenance of isotonicity.

7) **Carbohydrates:** These are sources of carbon and energy in a culture media. They also have some specific functions; **for example**, lactose is added to Cystine Lactose Electrolyte Deficient (CLED) medium and MacConkey agar for separating the lactose- and non-lactose fermenting organisms.

8) **Blood or Serum:** In few cultivations blood and serum are also required, that are used for enriching the culture media. Usually, 5-10% defibrinated sheep blood is used. Serum is also used in few cases.

9) **Agar:** It is a long-chain polysaccharide prepared from seaweed (Algae–Geladium species). It contains a small amount of protein-like material and a variety of inorganic salts.

3.2.2.2. Types of Media

The following are major types of media commonly used:

1) **Liquid Media:** These media allow the bacteria to move about freely. Bacterial growth in liquid media is indicated by turbidity, and by surface growth in case of some organisms. Liquid media are used for biochemical testing, blood culture,

motility testing, and as enrichment media. Liquid media can be stored in test tubes, bottles, or flasks. The major **disadvantage** of liquid media is that purity of growth is not guaranteed. **Table 3.2** enlists the commonly used liquid media:

Table 3.2: Composition and Uses of Liquid Media

Medium	Composition	Uses
1) **Peptone Water**	Peptone – 1% NaCl – 0.5% Water – 100ml (pH 7.4)	Routine culture, Sugar fermentation tests
2) **Nutrient Broth**	Peptone water + Meat extract – 1%	Routine culture
3) **Glucose Broth**	Nutrient broth + Glucose – 0.5%	Blood culture, Culture of certain organisms, **e.g.,** *Streptococci*
4) **Enrichment Media**		
i) Alkaline Peptone Water	Peptone water (pH 9.0)	Culture of *Vibrio*
ii) Selenite F Broth	Peptone water, Sodium selenite	Culture of faeces for *Salmonellae* and *Shigellae*
iii) Tetrathionate Broth	Nutrient broth, Sodium thiosulphate, Calcium carbonate, Iodine solution	Culture of faeces especially for*Salmonellae*
iv) Robertson's Cooked Meat Broth (RCM)	Nutrient broth, Predigested cooked meat of ox heart	Culture of anaerobic bacteria

2) **Semi-Solid Media:** These media are prepared by using 0.5% or less concentrations of agar. They are of soft custard-like consistency and are used for demonstrating bacterial motility and separating motile strains from the non-motile ones (by using U-tube and Cragie's tube). Some of the transport media (**e.g.,** Stuart's and Amies media) are semisolid in nature. Hugh and Leifson's oxidation fermentation test media and mannitol motility media are also semi-solid.

3) **Solid Media:** Organisms grown on these media, mature and multiply at the inoculation site and form visible colonies. Appearance of colonies and changes occurring in the surrounding media helps in identification of the bacterial species.

By adding a solidifying agent which does not change the nutritional content of the media (**agar** being widely used), liquid media can be made solid. An **ideal agar** should have the following **characteristics**:

i) It should gel at a concentration of 1%,
ii) It should melt at 98°C,
iii) It should set on cooling at 42°C, and
iv) It should easily soluble and maintain clarity in solution.

Gelatin (a protein obtained from the collagen of skin and bone) is another commonly used solidifying agent. It gels at a concentration of 12-15% in nutrient broth, melts at 22°C, and denatures on prolonged exposure to 100°C. Some bacteria liquefy gelatin at 37°C, thus helping in their identification.

Following are the **advantages of agar** over gelatin:

i) It is solid at 37°C,
ii) It can be re-melted once solidified, and
iii) It is bacteriologically inert, thus is not attacked by bacteria.

Table 3.3 enlists the commonly used solid media:

Table 3.3: Composition and Uses of Solid Media

Medium	Composition	Uses
1) **Simple Medium** Nutrient agar	Nutrient broth, Agar (2-3%)	Routine culture
2) **Enriched Media**		
i) Blood agar	Nutrient agar, Sheep blood (5-10%)	Routine culture
ii) Chocolate agar	Heated blood agar (55°C × 2hr)	Culture of *Neisseria* and *H. influenzae*
iii) Loeffler's Serum Slope (LSS)	Nutrient broth, Glucose, Horse serum	Culture of *C. diphtheriae*
3) **Indicator Medium** MacConkey's medium	Peptone, Lactose, Sodium taurocholate, Agar, Neutral red	Culture of Gram negative bacilli
4) **Selective Media**		
i) Deoxycholate citrate agar (DCA)	Nutrient agar, Sodium deoxycholate, Sodium citrate, Lactose, Neutral red	Culture of *Salmonellae* and *Shigellae*
ii) Bile Salt Agar (BSA)	Nutrient agar, Sodium taurocholate (0.5%) pH 8.2	Culture of *Vibrio cholerae*

4) **Anaerobic Media:** These media are utilised for the growth of anaerobic bacteria requiring low oxygen content, reduced redox potential, and extra nutrients. Anaerobic media is enriched with nutrients like hemin and vitamin K. They are also reduced by physical or chemical methods. On boiling the media, dissolved oxygen (if present) is removed. On adding 1% glucose, 0.1% thioglycolate, 0.1% ascorbic acid, 0.05% cysteine, or red hot iron filings, the media gets reduced. The media should be boiled in water bath to remove any dissolved oxygen and then sealed with sterile liquid paraffin prior to use. **For example**,
   i) Robertson cooked meat, commonly used to grow *Clostridium* spp. media.
   ii) Sodium thioglycolate, glucose, cysteine, yeast extract, and casein hydrolysate are present in thioglycolate broth.
   iii) Methylene blue or resazurin is a redox potential indicator that is added in the media. Methylene blue is colourless under reduced conditions.

5) **Simple or Basal Media:** These media support non-fastidious bacteria. Peptone water, nutrient broth, and nutrient agar are some of the **examples**. Simple media are used for the primary isolation of microorganisms.

6) **Complex Media:** All media other than simple media are complex. These media have added ingredients for providing special nutrients essential for bacterial growth.

7) **Synthetic Media:** These media are prepared using pure chemicals, and are used for special studies such as metabolic requirements. Dubo's media with tween 80 is an **example**.

8) **Enriched Media:** These media are used for growing nutritionally fastidious bacteria. On adding extra nutrients (in the form of blood, serum, egg yolk, etc.), basal media becomes enriched media. Some **examples** of enriched media are:
   i) **Blood Agar:** It is prepared by adding 5-10% (by volume) to a basal media such as nutrient agar or other blood agar bases. Since sterilisation of blood is not possible, it should be collected from the animal aseptically. Animals are made to bleed and the blood is collected in sterile containers having anticoagulant or glass beads. Blood from sheep, rabbit, horse, and ox are preferably used. Human blood is not used as it may contain antibiotics (which inhibit bacterial growth).

After autoclaving the blood agar base, blood is added to the media at temperature above the solidifying point of agar. The mixture is then poured on the plates and solidified. Blood agar demonstrates the haemolytic properties of certain bacteria.

ii) **Chocolate Agar:** It is also known as **heated** or **lysed blood agar**, and is prepared in the same manner as the blood agar with the exception that blood is added to hot molten blood agar base. This results in the lysis of blood cells, thus releasing their contents in the medium and turning it brown (hence named so). Chocolate agar is used for cultivating *Hemophilus sp.* and *Neisseria sp.* Animal blood is used for obtaining serum for medium which should be filtered through membrane or seitz filter prior to use.

9) **Enrichment Media:** These media is used as broth media to increase the relative concentration of certain microorganisms in the culture prior to plating on solid selective medium. Enrichment media are liquid media that also inhibit commensals (contaminating organisms) in the clinical samples. Selenite F broth, tetrathionate broth, and alkaline peptone water are used for recovering pathogens from faecal samples.

10) **Selective Media:** These media inhibit the unwanted commensals or contaminating bacteria. They also recover pathogens from a mixture of bacteria. On adding certain inhibitory agents which do not affect the desired pathogens, the agar media can be made selective. Addition of antibiotics, dyes, chemicals, alteration of pH, or a combination of these are other approaches by which any media can be made selective. **For example,**

i) **Thayer Martin Agar** containing antibiotics (vancomycin, colistin, and nystatin) is used to recover *N. gonorrhoeae*.

ii) **Mannitol Salt Agar** and **Salt Milk Agar** containing 10% NaCl is used to recover *S. aureus*.

iii) **Potassium Tellurite Media** containing 0.04% potassium tellurite is used to recover *C. diphtheriae*.

iv) **MacConkey's Agar** containing bile salt that inhibits gram-positive bacteria is used to recover *Enterobacteriaceae* members.

v) **Pseudosel** or **Cetrimide Agar** containing cetrimide (antiseptic agent) is used to recover *P. aeruginosa*.

vi) **Crystal Violet Blood Agar** containing 0.0002% crystal violet is used to recover *S. pyogenes*.

vii) **Lowenstein Jensen Medium** made selective by adding malachite green is used to recover *M. tuberculosis*.

viii) **Wilson and Blair's Agar** made selective by adding dye brilliant green is used to recover *S. typhi*.

ix) **TCBS Agar** (selective media) having elevated pH (8.5-8.6) is used to recover *V. cholerae* from fecal samples.

11) **Differential Media:** These media have substances which distinguish the differing characteristics of bacteria. **For example,**

i) **Mannitol salts agar** (mannitol fermentation forms yellow colonies).

ii) **Blood agar** (various kinds of haemolysis, i.e., α, β, and γ haemolysis).

iii) **MacConkey's agar** (lactose fermenter forms pink colonies, while non-lactose fermenter forms pale or colorless colonies).

iv) **TCBS** (sucrose fermentation forms yellow colonies of *Vibrio cholerae*).

12) **Indicator Media:** These media have an indicator which changes colour on bacterial growth. **For example,**
   i) **MacConkey's agar** contains bile salts (selective agent), lactose (sugar), peptone, neutral red (pH indicator), agar, and water. This media is commonly used for culturing and identifying gram-negative bacilli (especially *Enterobacteriaceae* members). Lactose fermenting bacteria form pink colonies, while non-lactose fermenting bacteria form colourless colonies on this medium.
   ii) **TCBS medium** containing sucrose develops yellow colonies of *Vibrio cholerae*.
   iii) **KT agar** develops black colonies when potassium tellurite is reduced to metallic tellurium by *Corynebacterium diphtheria*.
   iv) **Wilson and Blair's medium** develops black colonies when H_2S is produced by *Salmonella typhi*.
   v) **Bile esculin agar** develops black colonies when esculin is reduced to esculetin by *Enterococcus fecalis*.

13) **Transport Media:** These media are used for immediately transporting the collected clinical samples to the laboratory to prevent overgrowth of commensals. These media prevent drying (desiccation) of sample, maintain the pathogen to commensal ratio, and inhibit overgrowth of unwanted bacteria. **Stuart's** and **Amie's transport media** are semisolid in nature. On adding charcoal, the inhibitory factors are neutralised. **For example,**
   i) **Cary Blair transport medium** and **Venkatraman Ramakrishnan** (VR) **medium** are used to transport faeces from suspected cholera patients.
   ii) **Sach's buffered glycerol saline** is used to transport feces from suspected bacillary dysentery patients.
   iii) **Pike's medium** is used to transport *Streptococci* from throat samples.

14) **Sugar Media:** These media are used for identifying bacteria. The term **sugar** in microbiology denotes a **fermentable substance**. Glucose, lactose, sucrose, and mannitol are used for fermentation tests. Sugar media contain 1% sugar in peptone water and Andrade's indicator (0.005% acid fuchsin in 1N NaOH). Gas production can be detected using a small Durham's tube which is kept inverted in the larger sugar tube. As acid is produced by the bacteria, the colourless media turns pink; and production of gas is indicated when gas bubbles accumulate in the Durham's tube. Certain bacteria demand serum for their growth, **e.g.,** Hiss's serum sugars for *Pneumococcus*.

3.2.2.3. Preparation

Culture media are either prepared in laboratories using dried or dehydrated products, or commercially available ready-made media are used. Culture media in routine laboratories are no longer prepared using raw materials. The reason for using dehydrated products or ready-to-use culture media are:

1) They can be easily prepared,
2) They perform well,
3) They are highly standardised,
4) They are reproducible, and
5) They are economic.

Before the culture medium is autoclaved, its pH should be adjusted using pH indicators (**e.g.,** phenol red, neutral red, bromothymol blue, bromocresol purple, etc.). Commercially available dehydrated media should be reconstituted as per the

manufacturers' recommendation. The culture media are mostly sterililised by autoclaving; however, media containing thermolabile components (such as glucose, antibiotics, urea, serum, and blood) are not autoclaved, but the components are filtered or are added after the media has been autoclaved. Some highly selective media (**Wilson and Blair's medium** and **TCBS agar**) are not sterilised. A representation from each lot should be tested for performance and contamination before use. After preparation, the media are refrigerated for 1-2 weeks at 4-5°C. Some liquid media are stored at room temperature for weeks in screw-capped bottles or tubes, or cotton plugged.

3.2.3. Physical Parameters for Growth

The physical conditions maintained for bacterial growth in culture media are:

1) **Heat Treatment:** Spore-forming bacteria, **e.g.**, *Bacillus* spp., *Lactobacillus* spp., etc., require heat application. A mixed culture, heated at 80°C for 10 minutes can be used to choose the bacteria forming endospore, before being used for inoculation of the culture medium. This leads to survival of spore-forming bacteria, while killing the others (vegetative cells).

2) **Incubation Temperature:** For efficient growth, most microbes require a particular favourable temperature, known as **optimum temperature**. Incubation of cultures at very low temperatures ranging from 0-5°C helps in the identification of psychrophilic or psychrotrophic bacteria. Such low temperatures kill mesophilic (20-40°C) and thermophilic (45°C and above) microbes, facilitating the survival of only psychrophilic bacteria.

3) **pH:** It is an essential factor influencing microbial growth. Acidic pH (below 7) facilitates the growth of most *Lactobacilli* spp., while alkaline pH is required for the growth of many other bacteria, **e.g.**, *Vibrio cholera*. The optimum pH to grow most of bacteria ranges between 6.5 and 7.5 (i.e., near neutrality).

4) **Cell Size and Motility:** Microbes have mostly a definite size and are motile. Due to its size a *Treponema* of size 0.15mm is isolated by placement of gingival scrapings on the surface of an agar plate. *Treponema* penetrates the filter and swims through the agar medium which is solid in nature. The presence of *Treponema* in agar is indicated by a hazy appearance.

5) **Effect of Oxygen:** Approximately 21% oxygen is required by aerobic bacteria, in contrast to the anaerobic bacteria that do not require oxygen for their growth (as oxygen proves toxic to them). However, the growth of facultative anaerobic bacteria is not inhibited by oxygen. Though oxygen is required in very small amount for microaerophilic bacteria to grow, yet they are unable to tolerate the amount of oxygen present in the atmosphere, because the enzymes get inactivated at those oxygen levels. On the contrary, chemical reactions involving oxygen in molecular forms are catalysed by several cellular enzymes. At times, super oxide radical (O_2) is formed by adding a single electron to an oxygen molecule.

$$O_2 + e^- \longrightarrow O_2^-$$

Also, production of chemical substances that are even more toxic, **e.g.,** hydrogen peroxide (H_2O_2) and hydroxyl radical, inactivates cellular components.

$$2O_2 + 2H^+ \longrightarrow O_2 + H_2O_2$$

$$O_2^- + H_2O_2 \longrightarrow O_2 + OH^- + OH$$

Facultative microbes and aerobic bacteria have protective mechanism against oxygen due to the presence of super oxide dismutase. The H_2O_2 produced can be dissipated by the enzymes **catalase** and **peroxidase**.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

$$H_2O_2 + \text{reduced substrate} \xrightarrow{\text{Peroxidase}} 2H_2O + \text{oxidised substrate}$$

6) **Energy:** Microorganisms are divided into the following categories based on their energy source:
   i) **Photoautotrophs:** These microorganisms utilise light as their energy source and CO_2 as carbon source, **e.g.,** higher plants, algae, and photosynthetic bacteria.
   ii) **Photoheterotrophs:** These microorganisms utilise light as their energy source and an organic compound as the carbon source, **e.g.,** certain purple and green bacteria.
   iii) **Chemoautotrophs:** These microorganisms utilise a chemical energy source and CO_2 as the carbon source. They are also termed **chemolithotrophs** (*lithos* - rock or stone) as they grow in the absence of light in mineral media.
   iv) **Chemoheterotrophs or Chemoorganotrophs:** These microorganisms utilise a chemical energy source and an organic substance as the carbon source. They are the commonest of all the nutritional types.

7) **Temperature:** Most of the microorganisms grow well at **human body temperature**, i.e., 37°C. Based on their preferred temperature range, microorganisms are categorised into **psychrophiles** (cold-loving), **mesophiles** (moderate temperature-loving), and **thermophiles** (high temperature-loving). Mesophiles are the most common type and include most of the common spoilage and disease producing organisms as they have an optimum growth temperature of 30-45°C. The minimum, maximum, and optimum temperature ranges for the growth of various bacteria are given in **table 3.4**:
   i) **Minimum Growth Temperature:** It is the lowest temperature for the growth of microorganisms.
   ii) **Maximum Growth Temperature:** It is the highest temperature for the growth of microorganisms.
   iii) **Optimum Growth Temperature:** It is the temperature at which the microorganisms multiply rapidly.

Table 3.4: Temperature Ranges for Bacterial Growth

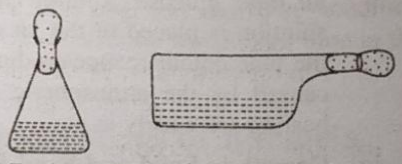
Group	Growth Temperature (°C)		
	Minimum	Maximum	Optimum
1) Psychrophiles			
i) Obligate	5 and below	19-22	15-18
ii) Facultative	5 and below	30-35	25-30
2) Mesophiles	10-15	35-47	30-45
3) Thermophiles	40-45	60-85	55-75

8) **Osmotic Pressure:** Microorganisms grow and thrive well in **isoosmotic** (osmotic pressure similar to that within the bacterial cell) environment, and not in **hyperosmotic** (osmotic pressure higher than that within the bacterial cell) or **hypoosmotic** (osmotic pressure lower than that within the bacterial cell) environment. The terms isoosmotic, hyperosmotic, and hypoosmotic are used synonymously with isotonic, hypertonic, and hypotonic, respectively. Bacterial growth is inhibited by both higher and lower osmotic pressure environments.

3.2.4. Cultivation of Bacteria (Aerobes and Anaerobes)

To cultivate a specific microbe, following methods are used:

1) **Methods of Culturing Aerobic Bacteria:** An atmosphere having oxygen in ample amount facilitates the growth of aerobic or facultative bacteria. For this, a large surface area of medium is used to increase the exposure. Numerous devices, **e.g.,** Fernbach flask, Kolle flask, and Roux bottle are used to provide increased aeration (**figure 3.22**). Oxygen is required for the growth of aerobic bacteria and hence they can easily be grown by incubation in surroundings containing air (i.e., 21% oxygen).



(a) Fernback flask (b) Roux bottle

Figure 3.22: Culture Vessels Used for the Growth of Aerobic Bacteria

Generally, incubation of medium under normal atmospheric conditions proves satisfactory, when the growth of aerobic or facultative bacteria is desired in tubes or small flasks. However, when the growth of these microbes is desired in huge quantities, increasing the exposure of medium to the atmosphere proves beneficial. To achieve this, distinctive containers are designed, such that they dispense the medium in shallow layers, thereby facilitating their growth in increased quantities. Constant shaking of inoculated liquid cultures that have been inoculated also increases the exposure to air.

2) **Methods of Culturing Anaerobic Bacteria:** There are two categories of methods by which bacteria can be cultivated under anaerobic conditions; methods in which culture media are placed in air-tight containers and oxygen is removed (**i-iii**), using media in which oxygen diffuses with difficulty (**iv**), and methods in which a non-toxic reducing agent is added to the culture media (**v-vii**). These methods are discussed below:

   i) **Boiling:** In this technique, the culture media is taken in bottles fitted with a screw cap having a central aperture and a rubber washer. The bottle is then placed in a water bath after loosening the screw cap, and boiled for 10 minutes. Then the bottle is cooled after tightening the screw cap. The microorganism is inoculated using a hypodermic syringe, the needle being plunged through the rubber washer.

   ii) **Use of Alkaline Pyrogallol:** After filling a desiccator with alkaline pyrogallol, culture media are placed in it for oxygen removal. The method can be modified by using a petri dish having a double compartmented lid; one compartment contains sodium hydroxide solution and the other contains pyrogallol solution. The microorganism to be grown is streaked on an agar plate, which is then placed downwards over the lid, the two solutions are gently mixed, and the assembly is incubated.

   iii) **McIntosh and Fildes Jar:** This jar (made up of strong glass) is fitted with a gas-tight metal lid securely clamped in place. This lid has two terminals for conveying electric current to a coil within the jar; the coil further provides heat to a piece of spongy palladium deposited on asbestos and surrounded with wire gauze. Inlet and outlet taps are also present in the lid. When the jar is to be used, the culture media are placed within; hydrogen is passed through the taps; current is switched on to heat the spongy palladium; and the oxygen present combines with hydrogen in the presence of the heated catalyst. Care should be taken that the reaction does not become explosive by the external gauze, and thus the jar is placed in a protective box.

Hydrogen is passed through the taps for further 30 minutes; after which the taps are closed and the jar is incubated at suitable temperature. An indicator of anaerobiosis consisting of a mixture of methylene blue and glucose, and made alkaline with sodium hydroxide is added in the jar. This is heated when the alkaline glucose solution turns methylene blue colourless. This colourless solution is placed in the jar and should remain colourless during incubation; if the blue colour reappears during incubation, the jar is believed to have leakage caused by the atmospheric oxygen. Nowadays, catalysts not requiring prior heating are widely in use.

iv) **Growth in Solid and Semi-Solid Media:** Under anaerobic conditions, the solid media are poured in a test tube and the microorganisms are inoculated at the bottom using a long wire; this is known as a **stab culture**. Oxygen is removed by boiling it off when the media are sterilised in the tube. Diffusion of this oxygen to the bottom of the tube is prevented, owing to the height and nature of the layer medium above. The solid culture media were modified using a nutrient broth in which agar was added in a quantity sufficient to make it semi-solid; this is called **sloppy agar**. These media are heated and cooled before inoculation. Anaerobic conditions should be maintained at the container bottom, and the viscosity of the media should be such that diffusion of oxygen is prevented.

v) **Metallic Iron:** It is used in the form of wire or small iron nails for producing anaerobiosis. The media are heated for 10 minutes on a boiling water bath. Iron is added to the media after sterilisation by heating for 45 seconds in a bunsen flame. The normal aerobic media used for studying sugar decomposition by aerobes and facultative organisms can be adapted for studying their decomposition by anaerobes.

vi) **Cooked Meat:** Sufficiently minced and cooked muscle of ox-heart is added to a conventional nutrient broth placed in a 30ml container having a screw cap. The meat should form a 1.5cm deep layer at the bottom of the liquid media. Muscle tissue contains two reducing systems; in one system reduction occurs by irreversible oxidation of lipids, and other is the reversible parahaematin-haemochromogen system. **Robertson** created this medium, therefore it is also known as **Robertson's cooked meat medium**.

vii) **Thioglycollic Acid:** Since 1926, thioglycollic acid ($CH_2SHCOOH$) is being used as an additive to nutrient broths for cultivating and detecting anaerobes.

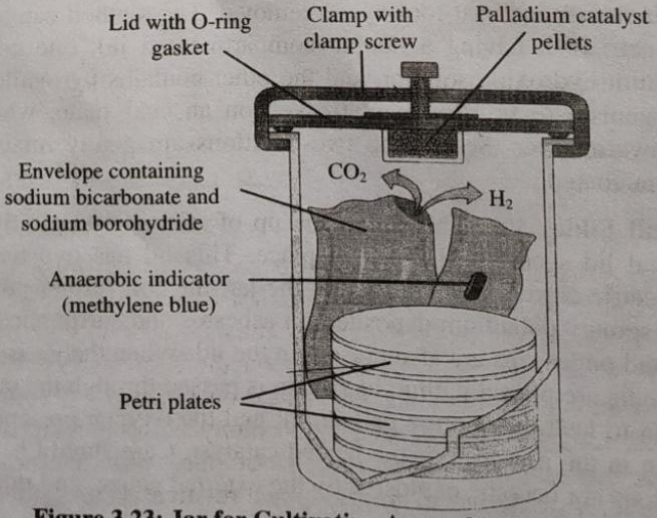


Figure 3.23: Jar for Cultivating Anaerobic Bacteria on Petri Plates

Gaspak method is used for preparing anaerobic jars (**figure 3.23**). Gaspak is commercially available as a disposable envelope. It contains chemicals generating hydrogen and carbon dioxide on addition of water. The envelope also contains a cold catalyst which allows hydrogen to combine with oxygen for producing an anaerobic environment. Reduced methylene blue used for verifying the anaerobic condition within the jars remains colourless under anaerobic conditions while turns blue in the presence of oxygen.

3.2.5. Isolation Methods for Pure Cultures of Bacteria

Culture is a procedure during which microorganisms are cultivated in a medium; if a culture has the population of a single type of microorganism; it is known as a **pure culture**.

Usually, isolation and growth of pure cultures of single-celled microbes carried under aseptic conditions needs sterilised instruments in addition to filtered and still air. The growth of microbes on the surface of a petri dish usually provides their isolated colonies. An appropriate growth medium, commonly gelled with agar is present in a petri dish (or plate) to facilitate the growth of desired microbe. Manipulation of the inoculum (initial sample) with an inoculation loop or needle is done, so that the cells are diluted and allowed to spread on the surface of the plate to achieve the isolation of a pure culture.

The objective of this procedure is to ultimately obtain some areas on the petri dish that have isolated single cells. Incubation of the culture under suitable environmental settings is carried out till the cells grow and bacterial colonies become visible. Colonies that have been isolated properly have maximum chances of being cultivated from single cells, and therefore they are regarded as pure cultures. A pure culture can also be prepared by high dilution from a liquid culture into a liquid medium. At sufficient dilution only a fraction of the inoculated culture tubes grow, and there are high chances that these cultures are originated from a single cell.

3.2.5.1. Streak-Plate Technique

In this method, a fraction of the mixed culture is placed on the surface of a medium containing agar and is streaked across the surface using a transfer loop. As a result of this manipulation, the bacterium present on the surface of agar 'thins out', facilitating the separation of some individual bacteria from each other. Different methods of streaking a culture plate to obtain isolated colonies are illustrated in **figure 3.24**.

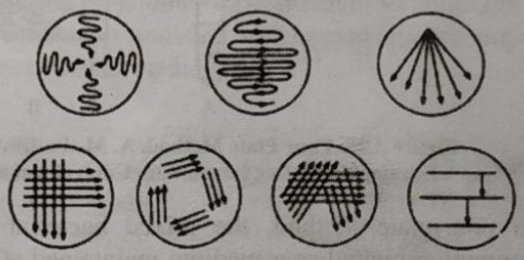


Figure 3.24: Various Methods of Streaking

It is recommended that second plate should be streaked using the same loop/needle without re-inoculation. These plates are then incubated for allowing the colonial growth. This method relies on the **principle** that streaking establishes a dilution gradient across the face of the petri plate as bacterial cells deposit on the agar surface. Due to this dilution gradient, confluent growth does not take place on the part of medium where bacterial cells are deposited. It is assumed that each colony is the progeny of a single microbial cell, thus each colony represents a clone of pure culture. These isolated colonies are separated with a sterile inoculating loop and re-streaked on a fresh medium to maintain purity.

Advantages

1) Numerous bacterial cells spread over the medium surface.
2) Individual bacterial cells deposit at some distances from each other.
3) Distinct colonies are formed, which do not touch each other.
4) Cloning of single bacteria of pure culture is possible.

3.2.5.2. Pour-Plate Technique

In this method, diluted samples mixed with melted agar medium are plated (**figure 3.25**). This method relies on the **principle** that the inoculum is diluted in successive tubes containing liquefied agar medium for thorough distribution of bacterial cells in the medium.

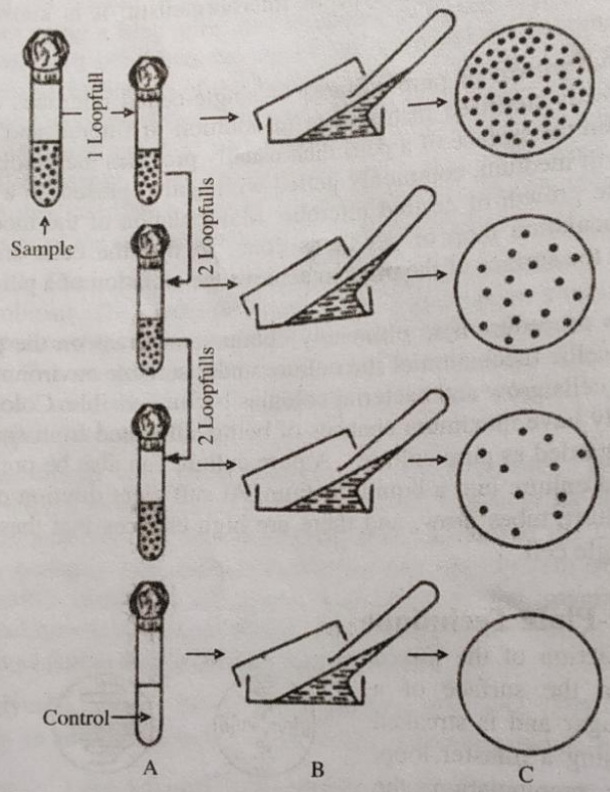


Figure 3.25: Pour Plate Method. A. Media/Dilution; B. Pouring of the Plate; and C. Colony Development after Incubation. Control consists of the Sterilised Plating Medium Alone

In pour-plate method, the mixed bacterial culture is diluted directly in test tubes containing melted agar medium maintained at 42-45°C (agar solidifies below 42°C). The bacteria and the melted medium in tubes are mixed properly.

The contents of each tube are poured in separate petri plates, solidified, and then incubated. On development of bacterial colonies, it is observed that isolated colonies develop within the agar medium (sub-surface colonies) as well as on the medium (surface colonies). These isolated colonies are picked up by inoculation loop and streaked on another petri plate to maintain purity.

The pour plate method has following **disadvantages**:

1) The sub-surface colonies are dug out of the agar medium, thus disturbing the other colonies.

2) The isolated bacteria should resist temporary exposure to 42-45°C temperature of the liquid agar medium, thus this method is not preferred for isolating psychrophilic microorganisms.

However, the pour-plate method apart from being used for isolating pure cultures is also used for estimating the number of bacterial cells viable in a culture.

3.2.5.3. Spread-Plate Method

In this method (**figure 3.26**), unlike the pour-plate technique the mixed culture or microorganism is not diluted in the melted agar medium; but diluted in a series of test tubes containing sterile liquid (water or physiological saline).

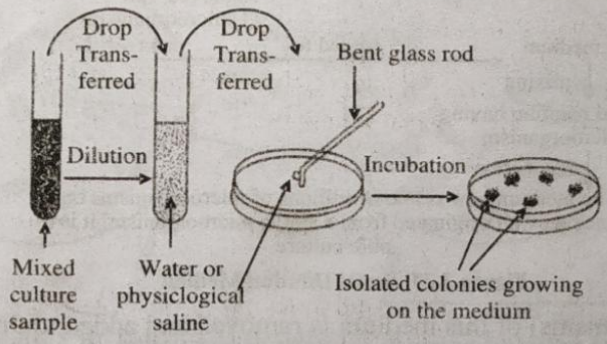


Figure 3.26: Spread Plate Method

A drop of diluted liquid from each tube is placed on the centre of an agar plate and spread uniformly on the surface using a sterilised bent glass rod. Thereafter the medium is incubated.

After the development of bacterial colonies on the agar plates, it is observed that in some plates well-isolated colonies have grown. This is because when the drop of diluted liquid is spread on the agar plate, individual microorganisms separate out.

The isolated colonies are picked up and transferred on another fresh medium to maintain purity. In this method, only surface colonies develop and the microorganisms are not required to withstand the temperature of the melted agar medium.

3.2.5.4. Serial Dilution Method

This method is used for obtaining pure cultures of those microorganisms that grow only in liquid media and are still to be cultivated on solid media.

A series of dilutions aids in isolating a pure form of microorganism, from a mixed culture. The inoculum is undergone serial dilution in a sterile liquid medium, and a large number of tubes containing sterile liquid medium are inoculated with aliquots of each successive dilution.

Serial dilution aims to inoculate a series of tubes containing such dilute microbial suspension that some tubes show growth of only one individual microbe.

Let us assume a culture of 10ml liquid medium containing 1,000 microorganisms (**figure 3.27**), i.e., 100 microorganisms/ml of the liquid medium.

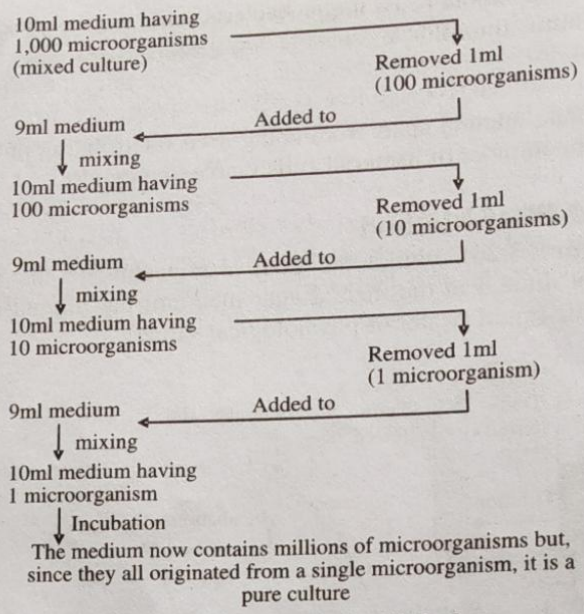


Figure 3.27: Serial Dilution Method

If 1ml (100 microorganisms) of this medium is removed and added to 9ml of fresh sterile liquid medium, 10ml medium with 100 microorganisms (i.e., 10 microorganism/ml) is obtained. If again 1ml (10 microorganisms) of this suspension is mixed with 9ml of another fresh sterile liquid medium, 10ml medium with 10 microorganisms (i.e., 1 microorganism/ml) is obtained.

If this tube shows microbial growth, it is the result of introducing a single microorganism in the medium; and it is the pure culture of that microorganism.

3.2.5.5. Single Cell Isolation Methods

In this method, an individual desired cell is picked up from the mixed culture and allowed to grow. For this purpose following **two methods** are used:

1) **Capillary Pipette Method:** In this method (**figure 3.28**), a few small drops of a dilute culture medium are placed on a sterile glass-cover slip using a sterile pipette drawn to a capillary. Each drop is then microscopically examined until a drop containing only one microorganism is found. This drop is picked up using a sterile capillary pipette and added to a fresh medium, where the individual microorganism starts multiplying to yield a pure culture.

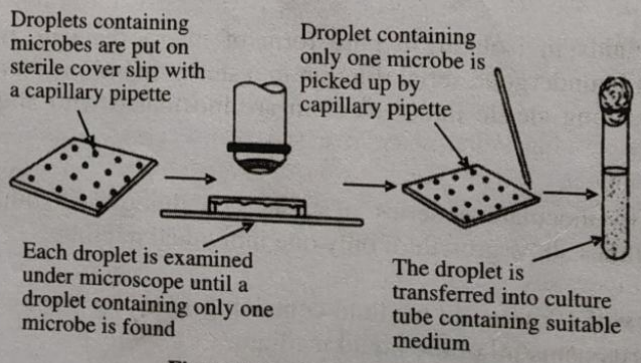


Figure 3.28: Capillary Method for Obtaining a Single Microbial Cell

2) **Micromanipulator Method:** A single bacterial cell can be picked from a mixed culture by using the device 'micromanipulator' under the microscope. The micromanipulator offers the advantage of controlling the movements of a **micropipette** or a **microprobe** (a fine needle) to its operator, for isolation of a single cell (**figure 3.29**). The use of a microscope shows the point where the microprobe touches the agar gel close to a bacterial cell, thus the microbe floating in the small amount of water exude from the gel. Next, the microscope stage is moved to the left, while the microprobe remains fixed; this allows the bacterium to follow the probe and become separated from the other cells. The agar block is subsequently dissected and the small piece containing the isolated bacterium is transferred to a sterile culture medium.

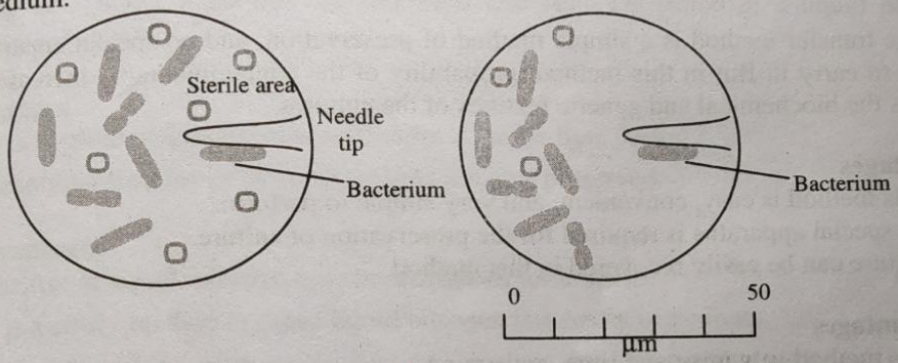


Figure 3.29: Schematic Illustration showing Isolation of a Single Bacterium from a Mixture of Cells on an Agar Gel

The **advantages** of this method are that the cultures are obtained from a single cell and strains within the species can be obtained. The **disadvantages** are that the equipment is expensive, its operation is tiresome, and it requires trained operators, thus this method is used in highly specialised cases.

3.2.6. Preservation Methods for Pure Cultures

The viability and purity of the microbe is necessary once it has been isolated and has been grown in the pure culture. This can be fulfilled by preserving the pure cultures from contamination. In laboratories for continuous growth, viability, and maintaining purity of microorganism, the microbial culture is transferred periodically onto or into a fresh medium (sub-culturing). The transfer of subject should be done under aseptic conditions to prevent contamination.

Keeping the culture away from the contamination and maintenance of its purity is really a tough task; but it is essential as repeated sub-culturing is time-consuming and there is a risk of genetic changes. For such reasons, methods which do not require regular sub-culturing are developed.

Preservation of microbial culture is required in all microbiology laboratories, **e.g.,** fermentation industry, brewing industry, culture collection centres, pathological labs, etc. To maintain the culture, one of the following **methods of preservation** can be used:

1) Periodic transfer of fresh media,
2) Oil overlay method,
3) Storage at low temperature,
4) Storage in sterile soil,
5) Lyophilisation or freeze drying,
6) Cryopreservation, and
7) Storage in silica gel.

3.2.6.1. Periodic Transfer of Fresh Media

Almost in all microbiology laboratories, microorganisms are preserved on agar slants. The agar slant is incubated for one or more days (as required), and then stored at low temperature in refrigerators. This agar slant media is continuously transferred to a fresh media on a definite time interval.

The time period at which the transfers are made to fresh media depends on the microorganisms and the conditions of growth. This time interval may range from 1 day or 1 week to 6 months. The procedure of transfer of media is performed, as long as the preservation of culture is required.

Periodic transfer method is a simple method of preservation, and no special apparatus is needed to carry it. But in this method, probability of the contamination is increased that changes the biochemical and genetic features of the cultures.

Advantages

1) This method is easy, convenient, and very simple to perform.
2) No special apparatus is required for the preservation of culture.
3) Culture can be easily recovered in this method.

Disadvantages

1) This method is tedious and time-consuming.
2) If the culture is not transferred at a given period of time, then mites may develop in it.
3) Biochemical characteristics and genetic features of microbes may change.
4) Risk of contamination is increased in this method.

3.2.6.2. Oil Overlay Method

This method was first used in **1947** by **Buell** and **Weston**, and is almost similar to periodic transfer method; however differs only in usage of paraffin oil in this method (thus the name 'oil overlay'). The oil used in the preservation of culture must have specific gravity in the range of 0.865 to 0.890.

The oil to be used should be sterilised by dry heat sterilisation method in a hot air oven. The sterilised oil is poured over the culture containing nutrient agar slant, up to 1cm above the slant. The agar slant is stored at low temperature or room temperature.

The water-loss as well as the gas exchange, taking place within the organism, and its surrounding becomes slow by the addition of oil.

Advantages

1) This method is used preferably for anaerobic organisms.
2) Growth of mites and other insects is not seen in this method.
3) It is a convenient and simple technique.

Disadvantages

1) Some microorganisms lose their ability to form spores.
2) Due to the periodic transfer of culture, chances of variations in genetic and biochemical characteristics of microbes increase.

3.2.6.3. Storage at Low Temperature

This method of preservation is preferred for short time preservation. In this method, refrigerators or cold rooms are used at the temperature of 4°C. If long period preservation is required, sub-culturing of the culture is essential.

Use of liquid nitrogen is another method for long term preservation of culture. The liquid nitrogen method of preservation can be used successfully for several microbes; in this method cells remain viable for 10-30 years without changing their property.

Procedure

A dense suspension of microbes is prepared in a medium that have a protective agent (**e.g.,** glycerol or dimethyl sulphoxide), which is helpful in prevention of cell damage due to the formation of ice crystals.

The cell suspension is sealed into the small vials or ampoules and kept for controlled freezing at −150°C. After this the ampoules and vials are stored in a liquid nitrogen refrigerator (−196°C).

Advantages

1) This method maintains preservation for a longer time period.
2) Genotypic stability of microorganisms remains preserved.

Disadvantages

1) The use of liquid nitrogen may be hazardous for humans.
2) It is a costly method because liquid nitrogen is a costly ingredient.

3.2.6.4. Storage in Sterile Soil

Sterile soil method is preferred for spore-forming microbes, **e.g.,** species of *Streptomyces*, *Aspergillus*, *Bacillus*, *Penicillium*, etc. This method involves storage of pure cultures (spores) of microbes in a medium of sterile soil followed by its preservation by refrigeration up to several months.

Procedure

1) A suspension containing spores is prepared in a special medium.
2) A mixture containing sand (78%), soil (20%), and calcium carbonate (2%) is prepared and distributed into tubes (a few grams per tube). This mixture is sterilised at a temperature of 130°C for 8-15 hours.
3) In the sterile mixture (prepared in step 2), the spore suspension (prepared in step 1) is added and the mixture is allowed to grow for around 10 days.
4) Mixture containing tubes are inoculated under vacuum in a desiccator. This is done to allow the evaporation of excess water, and after this the tubes are sealed for preservation.

3.2.6.5. Lyophilisation

Lyophilisation method was developed and first used by **Raper** and **Alexander** of the **National Research Laboratory**, at Peoria. In this technique, water is removed from a freezed product and is placed under a vacuum, allowing the ice to change directly from solid to vapour without passing through a liquid phase (process of either evaporation or sublimation); therefore this technique is also known as **freeze-drying**.

Principle

This method is preferred for those microbes that are killed by regular drying process. A suspension of dense cells is placed in small vials and frozen at temperature of –60 to –78°C. Immediately after maintaining the temperature, the vials are connected to a high

vacuum line. The ice present in the frozen suspension is evaporated (sublimes) under the pressure due to the vacuum. This procedure results in dehydration of bacteria without damaging the delicate cell structures adversely.

Procedure

1) The bacterial cells are allowed to grow in a petri dish with a suitable media.
2) 4ml of 10% skimmed milk is overlaid on the bacterial growth to cover it.
3) This skimmed milk is a suspending fluid that must be sterilised at 121°C for 15 minutes. The skimmed milk can be replaced with inositol broth (sterilised at 121°C for 15 minutes) or inositol serum (sterilised by filtration). Suspending agents are used for protection of bacteria from mechanical or chemical damages as well as for prevention of over-drying.
4) Previously, cotton-plugged and autoclaved glass ampoules (110mm × 5.5mm) are filled with 0.2ml of suspension (suspending fluid with bacteria).
5) These ampoules are placed in a rotating mixture of dry ice (solid CO_2) and alcohol; and the temperature of this mixture is maintained up to –78°C.

Drying of the media is performed in **two steps**:

1) **Primary Drying:** Ampoules are placed into the drying cabinets of freeze dryers and the vacuum pump is started to remove all water from the frozen suspension.

   Constriction of Ampoules and Packing

   With the help of a sterile pipette, a cotton plug is pushed in a downward direction up to half the length of the ampoules. A short capillary of 2mm in diameter is created by constricting the ampoules slightly above the cotton plug, by a narrow flame. Care should be taken that the surface of the cotton plug does not undergo partial burning and blackening (charring).

2) **Secondary Drying and Sealing:** During secondary drying, moisture content of the media should be decreased up to 1%. Sealing of ampoules should be carried out under vacuum with precautions, under the vacuum.

The lyophilised ampoules are stored in dark at the freezing or room temperature (**figure 3.30**).

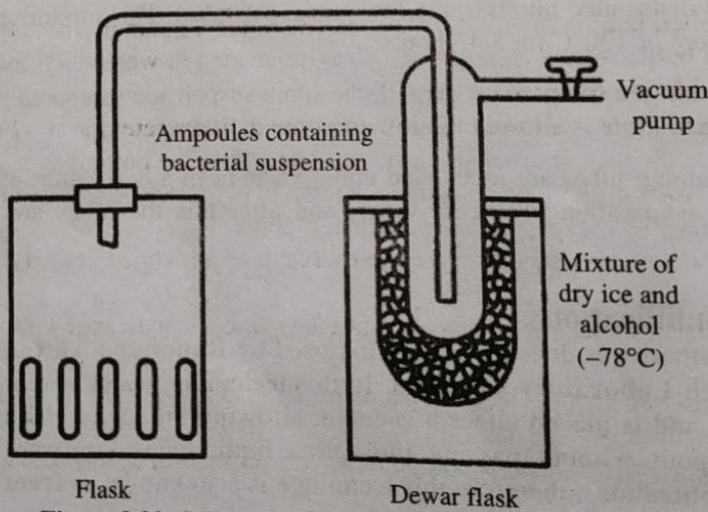


Figure 3.30: Schematic Diagram of Lyophilisation

Advantages

1) By lyophilisation, microbes can be preserved in sustainable and viable form for a long period, i.e., up to 20 years, without sub-culturing.

2) Genetic stability is maintained in the lyophilised strain. Risk of contamination is minimised in this process; and there is no need of very low temperature.
3) Preservation of ampoules utilises very less space and can be kept in small areas. Also, it is safe to carry the ampoules from one place to the other.
4) The process of lyophilisation can also be used to preserve proteins, viruses, and sera for a long time period (like bacteria, fungi, and its spores are preserved).

Disadvantage
The only drawback of this method is that it is expensive and only trained or skilled hands can run it.

3.2.6.6. Cryopreservation

Pure cultures are able to survive when stored for a long time with the aid of cryopreservation (i.e., freezing in liquid nitrogen at −196°C or in the gas phase above the liquid nitrogen at −150°C). In this method, the microorganisms of culture are rapidly frozen in liquid nitrogen at −196°C. Some stabilising agents, **e.g.,** glycerol or Dimethyl Sulfoxide (DMSO) are also used for the promotion of cell viability, survival, and prevention of cell damage due to formation of ice crystals.

Advantages
1) This method is used for preservation of several species that cannot be preserved by lyophilisation.
2) Maintenance of viability of most species for 10-30 years without altering their characteristics is possible by this method.

Disadvantage
High cost is the main disadvantage of this method.

3.2.6.7. Storage in Silica Gel

Silica gel powder maintained at low temperatures can be used to store both bacteria and yeast for up to 12-24 months. The fundamental **principle** of this method is that quick desiccation at low temperature increases the viability of cells for a longer time period.

Procedure
1) Silica gel is filled in a bottle, and sterilised at 180°C for 3 hours in a hot air oven.
2) The sterilised bottle is kept on a tray which is embedded in water such that the level of water is above the silica gel.
3) In the next step, the bottle is placed in cold water at a temperature of –17 to –24°C that will lead to the formation of silica gel crystals within the bottle.
4) With 5% skimmed milk, a suspension of spores is prepared; which is added to the silica gel crystals, by using a sterilised Pasteur pipette.
5) Then the temperature is raised gradually, as a result of which ice crystals around the bottle melt.
6) Ice crystals present in the bottle also melt and the spore suspension is mixed properly in silica gel.
7) The bottle should be stored at temperature of 25°C, for a period of 10-14 days to facilitate drying of the silica gel.
8) The dried crystals should be preserved at room temperature or at low temperature.

Advantages
1) It is a simple method of preservation.
2) Entry of mites into the culture can be prevented.
3) This process does not require any special equipment.

Disadvantage
Contamination results due to frequent recovery of culture.

3.2.7. Quantitative Measurement of Bacterial Growth

The term **growth** in microbiology indicates **the magnitude of total population**. Microbial growth measurement is essential for:

1) Determining the growth and reproduction rate,
2) Determining the suitability of water, milk, and food for human consumption,
3) Counting microflora of soil,
4) Monitoring the fermentation process,
5) Checking the suitability of raw material for preparing pharmaceutical products, and
6) Checking the microbial titre in commercial products (**e.g.,** bio-fertiliser, vaccine, probiotic tablets, etc.).

Quantitative measurement of bacterial growth can be carried out using the following **techniques**:

1) **Total Count/Direct Methods**
   i) Direct microscopic count/Breed method,
   ii) Counting chamber method/haemocytometer method,
   iii) Electronic cell counter/coulter method, and
   iv) Proportional count method.
2) **Viable Count/Indirect Method**
   i) Plate count method, and
   ii) Membrane filter count method.

3.2.7.1. Total Count

For counting the microorganisms they should be detected first. The oldest counting technique involves using a microscope to magnify individual cells so that they become visible to naked eye. Direct counting techniques do not rely on cell population growth. Different direct counting **methods** are discussed below:

1) **Direct Microscopic Count/Breed Method:** In this method, 0.01ml of bacterial cell suspension is uniformly smeared over a glass-slide within 1sq.cm specific area. It is then stained and examined under 'oil immersion objective'. The entire 1sq.cm area cannot be examined at the same time, thus few microscopic areas are observed. The total number of microscopic fields per 1sq.cm area is the total number of cells.

   The total number of bacterial cells can be obtained by the following calculations:

   i) Area of microscopic field = πr^2

      Where, r (radius of oil immersion objects) = 0.08mm

      Area of microscopic field = $3.14 \times (0.08)^2$
      Area of microscopic field = 0.02sq. mm

   ii) Area of the smear = 1sq. cm = 100sq. mm
      Number of microscopic fields = 100/0.02 = 5,000

   Total number of cells/1sq. cm = Average number of cells per field × 5,000, i.e., Number of cells/0.01ml of suspension. On multiplying this number with 100, number of cells/ml of suspension is obtained.

   Applications

   i) This method quickly analyses milk in dairy to determine the contamination level before pasteurisation.
   ii) This method also determines relative effectiveness of antibiotics against pathogenic bacteria.

   Disadvantage

   This method does not determine the number of viable cells.

2) **Counting Chamber Method:** In this method, special microscope slides having grid or lines etched on its surface are used for easy and accurate counting. In **Petroff-Hauser chamber** or **haemocytometer** (named so, as originally it was used for counting blood cells), a film of known depth is introduced between the slide and cover slip. When counting, the number of cells per ml of original sample is determined by multiplying the sample's dilution factor.

   Haemocytometer creates a volumetric grid divided into cubes of different sizes for accurate counting of the number of particles in a cube and calculating the sample concentration.

   Advantages
   i) This method is fast.
   ii) This method needs minimum equipment and other requirements.
   iii) This method can study bacterial morphology.

   Disadvantages
   i) This method cannot calculate the number of viable cells.
   ii) This method may involve statistical errors.

3) **Electronic Cell Counter\Coulter Method:** In this method, electronic cell counter is used to count the number of cells. The bacterial cell suspension is passed through a capillary tube with microscopic diameter through which only one cell can pass at a time. A light beam is directed to a photodetector instrument through a capillary tube. The disturbances in light beam due to passage of bacterial cell allow reading of a single unit, which is then displayed by a digital reader.

   Advantages
   i) This method accurately counts thousands of cells in a few seconds.
   ii) This method checks large amount of suspension in a very less duration.

   Disadvantages
   i) Large non-living particles may clog the pore.
   ii) This method does not provide a viable count as dead cells are also counted.

4) **Proportional Count Method:** In this method, a standard suspension of particles (**e.g.,** plastic bead) and an equal amount of bacterial cell suspension are mixed. The resultant suspension is spread over the slide and stained. The number of fields gives the average count of the particles and the cell.

3.2.7.2. Viable Count

A **viable cell** is defined as a cell which is able to divide and increase cell numbers. The normal way to perform a viable count is to determine the number of cells in the sample which is capable of forming colonies on a suitable medium. The **methods** employed for performing viable count are:

1) **Plate Count Method:** This method is commonly used to measure bacterial population. This method has an **advantage** that it measures the number of viable cells. The main **drawback** of this method is that it takes usually 24 hours or more for the formation of visible colonies. This can be offending in cases (**e.g.,** in quality control of milk) where a particular lot cannot be held for so long.

   In plate count method, it is assumed that each living bacteria grows and divides producing a single colony. However, this is not always true since bacteria mostly grow in chains or as clumps. Thus, a colony often results from short segments of a bacterial chain or from a bacterial clump (and not from single bacteria). To reflect this reality, plate counts are often reported as **Colony-Forming Units** (CFU).

When a plate count is performed, only a limited number of colonies should form. This is because in case large numbers of colonies are formed, some cells are over-crowded and are prevented from developing; thus resulting in inaccurate counts. Although the FDA convention is to count plates having 25-250 colonies, many microbiologists count plates having 30-300 colonies. To ensure that some colony counts will be within this range, the original inoculum is diluted several times in a process called **serial dilution** (**figure 3.31**).

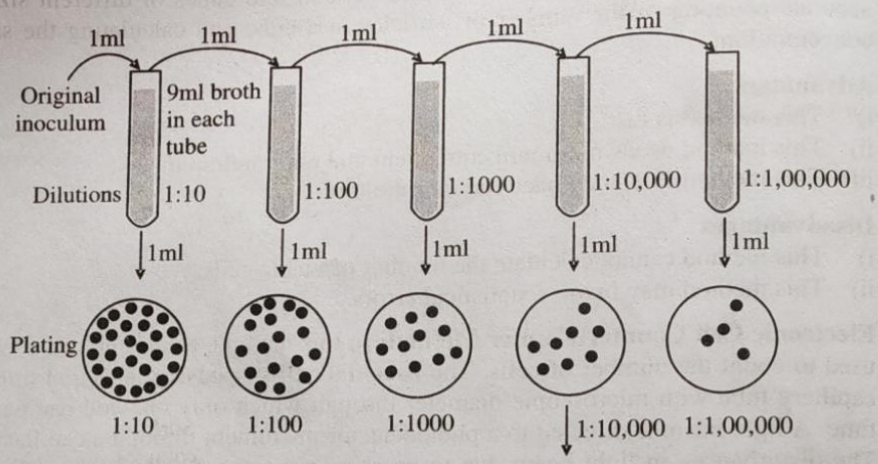


Figure 3.31: Plate Counts and Serial Dilutions. In serial dilutions, the original inoculum is diluted in a series of dilution tubes. In this figure, each succeeding dilution tube will have only one-tenth the number of microbial cells as the preceding tube. Then samples of the dilution are used to inoculate Petri plates, on which colonies grow and can be counted. This count is then used to estimate the number of bacteria in the original sample.

Serial Dilutions

Let us take an **example** of a milk sample having 10,000 bacteria per millilitre. If 1ml of this sample is plated out, 10,000 colonies are formed on the plate of medium. But, this does not produce a countable plate. If 1ml of this sample is transferred to 9ml of sterile water in a tube, 1000 bacteria are present in each ml of fluid. If 1ml of this sample is inoculated on a plate, still many colonies are formed. Therefore, another serial dilution is made and 1ml sample containing 1000 bacteria is transferred to a second tube containing 9ml of water. Each ml of this tube now contains 100 bacteria. When 1ml of this tube is again plated out, 100 colonies are formed which can be easily counted.

There are **two ways** of per forming plate count (**figure 3.32**):

i) **Pour Plate Method:** In this method, petri dishes of 9-10cm in diameter, and two agar media for each dilution are used. 1ml of the test fluid is used or its dilution is taken aseptically in each petri dish; 15-20ml of sterilised agar medium (previously melted and kept below 45°C) is added to each petri dish, and then mixed. Soybean-casein digest agar medium is preferred for detection of bacteria. After the solidification of agar, it is incubated for at least 5 days at 30-35°C. In case of development of a large number of colonies, viable counts are calculated on the basis of counts obtained from plates, with not more than 300 colonies per plate for bacteria. If the counts obtained are reliable in a shorter incubation time (less than 5 days), these counts are considered, for viable count.

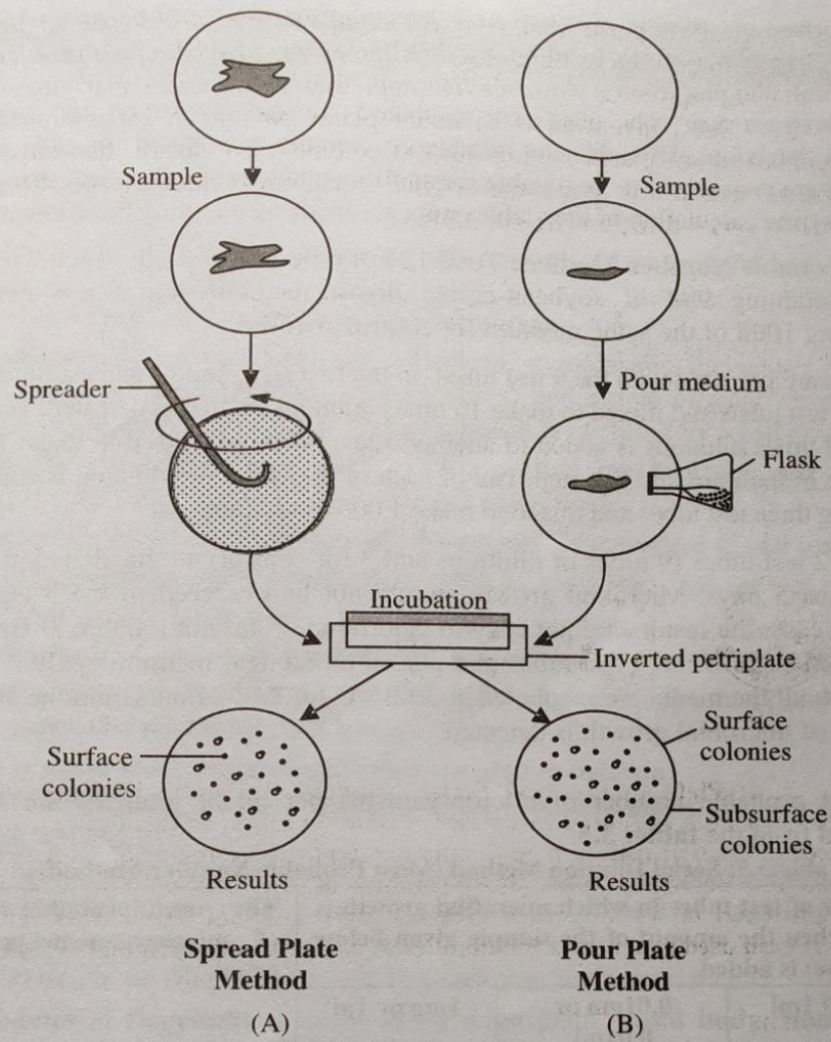


Figure 3.32: Viable Count by (A) Spread Plate Method and (B) Pour Plate Method

ii) **Spread Plate Method**: Around 0.05-0.2ml of the test fluid is placed on the dried surface of the solidified agar medium, and spread uniformly with a spreader. The test conditions should be same, as that for the Pour Plate Method, especially the conditions around petri dishes, agar media, incubation temperature and time, and calculation method.

Membrane Filter Count: This method is applicable to the sample containing antimicrobial substances. The membrane filters of an appropriate material with a pore size of 0.45µm or less are used. Filters of about 50µm pore size are recommended, however other sizes can also be used. The filters, filtration apparatus, media, and other apparatus required during the tests must be sterilised. 10ml of test fluid is measured in two places separately, and each sample is allowed to pass through a separate filter. The pre-treated test fluid is diluted if the bacteria concentration is high. This is done so that 10-100 colonies can develop per filter.

Each filter is washed three times or more after filtration with suitable liquids (**e.g.,** phosphate buffer, sodium chloride-peptone buffer, or fluid medium). The volume of the washings should be about 100ml each. If the filter used is not 50mm in diameter, an appropriate volume of washing should be used depending on the filter size. Polysorbate 80 or a suitable emulsifier should be added to the washings, if lipid is present in the sample.

After performing filtration for bacterial detection, the filters are placed on a plate of soybean-casein digest agar medium. Further for fungal detection, antibiotic is added to the medium and placed on a plate of either Sabouraud glucose agar, GP agar media, or potato-dextrose agar. The plates are incubated, for at least 5 days, at 30-35°C for bacterial detection. After this the number of colonies is counted. In case, if counts obtained are considered to be reliable for short incubation time (i.e., less than or equal to 5 days), the calculation of the viable count are made by adopting these counts.

3) **Most Probable Number Method:** Total 12 test tubes are used, in which 9 test tubes, each containing 9ml of soybean-casein digest medium and 3 test tubes each containing 10ml of the same medium for control are used.

Dilutions are prepared with the 9 test tubes. In the first step, 1ml of the test fluid is added to first 3 test tubes and mixed to make 10 times dilutions. In the second step, 1ml of each of the 10 times dilutions is added to another 3 test tubes and mixed to make 100 times dilutions. In the third and last step, 1ml of each of the 100 times dilution is added to the remaining three test tubes and mixed to make 1,000 times dilutions.

All the 12 test tubes (9 tubes of dilutions and 3 for control) are incubated at 30-35°C for at least 5 days. Microbial growth should not be observed in the 3 control test tubes. In case, the results are not easy to determine or are not reliable, 0.1ml fluid is taken from each of the 9 test tubes and placed on an agar medium or fluid medium. After that, all the media are incubated at 30-35°C for 24-72 hours, and the absence or presence of microbial growth is detected.

The most probable number of microorganisms per ml or gram of the sample is calculated from the **table: 3.5**:

Table 3.5: Serial Dilution Method (Most Probable Number Method)

The number of test tubes in which microbial growth is observed, when the amount of the sample given below (per test tube) is added.			**The most probable number of microorganisms per gram or ml.**
0.1gm or 0.1ml	**0.01gm or 0.01ml**	**1mg or 1µl**	
3	3	3	> 1100
3	3	2	1100
3	3	1	500
3	3	0	200
3	2	3	290
3	2	2	210
3	2	1	150
3	2	0	90
3	1	3	160
3	1	2	120
3	1	1	70
3	1	0	40
3	0	3	95
3	0	2	60
3	0	1	40
3	0	0	23

Note: The most probable number of microorganisms is possibly 100 or less per gram or ml of the sample, when the number of test tubes showing microbial growth is not more than two for test tube containing 0.1gm or 0.1ml of a sample.

3.3. SUMMARY

The details given in chapter can be summarised as follows:

1) Bacteria are a group of prokaryotic, unicellular microorganisms having wide range of shapes (from spheres to rods and spirals) and are of few micrometres in length. They are ubiquitous microorganisms.
2) Cell-envelope or the outer layer of bacteria consists of two components:
   i) A rigid cell wall, and
   ii) Underlying cytoplasmic or plasma membrane.
3) **Protoplasm** of the cell includes cytoplasm, cytoplasmic inclusions (mesosomes, ribosomes, inclusion granules, and vacuoles) and single circular **chromosome** of Deoxyribonucleic Acid (DNA). The protoplasm is enclosed by a cell envelop.
4) **Capsules** and **slime layers** are secreted outside the bacterial cell walls; these polymers are composed of polysaccharides and proteins.
5) Capsules are relatively **impermeable structures**, as compared to cell wall.
6) Capsule **cannot be stained with dyes** (such as India ink), and protect bacteria from phagocytosis and desiccation.
7) **Gelatinous** covering present around the cell wall is known as **slime layer**.
8) **Flagella** (singular flagellum) form the organ of locomotion in a number of bacilli and spirilla in addition to a few species of cocci.
9) **Four types of flagellar arrangements** are seen in bacteria:
   i) **Monotrichous** (a single flagellum protrudes from either of the ends or poles of bacterial cell).
   ii) **Lophotrichous** (numerous flagella protrude from a single pole of the bacterial cell).
   iii) **Amphitrichous** (either a single flagellum or a bunch of flagella protrude from both the poles of the bacterial cell).
   iv) **Peritrichous** (flagella protrude from all over the bacterial cell).
10) The **bacterial flagellum** is made up of three parts, **basal body**, **hook**, and **main filament** or **shaft**.
11) **Fimbriae** are thin, short, filament-like, non-flagellar appendages which project peritrichously from the cell surface.
12) **Cell wall** is a porous membrane and is not considered as a barrier for permeability of small substrates.
13) Cell wall of bacteria differs from other organisms due to the presence of **peptidoglycan** (poly-*N*-acetyl glucosamine and *N*-acetylmuramic acid).
14) Immediately below the cell wall, **plasma membrane** or **cell membrane** is present which is also known as cytoplasmic membrane or inner membrane.
15) Structure enclosed by the plasma membrane or cell membrane is known as **cytoplasm**. Cytoplasm is aqueous, semi-transparent, elastic, and thick.
16) This nuclear structure is labelled as a **nucleoid/nucleoplasm region/chromatin body/nuclear equivalent/bacterial chromosome**, as it is not a distinct nucleus.
17) **Ribosomes** are very small structures, present in the cytoplasm of a bacterial cell, ranging in number from 15,000-20,000.
18) In the cytoplasm of prokaryotic cells, some reserve deposits, known as **inclusions** are present.
19) On the basis of morphology, bacteria are classified into the following **three forms**:
   i) Spherical,
   ii) Cylindrical or rod-shaped, and
   iii) Spiral.

20) Given below are the different growth requirements provided to bacteria via different types of culture media:
    i) Water,
    ii) Carbon,
    iii) Nitrogen and sulphur,
    iv) Oxygen, and
    v) Organic growth factors.
21) Bacteria growing in different ecological niches have varied patterns of growth as described below:
    i) Exponential growth, and
    ii) Balanced growth.
22) On counting the bacteria of these cultures at definite time-interval and plotting a graph in relation to time, a growth curve is obtained known as **normal growth curve** of bacteria.
23) The bacterial growth curve consists of the following **four phases**:
    i) Lag phase,
    ii) Log (logarithmic) or exponential phase,
    iii) Stationary phase, and
    iv) Death phase or phase of decline.
24) In **binary fission**, newly formed bacterium has same copy of DNA as its parent, thus the newly formed bacterium is the cloned form of its original.
25) Sometimes, bacteria may also undergo sexual reproduction called **conjugation**.
26) **Budding** is a form of asexual reproduction in which some reproductive anatomical point in the parent organism gives rise to a new organism.
27) **Fragmentation** is an asexual means of reproduction in which a fragment of the parent gives rise to a new organism.
28) In case of unfavourable conditions, compartments are formed within the bacterial protoplasm followed by fragmentation resulting in minute bodies known as **gonidia**.
29) During unfavourable conditions some gram-positive bacteria (*Bacillus* and *Clostridium*) form resting spores which are actually **endospores** (formed within the **cells**).
30) **The transformation process** involves the transfer of genetic material in bacteria.
31) In **transduction process**, bacterial viruses or bacteriophages are responsible for the transfer of genetic material from one to the other lysogenic and lytic phages.
32) In **bacterial conjugation**, genetic material (DNA) is transferred by the contact between the donor and recipient bacterial cells.
33) Nutritional or culture media (singular medium) are **artificial mixture of various nutrients required for the growth and multiplication of microorganisms**.
34) To cultivate a specific microbe, following methods are used:
    i) Methods of Culturing Aerobic Bacteria
    ii) Methods of Culturing Anaerobic Bacteria
35) **Culture** is a procedure during which microorganisms are cultivated in a medium. If a culture has the population of a single type of microorganism, known as a **pure culture**.
36) In **streak-plate technique**, a fraction of the mixed culture is placed on the surface of a medium containing agar and is streaked across the surface using a transfer loop.
37) In **pour-plate technique**, diluted samples mixed with melted agar medium are plated. This method relies on the **principle** that the inoculum is diluted in successive tubes containing liquefied agar medium for thorough distribution of bacterial cells within the medium.

38) In **spread-plate method**, unlike the pour-plate technique the mixed culture or microorganism is not diluted in the melted agar medium; but diluted in a series of test tubes containing sterile liquid (water or physiological saline).
39) The **serial dilution method** is used for obtaining pure cultures of **those** microorganisms that grow only in liquid media and are still to be cultivated on **solid** media.
40) In **single cell isolation method**, an individual desired cell is picked up from the mixed culture and allowed to grow.
41) To maintain the culture, one of the following methods of preservation can be used:
    i) Periodic transfer of fresh media,
    ii) Oil overlay method,
    iii) Storage at low temperature,
    iv) Storage in sterile soil,
    v) Lyophilisation or freeze drying,
    vi) Cryopreservation, and
    vii) Storage in silica gel.
42) Quantitative measurement of bacterial growth can be carried out using the following techniques:
    i) Total count/direct methods
        a) Direct microscopic count/breed method,
        b) Counting chamber method/haemocytometer method,
        c) Electronic cell counter/coulter method, and
        d) Proportional count method.
    ii) Viable count/indirect method
        a) Plate count method, and
        b) Membrane filter count method.

3.4. EXERCISE

3.4.1. True or False

1) Lyophilisation method is preferred for those microbes that are not killed by regular drying process.
2) A viable cell is able to divide and increase cell numbers.
3) Atrichous is a condition in which flagella are present all over the bacterial cell.
4) A sex factor or fertility factor is a component of the circular DNA, possessed by the donor cells.
5) Generally, oxygen becomes the limiting factor for aerobic organisms.
6) Autotrophs are organisms which depend on an inorganic diet, using CO_2 or carbonates as a source of carbon for their survival.
7) Fragmentation is a sexual means of reproduction in which a fragment of the parent gives rise to a new organism.
8) During favourable conditions some gram-positive bacteria form resting spores which are actually endospores.

3.4.2. Fill in the Blanks

9) For efficient growth, most microbes require a particular favourable temperature which is known as_____.
10) In microbiology, the term growth indicates _____.
11) Cell wall of bacteria differs from other organisms due to the presence of _____.
12) Conjugation is a method of ____ reproduction.

13) Atrichous is a condition in which there is _____ of flagella.
14) A condition in which flagella protrude from all over the bacterial cell is known as_____.
15) Gelatinous covering present around the cell wall is known as_____.
16) The nucleoid can be visualised by a light microscope using _____, which is specifically used to visualise DNA.

Answers:

1) False 2) True 3) False 4) True 5) True 6) True
7) False 8) False 9) Optimum temperature 10) Magnitude of total population
11) Peptidoglycan 12) Sexual 13) Absence 14) Peritrichous 15) Slime layer
16) Feulgen staining

3.4.3. Very Short Answer Type Questions

1) What are pili or fimbriae?
2) How pili differ from flagella?
3) What are endospores?
4) Define following term:
   i) Lag phase
   ii) Pure culture
   iii) Exponential Phase
5) Define cryopreservation method.
6) Enlist various types of culture media.
7) What is transduction method?
8) Define transformation.
9) Enlist various methods of bacterial reproduction.
10) Give raw materials used for bacterial culture.
11) Define the budding process.
12) What is serial dilution method?

3.4.4. Short Answer Type Questions

1) Give classification of bacteria on the basis of their morphology.
2) Explain nutritional requirements of bacteria.
3) Write a note on growth of bacteria.
4) Explain growth curve of bacteria.
5) Enlist various methods of bacterial reproduction and explain budding method.
6) Write a note on bacterial cell wall and differentiate between the cell wall of gram-positive and gram-negative bacteria.
7) What are the physical parameters for growth of bacterial culture?
8) Write a note on types of culture media.
9) What is lyophilisation method?
10) Give a note on streak-plate method or pour-plate method.
11) What is serial dilution method?

3.4.5. Long Answer Type Questions

1) Explain ultrastructure of bacteria.
2) Write a descriptive note on culture media.
3) Write a detail note on bacterial reproduction.
4) What are the isolation methods for pure culture of bacteria?
5) Explain preservative methods for pure culture.
6) Write a detail note on quantitative measurement of bacterial growth.

CHAPTER 4

Microscopy

4.1. MICROSCOPY

4.1.1. Introduction

Microorganisms are so small that they cannot be seen with the unaided human eyes, thus microbiology led to the developments in microscopy. Simple magnifying lenses which magnify an image up to 10 times were used in ancient times; and the high power microscopy was developed in 1590 when **Zaccharias Janssen** (a Dutch spectacle maker) concluded that on combining two hand lenses an image can be markedly enlarged. The combination of lenses used by him had magnification of the order of 50 to 100x. This was the starting era of the compound microscopy.

During the 1660s, **Robert Hooke** developed a compound microscope with which he observed the structure of cork cells and described it in his book '**Micrographia**'. The images of cork structure he obtained have a magnification of 200x. During the observation of different specimens, Robert Hooke observed his samples by reflecting light from the sample surface.

Anton van Leeuwenhoek (a Dutch philosopher and contemporary of **Hooke**) developed his own simple microscope and used it to observe microorganisms. He observed his samples by transmitting light through them and suspending the microorganisms in various fluids, not immobilised or otherwise altered by drying. The microscope used by **Leeuwenhoek** is made up of a single convex (rather spherical lens), fixed on a stand of brass and silver.

4.1.2. Types of Microscopy

Microscope is an instrument which consists of lens or combinations of lens used to magnify the objects that cannot be seen by unaided eye. **Microscopy** is the science which deals with all aspects of microscope.

Microscopes used earlier were 'light' microscopes as they utilise light (either sunlight or an artificial light) for sample observation. At the present time, the following two types of microscopes are available:

1) **Light Microscopes:** These microscopes utilise light to see the object.
2) **Electron Microscopes:** These microscopes utilise an electron beam to see the object.

Different types of light microscopy are bright-field, dark-field, florescence, and phase contrast microscopy. Each of these techniques has different applications and advantages. Electron microscopy is used when images of higher quality and greater magnification are required.

4.1.3. Light Microscopy

Light microscope utilises a combination of optical lenses for obtaining magnification.

Principle

Light microscopy works on the principle of passing light through a sample and examining it under magnification (**figure 4.1**). The objective lens, eyepiece, condenser, and light source are the major optical parts of a light microscope. The **objective lens** magnifies the object; higher the magnification degree of the objective lens, smaller is the focal length. The magnified image appears directly behind the objective lens. The **eyepiece** delivers the image to the eye or camera. It also causes empty magnification of the image, i.e., it enlarges the image but does not increase the resolution.

The **condenser** focuses the light source on the sample and also eliminates stray light to provide a uniform illumination. The condenser lens is associated with an **iris diaphragm** which controls resolution *versus* the contrast and depth of field. Since resolution and contrast are antagonistic, on improving one feature the other is lost.

The **light source** from a lamp or other source illuminates the sample. The light source whose intensity can be controlled by adjusting the voltage is considered the best. Optimal contrast *versus* resolution is obtained by adjusting the voltage (i.e., intensity or brightness) of the lamp as well as the iris diaphragm.

Best resolution is achieved when all of these optical components are centred on an optical axis.

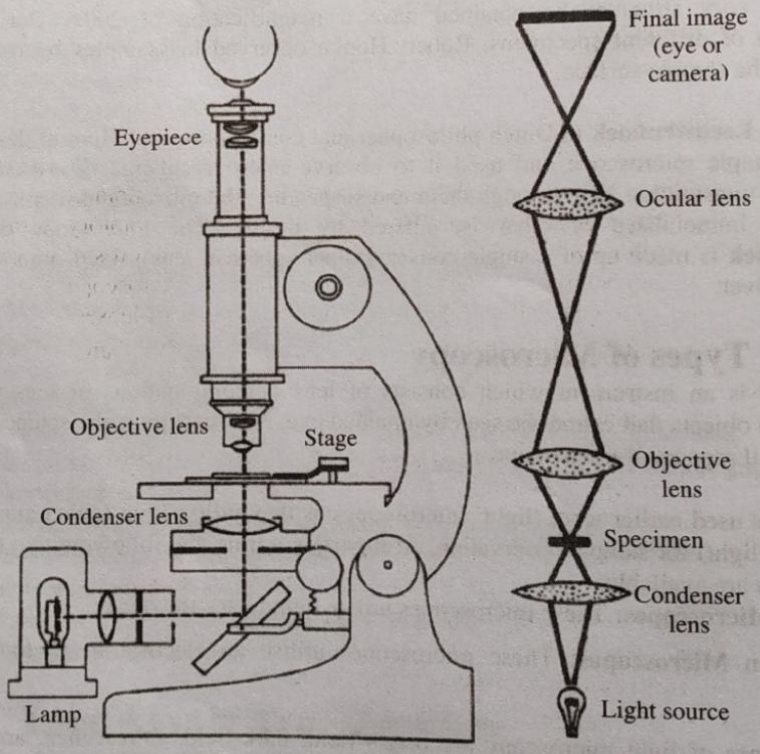


Figure 4.1: Components and Principle of a Light Microscope

Sample Preparation

Samples to be observed are placed on a glass microscope slide and covered with a glass cover slip. However, it is recommended that the sample should be prepared and stained prior to microscopic examination. The cells on the sample are preserved and stabilised by

the process of **fixation**. **Examples** of common **fixatives** include acids, organic solvents, formaldehyde, and glutaraldehyde. This treatment affixes the macromolecules in position. **For example,** glutaraldehyde chemically cross-links the primary amines of neighbouring proteins; organic solvents precipitate the proteins and other macromolecules. Once fixation has been done, the sample is embedded in a supporting medium. A common **embedding medium** for light microscopy is paraffin; some plastic resins can also be used.

Thick samples like tissues are cut into thin slices, using a **microtome** which makes successive sections of a specified thickness. The image developed depends on different sample components differentially interacting with and obstructing the light waves. Since biological samples are homogeneous (i.e., carbon-based polymers) and do not obstruct light, staining the cells with dyes is needed. The affinities of different dyes are different for different subcellular components; **e.g.,** dyes interacting with nucleic acids, differentially stain the cytoplasm and nucleus; therefore stained sub-cellular components differentially absorb the light waves and provide more contrast than the unstained samples.

Types of Light Microscopy

1) Bright field microscopy,
2) Dark field microscopy,
3) Fluorescence microscopy,
4) Phase contrast microscopy, and
5) Differential interference contrast microscopy.

4.1.3.1. Bright Field Microscopy

Bright field microscope is widely used in clinical and microbiological laboratories. It is named so as the object under observation is viewed against a bright field of view. Bright-field microscopes are commonly used for routine clinical laboratory tests. These microscopes are best for studying stained samples, **e.g.,** stained blood smears or stained bacterial smears. Given below are some **features** of bright field microscope:

1) Its illumination source is visible light passed through a series of lenses.
2) The microscopic field appears bright and the microorganisms under observation appear dark.
3) Its magnification power is about 1000-2000x.
4) It has a mechanical and an optical part. The **mechanical part** is made up of structural units except the lens system, while the **optical part** has an eye piece, an objective lens, and a condenser lens.
5) The eye piece (named so as it is placed near the eyes) is also known as **ocular lens**. Two sets of eye pieces (5x and 10x) are commonly used which can magnify an image 5 and 10 times, respectively. Three types of objective lenses with markings 10x (low power), 40x (high power), and 100x (oil immersion), which can magnify an image 10, 40, and 100 times, respectively are used.
6) The condenser lens illuminates the object by transmitting light from the illumination source.
7) Light from the source passes through the condenser lens and the object (placed on the slide) to enter the objective lens; here an inverted and magnified intermediate image is formed within the tube of microscope. This image then acts as an object for the ocular lens which again magnifies it and the final virtual image is seen through the eye piece (**figure 4.2**).

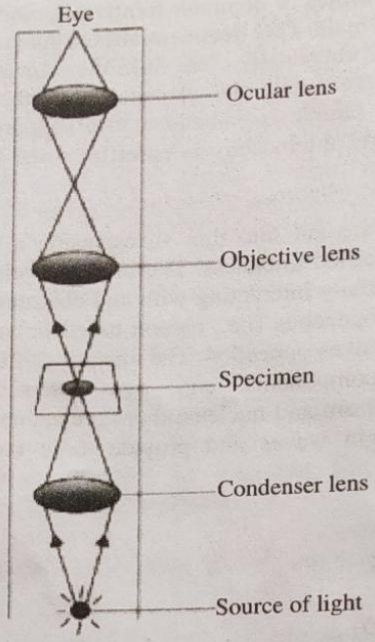


Figure 4.2: Components and Principle of Bright Field Microscope

8) The microscope efficiency depends on its magnification and resolving power.

9) **Magnification** is the product of the magnifying capacities of eye piece and the objective lens. **For example,** if the magnifying capacity of the eyepiece is 10x and that of the objective lens is 100x, the magnification of the microscope is 1000x, i.e., the object is magnified 1000 times.
Resolving power is the ability of a microscope to differentiate two adjacent points as separate, i.e., to distinguish fine structural details. This depends on the wavelength of the light used and the numerical aperture of the lens system. Shorter the wavelength, greater is the resolving power.

A compound microscope has a maximum resolution of 0.2μm. A microscope's resolving power can be calculated as:

$$RP = \frac{l}{2 \times NA}$$

Where, l = Wavelength, NA = Numerical aperture

Numerical aperture is the size of the light cone entering the objective lens from the object. This light cone is usually etched on the sides of objective lens.

Applications

1) It is used to examine either wet films or "hanging drop" for determining the motility of flagellated bacteria (**e.g.,** *Escherichia coli*, *Pseudomonas aeruginosa*, etc.) and protozoa (**e.g.,** *Trichomonas vaginalis*, *Giardia intestinalis*, etc.). The wet preparations are also used for detecting microorganisms in urine or faeces and for detecting fungi on skin.
2) It is used for determining the structural details.
3) It is also used for measuring the size of bacteria, fungi, and protozoa in stained samples.

4.1.3.2. Dark Field Microscopy

Dark field microscope is a special type of compound light microscope. Most essential part is the **dark field condenser** with a central circular stop; this part illuminates the object with a light cone. Dark field microscope makes use of reflected light (and not of transmitted light used in ordinary light microscope) **(figure 4.3)**. This microscope prevents the light from directly falling on the objective lens. Light rays passing through the object are reflected or scattered towards the objective lens. As a result, the microorganisms appear brightly stained against a dark background.

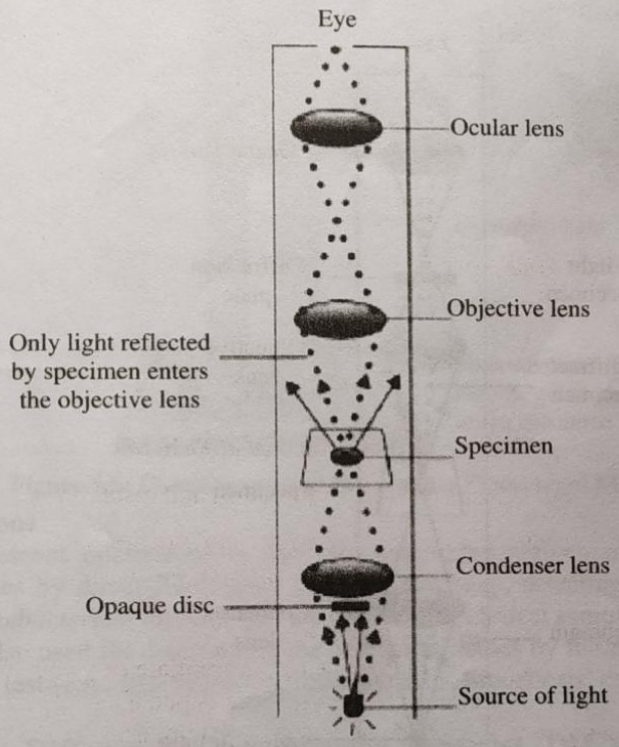


Figure 4.3: Components and Principle of Dark-Ground Microscope

Applications

1) It is used for observing very thin bacteria (such as spirochetes which cannot be viewed under ordinary illumination) as the reflection of light makes them appear big. This method is frequently used for rapid determination of *Treponema pallidum* in clinical samples.
2) It is also used for studying the motility of flagellated bacteria and protozoa.

4.1.3.3. Phase Contrast Microscopy

Phase contrast microscope utilises a special optical system, i.e., **phase contrast objective lens** and **phase contrast condenser lens**. This optical system enables observing the non-stained cellular structures, based on the refractive indices of the structures or the thickness of parts.

This working relies on the principle that light passing from one material to another having different refractive index (or thickness) will change its phase. These phase differences or wave irregularities are translated into variations in brightness and are detected by the eye.

In a phase contrast microscope, one set of light rays directly comes from the light source, and the other set comes from the light reflected or diffracted from the sample. On bringing together these two sets of light rays (i.e., **direct rays** and **reflected** or **diffracted rays**), an image of the specimen is formed on the ocular lens, containing areas relatively light (in-phase), through shades of grey to black (out-of-phase). The phase differences are amplified by using **annular rings** in the condenser and objective lenses; thus the in-phase light appears brighter than the out-of-phase light. The special phase condenser is made up of **annular diaphragms** mounted on a rotating disk fitted to the bottom of the condenser (**figure 4.4**).

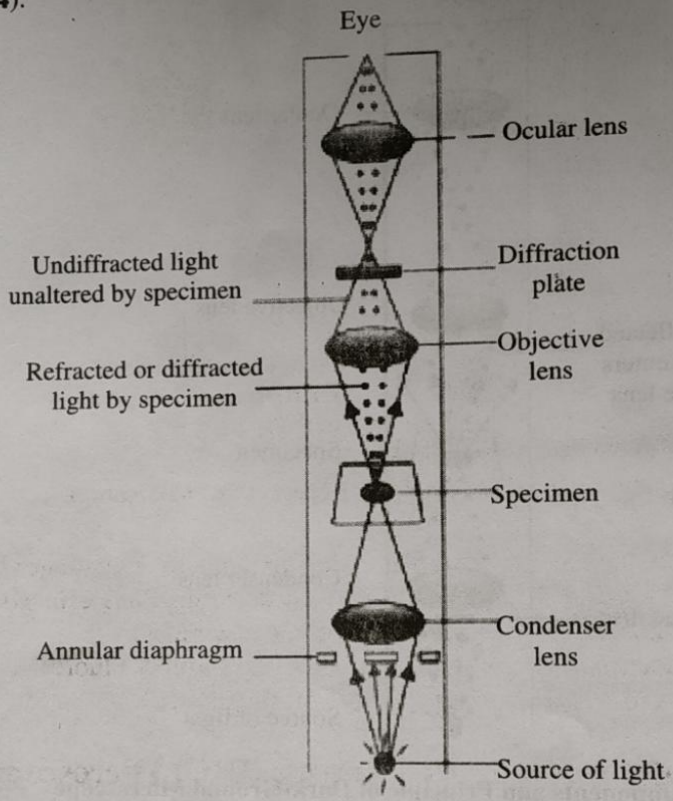


Figure 4.4: Components and Principle of Phase-Contrast Microscope

Applications

1) It is used for studying unstained living cells.
2) It is used for examining the internal structures of living microorganisms.
3) It is used for studying motility of flagellated bacteria and protozoa.
4) It is used for studying intestinal and other living protozoa such as amoebae an *Trichomonas.*
5) It is used for examining fungi grown in culture.

4.1.3.4. Fluorescent Microscopy

Fluorescent microscope works on the **principle** that a longer wavelength of light (i.e visible light) is emitted on exposing the samples stained with fluorescent dye to UV lig (**figure 4.5**). Bacteria stained with fluorescent dye appear bright against a da background.

In **fluorescent microscope**, a UV light source is equipped. The **bacteria to be** viewed a stained with **fluorescent dyes**, **e.g.,** auramine O, acridine orange, **and** rhodamine. T **resolving power** of this microscope increases due to the short wavelength of UV light.

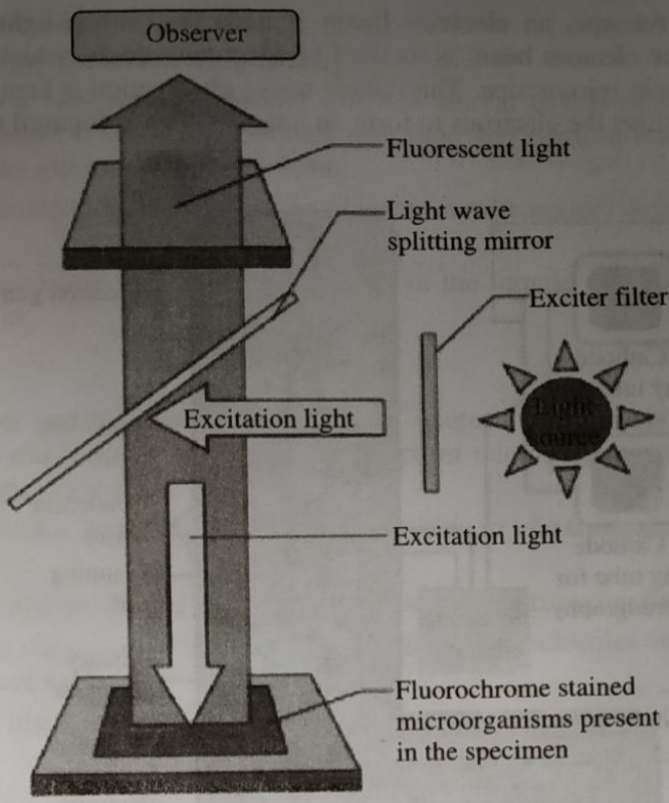


Figure 4.5: Components and Principle of Fluorescent Microscope

Applications

1) Fluorescent microscope is used for examining antigen of a pathogen in clinical samples by direct fluorescent antibody test (**e.g.,** detecting *Neisseria gonorrboeae*, *Corynebacterium diphtheriae*, etc., directly in clinical samples).
2) It is also used for determining the serum antibodies by Indirect Fluorescent Antibody (IFA) test (**e.g.,** IFA in leptospirosis, syphilis, brucellosis, etc.).

4.1.3.5. Differential Interference Contrast (DIC) Microscopy

Differential Interference Contrast (**DIC**) microscope utilises a **beam-shearing interference system** in which the reference beam is sheared by a minute amount (less than the diameter of an airy disk). This microscope works on the mechanism of rendering contrast in transparent samples. This forms a **monochromatic shadow-cast image** which displays the gradient of optical paths for both high and low spatial frequencies in the sample. The sample regions where the optical paths increase along a reference direction appear brighter (or darker), while the regions where path differences decrease appear in reverse contrast. The image contrast increases if the gradient of optical path difference becomes steeper.

Applications

1) It is used for viewing living cells.
2) It is used for quantitative studies of various cellular macromolecules.
3) It is also used for measuring sample's dry weight, thickness, and water content.

4.1.4. Electron Microscopy

Electron microscope utilises a technique in which **high resolution images** of biological and non-biological specimens are formed. This microscope is used in biomedical research work for determining the detailed structure of tissues, cells, organelles, and macromolecular complexes.

In electron microscope, an **electron beam** is used (and not a light beam as in light microscope). The electron beam is focused by **electromagnets**, which are similar to the lenses used in light microscope. The sample under observation is kept on the path of the beam, which scatters the electrons to form an image, which is focused on a screen (**figure 4.6**).

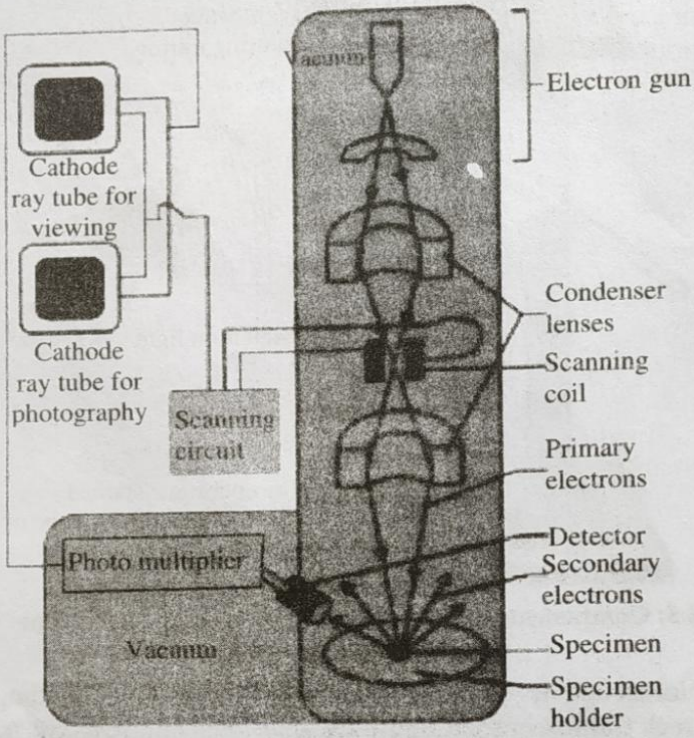


Figure 4.6: Components and Principle of Electron Microscope

Electron microscope has a **very high resolving power** (1,00,000 times than that of a light microscope) because it utilises electrons with wavelength 0.005nm as compared to 5000nm wavelength of the visible light.

Resolving power is half of the wavelength; thus electron microscope has a resolving power of about 0.1nm.

Types

1) Scanning electron microscopy, and
2) Transmission electron microscopy.

Applications

Electron microscope is used to study samples of very minute range, thus it is helpful in obtaining the following information:

1) **Topography:** Features of the sample surface.
2) **Morphology:** Shape and size of the cell organelles within the sample.
3) **Composition:** Amount of the elements and compounds of the sample.
4) **Crystallographic Information:** Arrangement of atoms within the sample.

Advantages

1) Electrons of short wavelength are used by the electron microscope as an illumination source for observing samples at a greater magnification.

2) It possesses highest magnification.
3) It possesses highest resolution power.
4) It works on a very simple principle which is similar to that of a light microscope, with the exception that the electromagnetic field and electron beam act in the same manner as the glass lens and light beam.
5) Scanning electron microscope (a type of electron microscope) develops 3-D image of the sample.
6) It also demonstrates the surface topography of the sample with sufficient clarity and depth of field.

Disadvantages

1) Construction and maintenance of electron microscope is expensive; however the investment and running cost of a basic electron microscope overlaps with those of confocal light microscope.
2) It is large, bulky, expensive, and highly sensitive to vibration and external magnetic fields.
3) It requires expertly trained and skilled operators, even for preparing samples.
4) The sample should be placed under vacuum as the molecules in the air deflect the electron beam used.
5) It produces black and white images and thus requires to be falsely coloured.

Limitations

1) Since the sample under observation is placed within a chamber of high vacuum, the cells in a living state are not examined.
2) The process of drying may change some morphological characters of the sample.
3) Thin sections of the sample should be used as the electron beam has a low penetration power, thus the internal structures of the cell are not revealed properly.
4) The lens used has a very small numerical aperture.

4.1.4.1. Scanning Electron Microscopy (SEM)

Scanning electron microscope utilises electron beam for producing **high-resolution images of sample surfaces**. It forms **3-D view** of the sample. It also gives the **surface topography** of the sample with sufficient clarity and depth of field.

The most common SEM mode is **detection of the secondary electrons emitted by the atoms excited by the electron beam**. The number of detectable secondary electrons depends on **sample topography** (the angle at which electron beam meets the sample surface). A special detector is used for scanning the sample and collecting the emitted secondary electrons, after which an image is formed revealing the sample topography.

Construction

The basic construction of SEM (**figure 4.7**) is discussed below:

1) **Electron Gun:** It produces an electron beam. A filament (cathode) of 0.1mm thin tungsten wire is heated at high temperature of about 2800K to emit thermoelectrons.
2) **Electromagnetic Lenses:** A magnetic lens is also used in an electron microscope. When a direct electric current is passed through a coil-wound electric wire, a rotationally-symmetric magnetic field is formed and a lens action is produced on an electron beam.

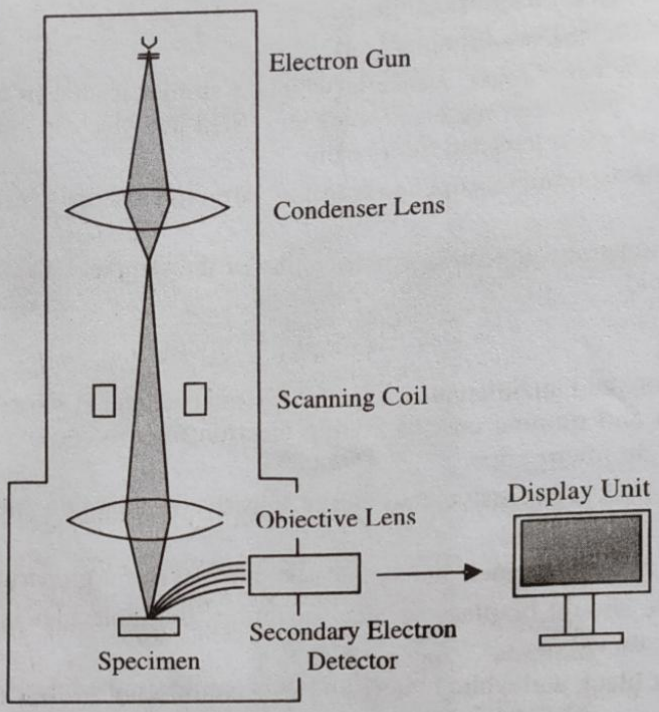


Figure 4.7: Basic Construction of a SEM

3) **Condenser and Objective Lenses:** A condenser lens is mounted below the electron gun to adjust the electron beam's diameter. **Two-stage lenses** combining the condenser and objective lenses are present below the electron gun. The electron beam coming from the electron gun is focused by the two-stage lenses, producing a small electron probe.

   If the action of condenser lens is strengthened, the electron probe becomes narrower; while if the action is weakened, the electron probe becomes broader. Between the condenser and objective lenses, the **aperture** is present. This aperture consists of a thin metal plate, having a small hole. The electron beam after passing through the condenser lens illuminates the aperture-plate, which directs a part of electron beam towards the objective lens. This lens is used for focusing and determining the final diameter of the electron probe.

4) **Specimen/Sample Stage:** Since, the sample is observed at a high magnification, the stage should firmly support the sample and should move smoothly. The sample stage can perform the following movements:

   i) Horizontal movement (X, Y),
   ii) Vertical movement (Z),
   iii) Specimen tilting (T), and
   iv) Rotation (R).

   X and Y movements are used for selecting a field of view; and Z movement is used for changing the image resolution and depth of focus. The construction of a sample stage is shown in **figure 4.8**.

5) **Secondary Electron Detector:** It detects the secondary electrons emitted from the sample. The tip of the detector is coated with a **scintillator** (fluorescent substance) and a high voltage of about 10kV is applied to it. This high voltage attracts the secondary electrons which hit the scintillator, producing light. A **light guide** directs this light to a Photo-Multiplier Tube (PMT), where it is converted into electrons to be amplified as an electric signal.

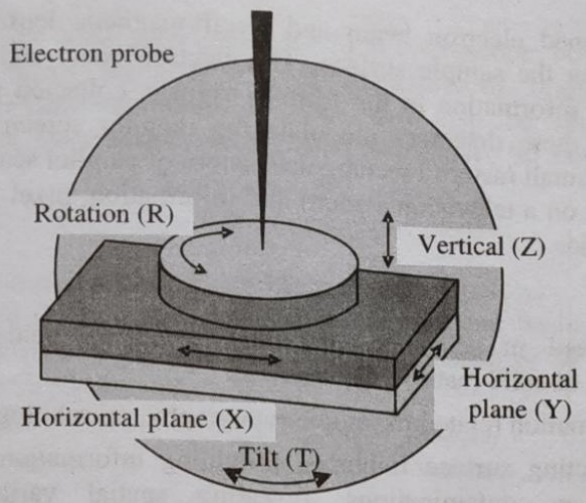


Figure 4.8: Construction of the Sample Stage

6) **Image Display and Recording:** The signals obtained from the secondary electron detector are amplified and transferred to the display unit. The scanning on display unit is synchronised with the electron-probe scan, thus brightness variation depends on the number of secondary electrons, appears on the monitor screen on the display unit. As a result, a SEM image is formed. Earlier Cathode-Ray Tube (CRT) was in use as a display unit; but in recent years, a Liquid-Crystal Display (LCD) is being used.

Working

The working of SEM is shown with a ray diagram in **figure 4.9**. A narrow electron beam from the electron gun is directed to the sample to rapidly scan its surface. As a result the sample releases secondary electrons, whose intensity depends on the shape and chemical composition of the irradiated object. A detector collects these secondary electrons and generates electrical signals, which are scanned to produce an image on a cathode ray tube.

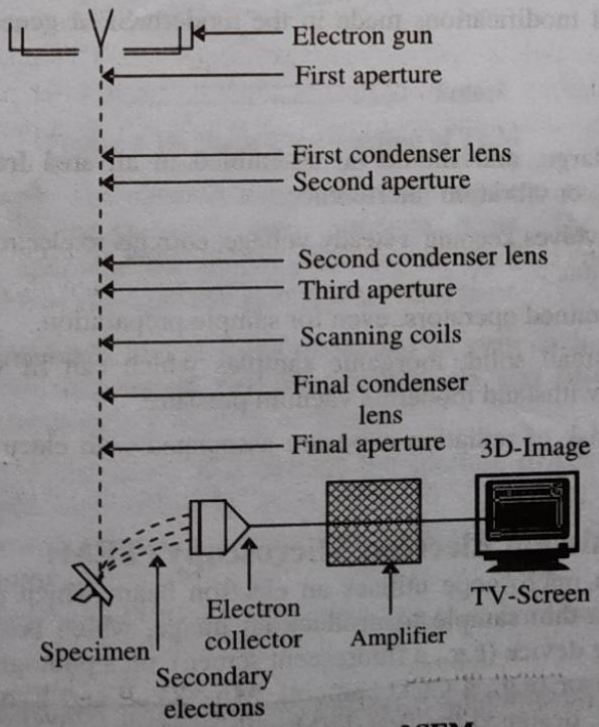


Figure 4.9: Working of SEM

SEM utilises a scanned electron beam and electromagnetic lenses for focusing and directing the beam on the sample surface. The electron beam and the sample surface interact and produce information in the form of signals, collected using detectors. The output signals from these detectors modulate the monitor screen to form an image, corresponding to the small raster (a rectangular pattern of parallel scanning lines followed by the electron beam on a television screen) and information, pixel by pixel, originating from the sample surface.

Applications

1) It is widely used in scientific and industry-related fields, especially where characterisations of solid materials is required.
2) It provides information related to sample topography, morphology, and composition.
3) It helps in detecting surface fractures, providing information in microstructures, analysing surface contaminations, revealing spatial variations in chemical compositions, providing qualitative chemical analyses, and identifying crystalline structures.
4) It also has practical industrial and technological applications such as semiconductor inspection, production line of miniscule products, and assembly of microchips for computers.
5) It is used as an essential research tool in life science, biology, gemmology, medical and forensic science, and metallurgy.

Advantages

1) It has a wide range of applications, and provides detailed 3-D and topographical imaging, and versatile information collected from detectors.
2) It can be operated easily with proper training; and advances in computer technology and related software make its operation user-friendly.
3) Its working is fast.
4) The technological modifications made in the modern SEM generate data in digital form.

Disadvantages

1) It is expensive, large, and should be assembled in an area free of any possible electric, magnetic, or vibration interference.
2) Its maintenance involves keeping a steady voltage, currents to electromagnetic coils, and cool water circulation.
3) It demands well-trained operators, even for sample preparation.
4) It is limited to small solid, inorganic samples which can fit within the vacuum chamber and can withstand moderate vacuum pressure.
5) It also carries a risk of radiation exposure associated with electrons emitted by the sample surface.

4.1.4.2. Transmission Electron Microscopy (TEM)

Transmission electron microscope utilises an electron beam which passes through and interacts with an ultra-thin sample to produce an image, which is then magnified and focused on an imaging device (**e.g.,** a fluorescent screen), on a photographic film layer, or is detected with a sensor (**e.g.,** a CCD camera). **Max Kroll** and **Ernst Ruska** designed the first TEM in **1931**; designed the first TEM with resolution power greater than that of light in **1933**; and the first commercial TEM in **1939**.

Principle

TEM relies on the same basic principle as the light microscope; however it has a greater resolution which allows studying the ultrastructure of organelles, viruses, and macromolecules. Specially prepared samples can also be observed using TEM. Combination of light microscope and TEM is often used to complement a research project.

Construction

The basic construction of TEM (**figure 4.10**) is discussed below:

1) **Electron Source (Gun):** This forms the foremost and basic part of a TEM. The electron gun consists of a V-shaped filament of **LaB6** or **W** (tungsten), wreathed with Wehnelt electrode (or cap).

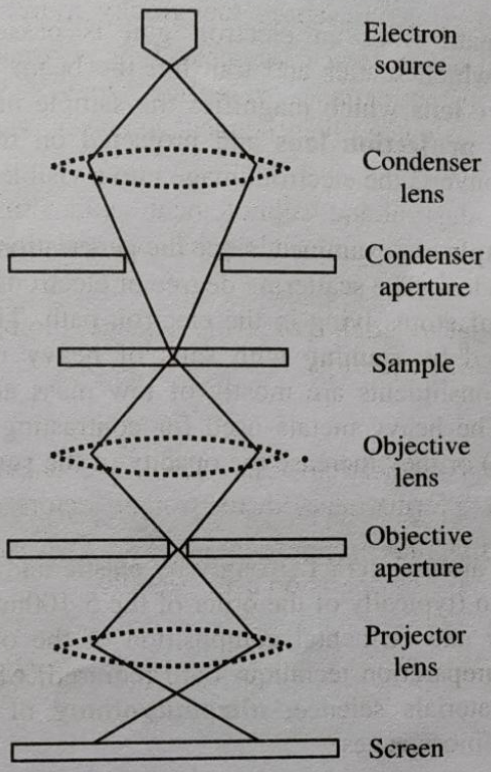


Figure 4.10: Basic Construction of TEM

2) **Condenser Lens:** The electron stream from the electron gun is focussed using the condenser lenses on a small, thin, coherent beam. The first condenser lens determines the **spot size** (the general size range of the final spot striking the sample), and the second lens **changes the spot size** on the sample.

3) **Condenser Aperture:** This is a thin metal disk or strip having a small circular hole. Aperture is used to **restrict the electron beams** and **filter out the unwanted scattered electrons** before forming an image.

4) **Sample:** The electron beam after passing the aperture strikes the sample to undergo **electron-sample interaction** in three different ways, i.e., un-scattered electrons (transmitted beam), elastically scattered electrons (diffracted beam), and inelastically scattered electrons.

5) **Objective Lens:** This is used to **focus the electron transmitted from the sample into an image**.

6) **Objective Aperture:** This is used to **enhance the contrast** by blocking out high-angle diffracted electrons.

7) **Selected Aperture:** The user **examines the periodic diffraction of electron** by ordered arrangements of atoms in the sample using this aperture.
8) **Projector Lens:** This lens is used to **expand the beam** on the phosphor screen.
9) **Screen:** TEM uses a **phosphor screen** made up of fine (10-100µm) particulate zinc sulphide for direct observation by the user.
10) **Image Pattern:** The image on striking the phosphor screen generates a light which allows the user to view the image. The darker areas of image represent those sample areas which transmit fewer electrons; while the lighter areas of image represent those sample areas which transmit more electrons.

Working

In TEM, an electron beam from an electron gun is passed through a series of **electromagnetic lenses**, which scatter and transmit the beam through the sample and then through the **objective lens** which magnifies the sample image. The image is then further magnified by the **projection lens** and projected on the fluorescent screen or photographic film. This converts the electron image into a visible form.

Very **thin sections of sample** are examined, since the penetration power of electron beam through the solid matter is low. The scattering degree of electrons by the sample is related to the number and mass of atoms lying in the electron path. The contrast of biological materials can be enhanced by staining with salts of heavy metals (like uranium or tungsten) because their constituents are mostly of low mass and the contrast of these materials is also weak. The heavy metals used for contrasting are either fixed on the sample (**positive staining**) or they increase the opacity of the surrounding area (**negative staining**).

Electrons and the sample atoms interact strongly by elastic and inelastic scattering. The sample should be very thin (typically of the order of the 5-100nm for 100keV electrons), depending on the density and elemental composition of the object and the resolution desired. Special sample preparation techniques are required; **e.g., electropolishing** and **ion-beam etching** in materials science, **ultramicrotomy** of stained and embedded tissues, or **cryofixation** in biosciences.

Electrons from the electron gun are emitted by thermionic, Schottky, or field emission. The latter is used when high gun brightness and coherence are required. A **condenser-lens system** having three or four stages allows the user to change the illumination aperture and the area of illuminated sample. The electron intensity distribution behind the sample is imaged on a fluorescent screen using a three to eight lens system. The image on the screen is recorded either by directly subjecting the photographic emulsion or an image plate within a vacuum chamber, or digitally to a CCD camera via fluorescent screen coupled with a fibre-optic plate.

Applications

1) It is ideally used in different fields like life sciences, nanotechnology, medical, biological and material research, forensic analysis, gemology, metallurgy, industry, and education.
2) It provides topographical, morphological, compositional, and crystalline information of the sample.
3) The images it produces allow the researchers to view the samples on a molecular level, thus making possible to study the ultrastructure and texture of sample.

4) It is used for semiconductor analysis and manufacturing of computer and silicon chips.
5) It is used in technology companies for identifying flaws, fractures and damages in micro-sized objects, so that with the data obtained the problems can be overcome and/or a more durable and efficient product can be formed.

Advantages

1) It has the most powerful magnification (potentially over 1 million times or more).
2) It has a wide range of applications and can be utilised in various different scientific, educational, and industrial fields.
3) It provides information on element and compound structure.
4) It produces high quality and detailed images.
5) It provides information on surface features, shape, size, and structure.
6) Its operation is easy with proper training.

Disadvantages

1) It is large and expensive.
2) Sample preparation is a tedious task.
3) Potential artefacts from sample preparation.
4) Its operation and analysis requires special training.
5) Only those samples which are electron transparent can withstand inside the vacuum chamber, and are small enough to fit in the chamber can be used.
6) It requires special housing and maintenance.
7) It produces black and white images.

4.2. SUMMARY

The details given in the chapter can be summarised as follows:

1) **Microscopes** used earlier were 'light' microscopes as they utilise light (either sunlight or an artificial light).
2) **Light microscope** works on the principle of passing light through a sample and examining it under magnification.
3) At the present time, the following two types of microscopes are available:
   i) **Light Microscopes:** These microscopes utilise light to see the object.
   ii) **Electron Microscopes:** These microscopes utilise an electron beam to see the object.
4) The **objective lens** magnifies the object; higher the magnification degree of the objective lens, smaller is the focal length.
5) The magnified image appears directly behind the objective lens and the **eyepiece** delivers the image to the eye or camera.
6) The **condenser** focuses the light source on the sample and also eliminates stray light to provide a uniform illumination. The condenser lens is associated with an **iris diaphragm** which controls resolution *versus* the contrast and depth of field.
7) **Numerical aperture** is the size of the light cone entering the objective lens from the object. This light cone is usually etched on the sides of objective lens.
8) **Magnification** is the product of the magnifying capacities of eye piece and the objective lens.
9) **Resolving power** is the ability of a microscope to differentiate two adjacent points as separate, i.e., to distinguish fine structural details.

10) The eye piece (named so as it is placed near the eyes) is also known as **ocular lens**.
11) **Bright field microscope** is widely used in clinical and microbiological laboratories. It is named so as the object under observation is viewed against a bright field of view.
12) **Dark field microscope** is a special type of compound light microscope which makes use of dark ground microscope.
13) **Phase contrast microscope** utilises a special optical system, i.e., **phase contrast objective** and **phase contrast condenser**.
14) The working of **phase contrast microscope** is based on the principle that light passing through one material to another of slightly different refractive index (or thickness) will undergo a change in phase.
15) **Fluorescent microscope** works on the principle that a longer wavelength of light (i.e., visible light) is emitted, when the samples stained with fluorescent dye are exposed to UV light.
16) **Differential Interference Contrast (DIC)** microscope is based on the **principle** of a **beam-shearing interference system** in which the reference beam is sheared by a minute amount (less than the diameter of an airy disk).
17) In **electron microscope**, an electron beam is used instead of light beam utilised in light microscope. The electron beam is focused by electromagnets, which are similar to the lenses used in light microscope. The sample under observation is kept on
18) The electron microscopy is of two types:
    i) Scanning electron microscopy, and
    ii) Transmission electron microscopy.
19) **Scanning electron microscope** utilises electron beam for producing **high-resolution images of sample surfaces**. It forms **3-D view** of the sample.
20) **Transmission electron microscope** utilises an electron beam which passes through an ultra-thin specimen and interacts with it to produce an image.

4.3. EXERCISE

4.3.1. True or False

1) Magnification is the product of the magnifying capacities of eye piece and the objective lens.
2) Electron utilise a beam of light to see the object.
3) Light microscopes utilise light to see the object.
4) The objective lens magnifies the object; higher the magnification degree of the objective lens, smaller is the focal length.
5) Numerical aperture is the size of the light cone entering the objective lens from the object.
6) Scanning electron microscope forms 2-D view of the sample.
7) TEM utilises a light beam which passes through an ultra-thin specimen and interacts with it to produce an image.

4.3.2. Fill in the Blanks

8) _____ is the science which deals with all aspects of microscope.
9) _____ is used to produce an electron beam.
10) _____ is the ability of a microscope to distinguish fine structural details.

11) The electrons are scattered and transmitted through the beam, the sample and then through the _____ which magnifies the sample image.
12) Electron microscope utilises a technique in which _____ of biological and non-biological specimens are formed.
13) The eye piece is also known as_____.
14) Scanning electron microscope utilises _____ for producing high-resolution images of sample surfaces.

Answers:

1) True	2) False	3) True	4) True	5) True
6) False	7) False	8) Microscopy	9) Electron gun	
10) Resolving power		11) Objective lens	12) High resolution images	
13) Ocular lens		14) Electron beam		

4.3.3. Very Short Answer Type Questions

1) What is microscopy?
2) Draw a clean diagram of light microscope with naming.
3) Write a note on DIC.
4) Give principle of light microscopy.
5) Give applications of phase contrast microscope.
6) Write a short note on dark field microscopy.
7) Write a note on bright field microscopy.

4.3.4. Short Answer Type Questions

1) What is microscopy? Give its types.
2) Write a note on phase contrast microscopy.
3) Give the principle, construction, working and applications of SEM or TEM.
4) Write a note on phase contrast microscopy.

4.3.5. Long Answer Type Questions

1) Write a detail note on electron microscopy.
2) Give the principle of light microscopy and define its various types.

CHAPTER 5
Identification of Bacteria

5.1. IDENTIFICATION OF BACTERIA

5.1.1. Introduction

It is really a tedious task to study microorganism in living state as their size is in microns and most of them are transparent and practically colourless when suspended in an aqueous medium. Therefore, different staining techniques along with light microscopy are used to identify, study, and classify the microorganisms into specific groups for various purposes.

The isolated bacteria are identified by one or few of the procedures mentioned below:

1) Staining of the isolated bacteria,
2) Biochemical testing,
3) Motility testing,
4) Serological tests,
5) Phage typing,
6) Identification disc testing,
7) Semi-automated and automated identification systems, and
8) Molecular techniques.

Microbes cannot be seen with unaided eye, even it is very difficult to see and identify them through microscopes. With the aid of staining it becomes easy to observe, identify, and reveal the presence of microscopic structures, as the specific macromolecules within the bacterial structures bind to the charged portions of the stain.

5.1.2. Stains

Dyes, stains, and different techniques of staining with the conjugation of microscopes are used to highlight structures in biological tissues and cells. Stains are used to study bulk tissues (by highlighting muscle fibres or connective tissue), cell populations (**e.g.,** classifying different blood cells), or organelles within the individual cells.

Various stains and dyes have been used to stain bacterial cells or any sub-cellular structure/component. These stains are broadly classified into three classes, i.e., **acidic**, **basic**, and **neutral**. Material fixation is a primary requirement for staining, and heat fixation is most commonly used method for this. For heat fixation, small amount of bacterial culture is spread over a clean glass slide to form a thin film. The film is kept to dry and then rapidly passed several times through the flame of a burner. As a result of heating, bacteria are killed, and protoplasm is coagulated, but the cell structure remains preserved. Sometimes fixative agents (**e.g.,** alcohol, acetone, etc.) are also used.

On the bacterial cells, slightly negative charge is present; therefore **basic dyes carrying a positive charge** (**e.g.,** crystal violet, methylene blue, carbol fuchsin, and safranin), give best results with them. In these dyes, crystal violet gives the best results because it penetrates the cells very rapidly and carbol fuchsin reacts most rapidly but tends to over-stain the cell.

Negatively charged or **acidic stains** (**e.g.,** eosin Y and nigrosine) do not react with bacterial cell and get deposited around the bacterial cells; this is also known as **negative staining**, because the cell remains unstained but background becomes coloured. Negative staining is useful in estimating the shape and size of bacterial cells, **e.g.,** India ink-mediated negative staining is often used to visualise the capsules.

Neutral stains (**e.g.,** Sudan III and Toluidine Blue) are specifically used to stain the fat inclusions and polyphosphate granules respectively.

5.1.3. Staining Techniques

Staining is a basic step of microscopy which is used to enhance the contrast of microscopic image. It is not limited to the study of biological materials, but is also used for the study of morphology of other materials, **e.g.,** the domain structures of block copolymers, or the lamellar structures of semicrystalline polymers.

Aims of Staining

1) Due to the semi-transparent cells, the refractive index of microbes and surrounding environment is almost equal. In such conditions, staining proves very useful to increase the visibility and to develop the better contrast with surrounding.
2) It becomes easy to analyse and study the shape, size, and other morphological characters of microorganisms.
3) Along with the morphological characters, intracellular structures can also be studied conveniently by applying staining.
4) Staining also has a clinical importance as it is used for primary level of identification, **e.g.,** whether bacteria are gram-positive/gram-negative or acid fast/non-acid fasts. These characters are helpful in diagnosis and treatment.

Preparation of Smear

A smear for staining is prepared under aseptic conditions. The loop being used should be passed through the flame before and after each use. A smear is prepared by the following **three steps**:

1) **Preparation of the Slide:** Oil is removed by proper cleaning and drying of the slide to be used.
2) **Preparation of the Smear:** Using a loop, 1 or 2 drops of the sample from the broth culture is spread in the centre of the slide (till it becomes the size of a nickel). In case a solid media culture is being used, a very small drop of water is first placed in the centre of the slide and then mixed with a loopful of bacteria collected from the solid media surface. The mixture is spread to attain the size of a nickel.
3) **Fixation:** At the fixation point, the organisms and cells are carefully attached to the slide. An electric fixing tray can be used to carry out the drying and fixing of smears in a single step. Before staining, the slides should be completely dried and fixed otherwise the stains applied will get washed away.

Note: If smears are prepared using a broth, they appear shiny even after drying.

Staining the Smears

1) Initially a single broth slide and solid medium slide is prepared, stained, and observed. If optimum results are obtained then other slides are prepared, otherwise some other technique is practiced.
2) Known culture smears (**e.g.,** *S. epidermidis*, *E. coli*, or *Bacillus sp.*) are placed on the staining rack over the sink.
3) After covering the smear area with crystal violet stain, it is left for 15 seconds, and then washed gently with a stream of water.

4) The smear is covered with Gram's iodine, left for 15 seconds, and then washed.
5) A drop of Gram's decolouriser is applied to the smear area to prevent further discharge of colour. The decolourising process is stopped by washing with water.
6) Safranin is applied to the smear, left for 15 seconds, and then washed with water.
7) The smear is either air-dried or placed on the drying and fixation tray.

Techniques of staining may be categorised into **three classes**:

1) Simple staining:
   i) Negative staining, and
   ii) Monochrome staining.
2) Differential staining:
   i) Gram's staining, and
   ii) Acid-fast staining.
3) Special staining.

5.1.4. Simple Staining

In simple staining, either alcoholic or aqueous solution of a single basic dye is used, and the dye is washed off with water after 30-60 seconds, before drying and examination. This method is useful in revealing the shape, size, and arrangement of the organism. Simple staining is also preferred to study the nature of the cellular contents of the exudates and the intracellular location of the cell organelles of bacteria.

Given below are some of the commonly used **simple stains**:

1) **Methylene Blue:** It is used to clearly mark the bacterial morphology, **e.g.,** *H. influenzae* in CSF, *Gonococci* in urethral pus.
2) **Dilute Carbol Fuchsin:** It is used to stain throat swab from patients of suspected Vincent's angina, (Borrelia are better stained), as a counter stain in Gram stain, and to study the morphology of *Vibrio cholerae* (comma-shaped).
3) **Polychrome Methylene Blue:** It is used to study Mc Fadyean reaction of *B. anthracis*, in which the blue bacilli is surrounded with purple capsular material.

Principle

In simple staining, **a single reagent is used** for staining the bacterial smear. Generally, basic stains having a positively charged (i.e., cationic) chromogen are used; this is because nucleic acids and some cell components of bacteria are negatively charged, thus the cationic chromogen gets strongly attracted and binds to them. Methylene blue, crystal violet, and carbol fuchsin are some of the most commonly used basic stains. Simple staining aims to **study the morphology and arrangement of bacterial cells**.

Materials Required

1) Basic stains (**e.g.,** crystal violet, methylene blue, and safranin),
2) Air-dried, heat-fixed bacterial smears,
3) **Cultures:** A nutrient agar **slant culture** of *E.coli* and *Bacillus cereus* and a nutrient broth culture of *S. aureus* (each stored for 24 hours),
4) Wash bottle, and
5) **Reagents:** Methylene blue, crystal violet, and carbol fuchsin.

Procedure

1) Separate bacterial smears of the organisms are prepared and heat fixed before staining.
2) The smear slide is placed on the staining tray, stain is applied over the smear, and left for a few seconds (15-30 seconds for carbol fuchsin, 20-60 seconds for crystal violet, and 60-120 seconds for methylene blue).
3) Excess stain is removed by washing the slide with tap water. The slide should be kept parallel to the stream of water, so that minimum loss of organisms from the preparation occurs.

4) The slide is blot dried with a bibulous paper, but not wiped off.
5) All the steps are repeated for the remaining two organisms, but with a different stain for each.
6) All the stained slides are placed on the microscope stage and focused using the 10x objective.
7) An area of the smear in which the cells are well spread in a monolayer is selected.
8) The area is centred, applied with immersion oil directly, and focused under the oil with 100x objective.

Result

The bacterial cells stain uniformly and the colour of the cell depends on the type of dye used. If methylene blue is used, some granules in the interior of the cells of some bacteria may appear more deeply stained than the rest of the cell, which is due to the presence of different chemical substances.

5.1.4.1. Negative Staining or Indirect Staining Technique

This technique is also used when the sample is to be viewed under a light microscope. Microbes and an acidic dye nigrosin (a black stain) or Congo red (a red dye) are mixed, spread as a thin film on a slide, and air-dried. Since the acidic dye has a negatively charged chromophore, it gets repelled by the negatively charged microorganisms and sits in the background. This is known as negative or indirect staining of the microbial cell, and clear or white cells are observed against a contrasting dark background. The size and shape of bacterial cell is less wrinkled and distorted as heat fixing and chemical reactions are avoided in this staining technique.

Principle

Negative (or indirect or relief) staining aims to study the morphology of bacterial cell. In this technique, the bacterial cell remains colourless while the background gets stained. An acidic stain (**e.g.,** Eosin stain, Nigrosine, Congo red, Rose Bengal Stain, etc.) is used, which however does not stain the cell. This is because the stain as well as the bacterial cell carry negative charges, thus repulsion occurs between two similar charges. Although the stain gets attracted to the positively charged glass slide and binds to it. **India Ink** can also be used for staining as its molecular size is greater than pores present on bacterial cells, so they remain on the outer edges of cell and stains its background.

Procedure

1) The dirt and grease is removed from the glass slide by washing it with detergent and water. The slide should be hold by its edges during the complete process.
2) Using an inoculating loop, a loopful of bacterial suspension is placed at the right edge of the clean slide.
3) A drop of nigrosine solution is mixed with the bacterial suspension. During mixing, the drop should not be spread.
4) A spreader slide is used to spread the mixture by holding the slide at an angle of 45° to form a thin film of bacterial suspension.
5) After drying the film, the slide is applied with immersion oil.
6) A thin area under oil immersion is focused and the unstained cells surrounded by the grey stain are observed.

Negative staining is used for bacteria which are stained with difficulty, **e.g.,** spirili. This technique does not involve harsh physical (heating) or chemical treatments, thus the bacterial morphology remains unaltered.

5.1.4.2. Monochrome Staining

Monochrome staining is used for determining bacterial morphology. Since **mono** means single and **chrome** means colour, this staining technique involves use of a single colour stain for colouring the bacterial cell.

Principle

In monochrome staining, the negatively charged bacterial cell attracts the basic or cationic stains (**e.g.,** methytene blue, crystal violet, etc.). These stains are positively charged due to the presence of chromophore (the colour-bearing cation).

Procedure

1) The dirt and grease is removed from the glass slide by washing it with detergent and water. The slide should be hold by its edges during the complete process.
2) A half-inch (approximately 1.5cm) target circle is drawn on the centre of the slide, and then it is inverted.
3) A loopful of bacterial suspension is placed on the centre of slide and uniformly spread throughout the target circle.
4) The slide is air-dried by evaporating the water.
5) After drying, the microorganisms are heat fixed by passing the slide through Bunsen burner flame.
6) The bacterial suspension is stained either on a staining rack near a sink or in a koplin jar.
7) After staining, the slide is left for a few seconds; 2-6 seconds for crystal violet, 60-120 seconds for methylene blue, and 15-30 seconds for carbol fuchsin.
8) The stain is gently washed away by keeping the slide under tap water.
9) The slide is air-dried, and observed under oil immersion objective.

5.1.5. Differential Staining

In differential staining process, more than one chemical stain are used for better differentiation between different microorganisms, structures, cells, and sub-cellular structures. This staining technique is also used for the detection of abnormalities in the proportion of different white blood cells in the blood, and the process or result is known as WBC differential. Most frequently used differential stain for bacteria are the **gram stain** and **acid-fast stain**.

5.1.5.1. Gram's Staining

Gram's staining (or Gram's method) is a technique which is used to differentiate bacterial species into **two large groups**, i.e., **gram-positive** and **gram-negative**, on the basis of physical and chemical properties of their cell-wall. Gram staining is also based on the composition of cell wall. In gram staining, crystal violet stain is used to stain the cell wall, iodine is used as a mordant, and a fuchsin or safranin is used to counter stain to mark all bacteria. Gram-positive bacteria take dark blue or violet stain, gram-negative organisms appear red or pink.

Principle

On colouring the bacteria with a basic dye (**e.g.,** crystal violet) followed by iodine treatment, the dye and the iodine combine to form a dye-iodine complex in the cytoplasm; as a result bacteria appear dark violet or purple in colour. Later on, treating with alcohol or acetone, certain bacteria resist the decolourising step and retain the previous violet colour; these bacteria are gram-positive. On the other hand, few bacteria on decolourisation lose the violet colour and become colourless. These bacteria when coloured with basic dyes (**e.g.,** fuchsin or safranin), take up the counter stain and appear pink; these bacteria are gram-negative.

Procedure

1) Bacterial smear is prepared and kept in heat for drying and fixation.
2) A drop of Hucker ammonium oxalate crystal-violet is added and retained for 60 seconds.
3) The smear is washed with water and gram iodine is added allowing retaining time for 60 seconds, followed by rinsing with tap water.

) Thereafter, the smear is decolourised with 95% alcohol, retained for few seconds, and washed again with water.
) Safranin is added for 60 seconds, rinsed with water, and blotted dry with tissue cautiously.
) At the end of staining, the gram-positive bacteria retain purple colour and gram-negative bacteria retain pink colour.
) Basic dyes penetrate the bacterial cell and react with the acidic compounds present in the protoplasm.

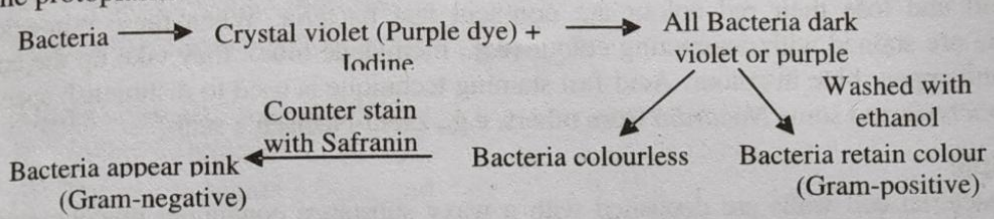


Figure 5.1: Procedure of Gram's Staining

Result

Table 5.1 lists the changes the gram-positive and gram-negative bacteria undergo when reated with different stains:

Table 5.1: Changes the Gram-Positive and Gram-Negative Bacteria Undergo

Gram Staining Stems	Gram-Positive	Gram-Negative
Step 1: Crystal Violet Primary stain added to sample smear	Stains cells purple or blue	Stains cells purple or blue
Step 2: Iodine Mordant makes the dye less soluble so it adheres to cell walls.	Cells remain purple or blue	Cells remain purple or blue
Step 3: Alcohol The decoloriser washes away stain from gram-negative cell walls	Cells remain purple or blue	Cells are colourless
Step 4: Safranin Counter-stain allows dye adherence to gram-negative cells	Cells remain a purple or blue	Cells appear pink or red

Difference between Gram-Positive and Gram-Negative Bacteria According to Staining

Table 5.2 shows the difference between gram-positive and gram-negative bacteria on the **basis of their appearance:**

Table 5.2: Difference between Gram-Positive and Gram-Negative Bacteria

Characters	Gram-Positive Bacteria	Gram-Negative Bacteria
1) **Staining Ability**	Absorb gram stain and appear purple coloured.	Do not absorb gram stain.
2) **Cell Wall**	Monolayered (only of murein)	Two layered, outer of glycolipids and inner of murein.
3) **Thickness of Cell Wall**	100-200 Å	70-120 Å
4) **Murein Percentage**	70 -80%.	10-20% only
5) **Lipids Quantity**	Less	More
6) **Mesosomes**	Present	Absent
7) **Teichoic Acids**	Present (mostly)	Absent
8) **Periplasmic Space**	Absent	Present
9) **Outer Membrane**	Absent	Present
10) **Peptidoglycan Layer**	Multilayered (thick)	Single-layered (thin)
11) **Resistance to Physical Disruption**	Marked	Much less
12) **Toxins Produced**	Primarily exotoxins	Primarily endotoxins
13) **Examples**	*Diplococcus pneumoniae*	*Escherichia coli.*

5.1.5.2. Acid-Fast Staining

In the cell wall of few bacteria, a waxy material is found in abundant that prevents the easy penetration of the stain into the cell, when stained using ordinary techniques. After staining with carbol fuchsin, on heating or by prolonged exposure of heat, the bacteria take up the dye and appear red. They prevent the decolourising action of the acid and retain their red colour, this process is thus known as 'acid-fast staining'. Those bacteria which are decolourised by the acid and lose their red colour are non-acid fast bacteria. When these non-acid fast bacteria are stained with contrasting colour (**e.g.,** methylene blue), they take up the counter stain and appear blue in colour. Acid-fast staining technique is used to distinguish species of *Mycobacteria* and some *Nocardia* from others, **e.g.,** Ziehl-Neelsen's stain.

Principle

Mycobacterial cell walls are deposited with a waxy substance containing mycolic acids (β-hydroxy carboxylic acids of up to 90 carbon atoms chain lengths). The property of acid fastness is dependent on the carbon chain length of mycolic acid found in any particular species.

Procedure

1) Air-dried, heat-fixed smears of bacterial species are prepared.
2) The smear is flooded with **carbol fuchsin stain** (a lipid soluble, phenolic compound with the ability to penetrate cell wall).
3) This flooded smear is covered with filter paper.
4) It is then steamed for 10 minutes, and more carbol fuchsin stain is added.
5) The slide is cooled and washed with distilled water.
6) Then the slide is flooded with **acid alcohol** (containing 3% HCl and 95% ethanol) and left for 15 seconds.
7) The slide is tilted over a sink at 45° angle and acid alcohol is added drop wise until the red colour stops flowing from the smear.
8) The slide after washing with distilled water is stained with **Loeffler's Methylene Blue stain** (a counter-stain adding blue colour to non-acid fast cells) and left for a minute. Then the slide is washed and dried. Oil immersion objective is used to view the slide.

Result

Table 5.3 illustrates the acid-fast staining process and the associated results:

Table 5.3: Ziehi-Neelsen Acid-Fast Staining Procedure

Acid-fast (+) Bacilli	Steps for Staining	Acid-fast (–) Bacilli
1) Remains colourless	Cells on slide	Remains colourless
2) Stains red	Primary stain (carbolfuchsin red)	Stains red
3) Remains red	Decoloriser (HCl, alcohol)	Becomes colourless
4) Remains red	Counterstain, (methylene blue)	Stains blue

5.1.6. Special Staining

Special staining techniques are used to colour certain specific parts of bacteria, **e.g.,** volutin granules (Albert's or Neisser's stain), capsules (Muir's method), spores (Schaffer and Fulton's), flagella, spirochaetes (Fontana's or Levaditi's method), rickettsias (Giemsa's or Machiavelli's stain), etc. Special staining is used for identifying chemical constituents, antigens, for staining bacteria in tissue sections, etc.

5.1.6.1. Albert's Staining for *C. diphtheria*

In suspected diphtheria cases, one of the smears is stained with Gram stain. If Gram stained smear shows morphology similar to that of *C. diphtheria* (thin gram-positive bacilli; straight or slightly curved; enlarged at one or both ends; and arranged at acute angles forming V-shapes), Albert staining is done which **detects the presence or absence of metachromatic granules**. Presence of a large number of metachromatic granules in *C. diphtheria* bacillus gives it a beaded or barred appearance. These granules are detected using Albert's stain.

Materials Required

1) **Albert stain I:**
   i) Toluidine blue 0.15gm,
   ii) Malachite green 0.20gm,
   iii) Glacial acetic acid 1.0ml,
   iv) Alcohol (95%) 2.0ml, and
   v) Distilled water 100ml.

2) **Albert stain II:**
   i) Iodine 2.0gm,
   ii) Potassium iodide 3.0gm, and
   iii) Distilled water 300ml.

Procedure

1) The heat-fixed smear is covered with Albert stain I and left for 2 minutes.
2) It is then washed with water.
3) Again the smear is covered with Albert stain II and left for 2 minutes.
4) It is then washed with water and dried.
5) The smear is examined to detect the presence of metachromatic granules (in *C. diphtheria*) which appear bluish black, while the bacilli body appear green or bluish green.

5.1.6.2. Capsule Staining

The capsule stain detects the presence of bacterial capsule, since viewing the water-soluble capsule of some bacterial cells using standard simple staining procedures or Gram's staining is often difficult. Capsule staining was developed for viewing bacterial capsules and yielding consistent and reliable results. On smearing the fresh sample with **Gram's** or **Leishman stain**, and **Negative stain** (using India ink or Nigrosin), the bacterial capsule appears clear halo.

Procedure

1) A loop full of India ink (used to view capsule which appears as unstained halo around the organisms distributed in a black background **e.g.,** *Cryptococcus*) is placed on the slide.
2) A small portion of the culture is emulsified in a drop of India ink.
3) A clean cover slip is placed over the preparation, taking care of avoiding bubble formation; and pressed down gently.
4) The slide is then examined under dry objective.

5.1.6.3. Endospore Staining

Bacterial endospores are metabolically inactive and highly resistant structures, produced by some bacteria themselves for resistance against unfavourable environmental conditions. **Primary stain** (malachite green) used for staining both vegetative cells and endospores, is subjected to heat so that it penetrates the endospore. After primary staining, the smear is decolourised with water to remove the malachite green from the vegetative cell, but not from the endospore. The decolourised cells are then **counter-stained** with safranin, as a result of which the vegetative cells appear pink and endospores appear dark green.

5.1.6.4. Flagella Staining

Flagella are fragile appendages which cannot be viewed using ordinary microscopes. Therefore, its surface is coated with a precipitate to form a colloidal substance. This precipitate forms a layer of stainable material.

Materials

1) 1% Osmic acid.
2) Mordant (10% tannic acid, potassium alum, and 10% ferric chloride), and
3) Fontana's silver solution.

After the process of flagella staining, the organisms appear black and flagella appear light brown, thus making their detection easy.

5.1.7. Biochemical Tests

Biochemical tests differentiate various microorganisms, based on the production of enzymes to observe a biochemical change with a substrate and the bacterial colony. Biochemical tests are rapid and utilise only a small amount of bacteria from a colony growing on a plate to obtain results.

Some of the commonly used biochemical tests are given below:

1) Sugar fermentation test,
2) Indole production test,
3) Urease production test,
4) Citrate utilisation test,
5) Catalase test,
6) Oxidase test,
7) Phenylalanine deaminase test,
8) Methyl red reduction test,
9) Voges-Proskauer (VP) or acetoin production test,
10) Nitrate reduction test,
11) Triple-sugar iron agar,
12) Hydrogen sulphide production test, and
13) Growth in KCN.

5.1.8. IMViC Test

This test is a series of four tests used for identifying *E. coli* and *Enterobacter aerogenes*. The letters in IMViC stands for:

'I' - Indole production test,
'M' - Methyl red reduction test,
'V' - Voges-Proskauer test,
'C' - Citrate utilisation test, and
'i' - Has no significance with the test systems.

Table 5.4 shows the IMViC test results for *E. coli* and *Enterobacter aerogenes*:

Table 5.4: IMViC Test Differences

Organism	Tests				
	I	M	V	C	Pollution Source
E. coli	+	+	–	–	Faecal
Ent. Aerogenes	–	–	+	+	Non-faecal

5.1.8.1. Indole Production Test

Amino acids form the basic constituents of proteins present within the living organisms. Some microorganisms obtain energy by degrading the amino acids into a variety of end products of ammonia, indole acid, and water. Many reactions involving amino acids' degradation are used for classifying the *Enterobacteriaceae*.

Certain amino acids-degrading bacteria, like *E. coli*, degrade tryptophan into indole and pyruvic acid via hydrolysis in the presence of tryptophanase enzyme.

Principle

Indole is a nitrogen-containing, degradation end-product of tryptophan amino acid. Indole test is important as only a few bacteria can form indole, which can be detected easily using **Kovac's reagent**. After this reagent is added and the contents are mixed, the tube is left undisturbed. The alcohol and the aqueous layer separate out from each other; and the alcohol layer starts reddening thus confirming the presence of indole in the culture. Therefore, formation of a red layer at the top of the culture renders the test positive. Pure tryptophan is usually not used in the test; rather tryptone containing tryptophan is used as a substrate.

$$\text{Tryptophan} \xrightarrow{\text{Tryptophanase}} \text{Indole} + \text{Pyruvicacid} + H_3N$$

$$\text{Indole} + \text{Kovac's reagent} \xrightarrow[\text{(Butanol)}]{\text{HCl}} \underset{\text{(Cherry red compound)}}{\text{Rosindole}} + H_2O$$

Materials Required

1) Bacterial culture (*E. coli*, *Klebsiella pneumonae, Enterobacter aerogenes*),
2) Tryptone broth,
3) Paper strip,
4) Oxalic acid,
5) Incubator,
6) Inoculation loop,
7) Indole tryptone broth*, and
8) Kuvac's reagent* *.

*Indole Tryptone Broth Medium	Quantity
Tryptone	10gm
Distilled water	1 litre
** Kovac's Reagent	
p-dimethylaminobenzaldehyde	15gm
Isoamyl alcohol	150ml
HCl	75ml

Procedure [Figure 5.2 (A)]

1) Tryptone broth is prepared and inoculated with bacterial culture.
2) The culture is incubated for 2 days at 37°C.
3) 0.5ml of Kovac's reagent is added after the bacterial growth is observed.
4) After 5 minutes of adding the reagent, a red layer forms at the junction of medium and reagent (as in case of *E. coli*). On the other hand, indole is diffused above the medium and on touching with a paper strip impregnated with oxalic acid the strip turns pink.

Result

Formation of a red coloured layer near the medium surface renders the indole test positive (*E. coli* is indole-positive bacteria); while formation of a yellow coloured layer renders the test negative (*K. pneumonae* and *E. aerogenes* are indole-negative bacteria).

5.1.8.2. Methyl Red (MR) Reduction Test

The enteric bacteria with respect to glucose fermentation and product formation are categorised into two groups (i.e., the **mixed acid fermenters** and **2,3-butanediol fermenters**). The mixed acid fermenters produce a large amount of acids and the butanediol fermenters produce **acetoin** (the neutral end product, also called acetyl methyl carbinol). Both these tests are performed simultaneously on the same medium [Methyl Red (MR) and Voges-Proskauer (VP)], thus it is also named **MR-VP test**.

E. coli and Proteus bacteria produce a large amount of lactic acid, acetic acid, succinic acid, and formic acid by glucose fermentation. Also carbon dioxide, hydrogen, and ethyl alcohol are formed. When the bacteria do not convert the acidic product to a neutral product, the medium pH is 5 or less. Such acids are **mixed-acid fermenters**.

MR-VP medium is a glucose broth having buffered peptone and dipotassium phosphate. It is inoculated with test microorganisms and then added with **methyl red indicator** which is yellow at 5.2 pH and red at 4.5 pH. The indicator turning red signifies that the organism is a mixed-acid fermenter, thus a positive test; while the indicator turning yellow renders the test negative.

Principle

MR-VP test detects the production of acid by bacteria during glucose fermentation and also maintains the culture medium at pH 4.5 or below.

Materials Required

1) Methyl red indicator,
2) Sterilised test tubes,
3) Conical flasks,
4) Glass rods,
5) Cotton plugs,
6) Test culture, and
7) MR-VP media.

Procedure [Figure 5.2 (B)]

1) Methyl red indicator is prepared by dissolving 0.1gm of methyl red in 300ml of 95% ethyl alcohol and 300ml of distilled water, followed by filtration.
2) MR-VP media (glucose phosphate broth) is prepared using the following ingredients:
   i) Dipotassium hydrogen phosphate (5gm),
   ii) Peptone (5gm),
   iii) Glucose (5gm), and
   iv) Distilled water (1000ml).
3) These ingredients are added in distilled water and gently warmed. The pH should remain unchanged. 5ml of media is distributed in plugged test tubes and sterilised for 15 minutes at 121°C.
4) The tubes (except the control tube) are inoculated with the test bacterial culture.
5) All the tubes are incubated for 2 days at 37°C.
6) 5-10 drops of methyl red indicator are added in each tube.
7) Formation of a red colour renders the test positive; while a yellow colour renders the test negative.

Result

Formation of red colour renders the MR test positive (*E. coli*, *Yersinia* sp., and *Listeria monocytogenes* are MR-positive bacteria); while formation of yellow colour renders the MR test negative (*Klebsiella* sp., and *Enterobacter* sp. are MR-negative bacteria).

5.1.8.3. Voges-Proskauer (VP) Test

All species of *Enterobacter* and *Serratia*, and some species of *Bacillus* and *Aeromonas* produce 2,3-butanediol and ethyl alcohol in large amounts. Such organisms are **butanediol fermenters**. Production of these non-acid end products results in less lowering of the pH in MR-VP media, and thus rendering the test negative.

Principle

Butanediol fermenters produces 2,3-butanediol, which cannot be detected by any suitable test. On the other hand, acetyl methyl carbinol or acetoin ($CH_3CHOHCOCH_3$) produces 2,3-butanediol which can be detected easily with **Barrit's reagent**. This test is valid as acetoin and 2,3-butanediol are present simultaneously. In VP test, acetyl methyl carbinol is formed from glucose. Small amounts of acetyl methyl carbinol present in the medium oxidises into diacetyl in the presence of alkali, atmospheric oxygen, and Barrit's reagent. The resultant diacetyl produces a red colour with guanidine residue in the media; thus formation of red or pink colour indicates positive VP test.

Materials Required

1) Nutrient broth culture of *E. coli*, *K. pneumonae*, and *E. aerogenes*,
2) MR-VP broth*,
3) Barritt's reagent*,
4) Methyl red,
5) Bunsen burner,
6) Test tubes, and
7) Inoculation needle.

*MR-VP Broth	
Peptone	7.0gm
Glucose	5.0gm
Potassium phosphate	5.0gm
Distilled water	1 litre
pH	6.9

(5ml broth is added in each culture tube and autoclaved at 15lb/inch^2 for 15 minutes)

***Barritt's Reagent**

VP I reagent (5% α-naphthol dissolved in absolute alcohol)
VP II reagent (40%KOH solution)

Procedure [Figure 5.2 (C)]

1) This first step involves:
   i) **Preparation of MR-VP medium or glucose phosphate broth** utilising the following constituents:
      a) Dipotassium hydrogen phosphate (5gm),
      b) Peptone (5gm),
      c) Glucose (5gm), and
      d) Distilled water (1000ml).

      These ingredients are added in distilled water and gently warmed, without changing the pH. 3ml of media is distributed in plugged test tubes and sterilised for 15 minutes at 121°C.

   ii) **Preparation of Barrit's Reagent:** Barrit's reagent is prepared using solution A (prepared by dissolving 6gm of α-naphthol in 100ml of 95% ethyl alcohol) and solution B (prepared by dissolving 16gm of potassium hydroxide in 100ml of water).

2) The tubes are inoculated with the bacterial culture.
3) The culture is then incubated for 2 days at 37°C.
4) 2 ml from each culture tube is transferred into clean separate tubes, using separate pipettes for each transfer.
5) 18 drops (0.6ml) of Barrit's solution A (α-naphthol) is added to each media-containing tube.
6) An equal amount of solution B is also added in the same tubes.
7) The tubes are vigorously shaken after every 30 seconds.
8) Formation of pink colour, which turns red in 1-2 hours after vigorous shaking indicates a positive test. This step is very important for achieving complete aeration.

Result

Formation of pink colour within 2-5 minutes which deepens into crimson colour in 30 minutes renders the VP test positive (*Klebsiella* sp., *Enterobacter* sp., *Eltor vibrios*, and *Staphylococcus* are VP-positive bacteria); while a colourless medium for 30 minutes renders the VP test negative (*E. coli* and *Micrococcus* are VP-negative bacteria).

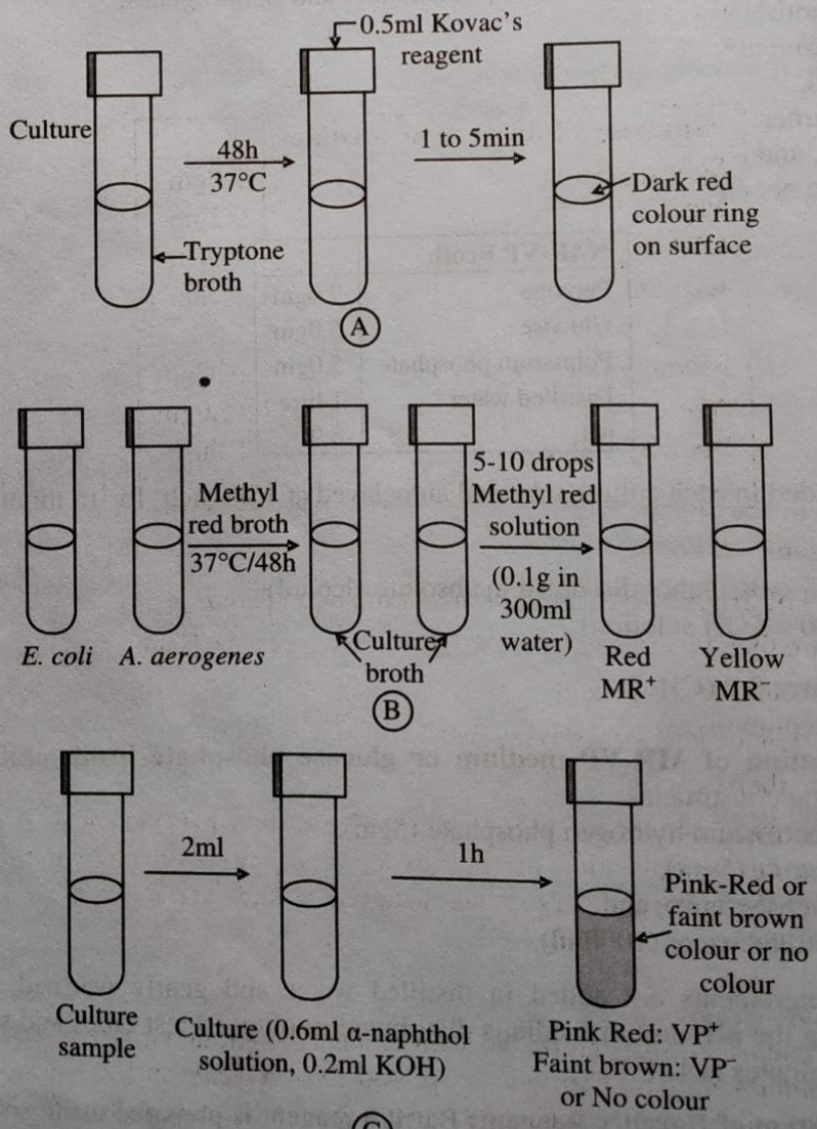


Figure 5.2: IMViC Test. Indole Production Test (A); Methyl Red Reduction Test (B); Voges Proskauer Test (C)

5.1.8.4. Citric Acid (Citrate) Utilisation Test

Salmonella typhi and *Escherichia aerogen* bacteria obtain carbon from citrate. This ability of utilising citrate as the carbon and energy source is used as a distinguishing test for some Grods.

Principle

Simmons citrate agar containing sodium citrate as the carbon source is used to detect whether or not citrate is utilised by the organism. This agar contains **Bromothymol blue indicator** which undergoes a colour change from green to blue when bacterial growth utilises citrate and turns the media alkaline. This colour change from green to blue indicates a positive test.

Materials Required

1) Simmon's citrate agar medium*,
2) Cultures of *E. coli* and *Enterobacter aerogenes*,
3) Inoculation needle,
4) Culture tubes, and
5) Incubator.

*Simmon's Citrate Agar Medium	
Sodium citrate	2.0gm
$MgSO_4$	0.2gm
$(NH_4)H_2PO_4$	1.0gm
$K_2H.PO_4$	1.0gm
NaCl	5.0%
Bromthymol blue	0.08gm
Agar	15.0gm
Distilled water	1 litre
pH	7.0

Procedure

1) A few slants of Simmon's citrate agar medium are prepared.
2) The slants are then inoculated by stabbing to the base of the slant.
3) The medium surface is streaked.
4) The tubes are incubated for 2 days at 37°C.
5) The tubes are then examined.
6) If bacterial growth occurs the culture turns blue due to pH change as it becomes alkaline after citrate utilisation (as in case of *E. aerogenes*); while if there is no visible growth the culture undergoes no change in colour (and remains green) due to unutilised citrate (as in case of *E. coli*).

Result

Formation of an intense blue colour on the slant renders the citrate utilisation test positive (*Klebsiella* sp., *Salmonella* sp., *Citrobacter* sp. and *Enterobacter* sp. are citrate-positive bacteria); while no colour change renders the test negative (*E. coli*, *Edwardsiella*, and *S. typhi* are citrate-negative bacteria).

5.2. SUMMARY

The details given in chapter can be summarised as follows:

1) Various stains and dyes have been used to stain bacterial cells or any sub-cellular structure/component.
2) Stains are broadly classified into three classes, i.e., **acidic**, **basic**, and **neutral**.
3) On the bacterial cells, slightly negative charge is present; therefore basic dyes carrying a positive charge (**e.g.,** crystal violet, methylene blue, carbol fuchsin, and safranin), give best results with them.
4) Negatively charged or acidic stains (**e.g.,** eosin Y and nigrosine) do not react with bacterial cell and get deposited around the bacterial cells; this is also known as **negative staining**, because the cell remains unstained but background become coloured.
5) **Neutral stains** (**e.g.,** Sudan III and Toluidine Blue) are specifically used to stain the fat inclusions and polyphosphate granules respectively.
6) Staining is a basic step of microscopy which is used to enhance the contrast of microscopic image.
7) In **simple staining**, either alcoholic or aqueous solution of a single basic dye is used, and the dye is washed off with water after 30-60 seconds, before drying and examination.
8) **Methylene blue**, **dilute carbol fuchsin**, and **polychrome methylene blue** are some examples of commonly used simple stains.
9) **Negative** or **indirect staining** technique is used when the sample is to be viewed under a light microscope.
10) In **monochrome staining**, the negatively charged bacterial cell attracts the basic or cationic stains (**e.g.,** methytene blue, crystal violet, etc.).
11) In **differential staining** process, more than one chemical stain is used for better differentiation between different microorganisms, structures, cells, and sub-cellular structures.
12) **Gram's staining** (or Gram's method) is a technique which is used to differentiate bacterial species into **two large groups**, i.e., **gram-positive** and **gram-negative**
13) On colouring the bacteria with a basic dye (**e.g.,** crystal violet) followed by iodine treatment, the dye and the iodine combine to form a dye-iodine complex in the cytoplasm, as a result bacteria appear dark violet or purple in colour.
14) The bacteria which do not retain the gram stain are called as **gram-negative bacteria**, while those retain the gram stain are called the **gram-positive bacteria**.
15) **Acid-fast staining** technique is used to distinguish species of *Mycobacteria* and some *Nocardia* from others, **e.g.,** Ziehl-Neelsen's stain.
16) **Special staining** techniques are used to colour certain specific parts of bacteria, **e.g.,** volutin granules (Albert's or Neisser's stain), capsules (Muir's method), spores (Schaffer and Fulton's), flagella, spirochaetes (Fontana's or Levaditi's method), rickettsias (Giemsa's or Machiavelli's stain), etc.
17) If Gram's stained smear shows morphology similar to that of *C. diphtheria*, Albert staining is done which **detects the presence or absence of metachromatic granules**.
18) The **capsule stain** detects the presence of bacterial capsule, since viewing the water-soluble capsule of some bacterial cells using standard simple staining procedures or after the Gram's stain is often difficult.

19) **Biochemical tests** differentiate various microorganisms. These tests are based on the production of enzymes to observe a biochemical change with a substrate and the bacterial colony.

20) **IMViC test** is a series of four tests used for identifying *E. coli* and *Enterobacter aerogenes*. The letters in IMViC stands for:
'I' – Indole production test,
'M' - Methyl red reduction test,
'V' - Voges-Proskauer test,
'C' - Citrate utilisation test, and
'i' - Has no significance with the test systems.

21) Indole is a nitrogen-containing, degradation end-product of tryptophan amino acid. **Indole test** is important as only a few bacteria can form indole, which can be detected easily using **Kovac's reagent**.

22) **MR-VP test** detects the production of acid by bacteria during glucose fermentation and also maintains the culture medium at pH 4.5 or below.

23) *Salmonella typhi* and *Escherichia aerogen* bacteria utilise citrate as the only carbon source. This ability of utilising citrate as the carbon and energy source is used as a distinguishing test for some Grods and the test is known as **Citric Acid (Citrate) Utilisation Test**.

5.3. EXERCISE

5.3.1. True or False

1) Acidic stains are used to stain the fat inclusions and polyphosphate granules respectively.
2) In simple staining, more than one reagent is used for staining the bacterial smear.
3) The bacteria which do not retain the gram stain are called as gram-positive bacteria.
4) In differential staining process, more than one chemical stain is used for better differentiation between different microorganisms, structures, cells, and sub-cellular structures.
5) In gram staining, crystal violet stain is used to stain the cell wall, iodine is used as a mordant, and a fuchsin or safranin is used to counter stain to mark all bacteria.
6) MR-VP test detects the production of acid by bacteria during glucose fermentation and also maintains the culture medium at pH 4.5 or below.

5.3.2. Fill in the Blanks

7) The bacteria which retain the gram stain are called _____.
8) In _____ staining the bacterial cell remains colourless while the background gets stained.
9) Biochemical tests are based on the production of enzymes to observe a _____ with a substrate and the bacterial colony.
10) IMViC test is a series of four tests used for identifying _____ and_____.
11) Cell wall of _____ consists of a waxy substance containing mycolic acids.
12) This ability of utilising citrate as the carbon and energy source is used as a distinguishing test for some Grods and the test is known as_____.

Answers:

1) False 2) False 3) False 4) True 5) True
6) True 7) Gram-positive bacteria 8) Negative 11) Mycobacteria
9) Biochemical change 10) *E. coli*, *Enterobacter aerogenes*
12) Citric Acid (Citrate) Utilisation Test

5.3.3. Very Short Answer Type Questions

1) What is monochrome staining?
2) Enlist various techniques of staining.
3) What are stains?
4) Give examples of stains used in simple staining.
5) Write the full form of IMViC test.
6) What is negative staining?

5.3.4. Short Answer Type Questions

1) Differentiate between gram-positive and gram-negative bacteria.
2) Give the principle and procedure of negative staining.
3) Write a note on acid-fast staining.
4) What are biochemical tests?
5) Write the principle and procedure of Gram's staining.
6) Give application and aim of staining.

5.3.5. Long Answer Type Questions

1) Write a detail note on differential staining technique.
2) What is simple staining? Explain various types of simple staining.
3) Write a note on IMViC test.

CHAPTER 6

Sterilisation

6.1. STERILISATION

6.1.1. Introduction

Sterilisation is the process of **effective killing** or **removal of transmissible agents** or **microorganisms** (**e.g.,** bacteria, fungi, virus, and prions), from any surface, food, equipment, medications, or biological culture medium.

Practically sterility is achieved by exposing the object to be sterilised to chemicals or physical agents for a specified time period. For sterilisation process various agents are used, **e.g.,** ionising radiation, temperature, chemical liquids or gases, etc. The efficiency and the success of sterilisation process, directly depend on the method of sterilisation used.

Importance of sterilisation can be summarised in the following points:

1) **Moist heat sterilisation** is the most efficient bactericidal technique or method. In pharmaceutical operations, this method is used to sterilise containers, closures, aqueous injections, ophthalmic preparations, surgical dressings, sheets, surgical and diagnostic equipment, etc.
2) **Dry heat sterilisation** is used for the sterilisation of medicinal and pharmaceutical products with some specific properties (such as moisture impermeable, moisture sensitive, and thermostable). Many other products like dry powdered drugs, suspensions of drug in non-aqueous solvents, oils, fats, waxes, soft hard paraffin, oily injections, implants, ophthalmic ointments, ointment bases, etc., are also sterilised by using dry heat method.
3) **Gaseous method of sterilisation** is preferred for thermolabile substances, **e.g.,** proteins, various heat sensitive drugs, hormones, etc.
4) **UV radiation** is used to sanitise garments or utensils used in pharmaceutical or medical operations or task. UV light is a most lethal component of sunlight.
5) **Gamma-rays** emitted from the source Cobalt 60 is used to sterilise hormones, antibiotics, catheters, sutures, plastic containers, etc.
6) **Filtration sterilisation method** is used for sterilising heat sensitive injections, ophthalmic solutions, biological products, air and other gases supplied to aseptic areas. Filtration method is also used in industry as part of venting system on fermenters, centrifuge, freeze driers, and autoclaves. For sterility testing, membrane filters are used.

6.1.2. Methods of Sterilisation

For sterilisation following methods are used:

1) **Physical Methods**
   i) Sunlight
   ii) Dry heat sterilisation:
      a) Incineration,
      b) Red heat,
      c) Flaming, and
      d) Hot air oven.

iii) Moist heat sterilisation:
   a) Autoclave,
   b) Tyndallisation, and
   c) Pasteurisation.

iv) Radiation sterilisation:
   a) By ionising radiation, and
   b) By non-ionising radiation

v) Ultrasonic and sonic vibration

2) **Chemical Methods**
   i) Sterilisation by heating with bactericide, and
   ii) Gaseous sterilisation.

3) **Mechanical/Filtration Methods**
   i) Depth filters,
   ii) Membrane filters, and
   iii) HEPA filters.

6.1.3. Physical Methods

Since Stone Age, humans have preserved foods using drying (desiccation) and salting (osmotic pressure) physical methods of microbial control.

The methods of microbial control should be selected based on their effects on the substances to be sterilised as well as microbes.

For example, heating inactivates some vitamins or antibiotics in a solution; repeated heating damages various laboratory or hospital materials (like rubber, latex tubing, etc.). Also economic methods should be selected, **e.g.,** use of pre-sterilised, disposable plastic ware should be less expensive than repeatedly washing and re-sterilising glassware.

6.1.3.1. Sunlight

Sunlight possesses germicidal activity due to the combined effect of UV and heat rays, thus effectively kills bacteria in water and other surfaces of earth in tropical countries. Sunlight can even kill typhoid bacilli in 2 hours; however the effectiveness depends on the UV ray amount passing through the ozone atmosphere and the amount of heat it can produce. This method is clinically not in practice.

6.1.3.2. Dry Heat Sterilisation

Heat sterilisation is the most reliable and widely used sterilisation method, in which enzymes and other essential cell constituents are destroyed. This process is more effective in hydrated state where hydrolysis and denaturation occur under high humidity conditions, thus requiring lower heat input. While in dry state, oxidation occurs which require higher heat input.

Principle

Oxidative process is involved in the killing of organisms by dry heat sterilisation. Oxidative process is less effective than the hydrolytic damage which involves exposure to steam.

Therefore, dry heat sterilisation usually employs **higher temperatures** ranging from **160-180°C** and requires **exposures time** of up to **2 hours** depending on the temperature used.

Procedures

Dry heat sterilisation is performed by the following methods:

1) **Incineration:** In this process, the objects are burnt in direct flame. This method is efficient and frequently used for sterilising and disposing the contaminants at a high temperature. Incineration is suitably used for disposable items, **e.g.,** animal carcasses, clinical waste, pathological waste materials, surgical dressings, and other contaminated materials.

2) **Red Heat:** In this procedure, the articles are exposed to flame till they become red hot. It is used for sterilising the tips and points of spatula, forceps, inoculating loop, knives, etc. Points of forceps, inoculating wires and loops are sterilised by holding them almost vertically in a Bunsen flame till they become red hot.

3) **Flaming:** In this procedure, the article is passed through the flame many times without allowing it to become red hot. Flaming is used to sterilise articles used in laboratories, **e.g.,** mouths of test tubes and flasks, glassware, cover slip, scalpels, needles, etc.

4) **Hot Air Oven:** It is the most common method to carry dry heat sterilisation (**figure 6.1**). The hot air oven consists of an insulated polished stainless steel chamber, surrounded by an outer case containing electric heaters to prevent development of cool spots inside the chamber. It is available up to 250L of capacity. On the rear side of the oven, a fan is present to provide circulating air that ensures more rapid equilibration of temperature.

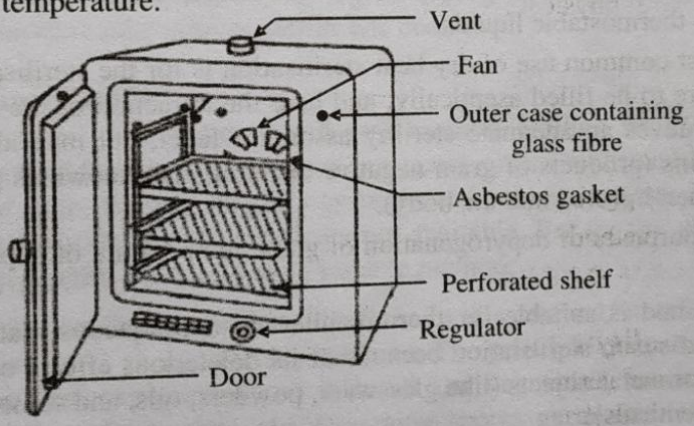


Figure 6.1: Hot Air Oven

To allow proper airflow, perforated shelves are present in the chamber. Thermocouples are used to monitor the temperature of both the oven air and articles in the chamber. To record the sterilisation cycle, a fixed temperature sensor is connected to a chart or digital recorder. For the prevention of interruptions of sterilisation cycle, proper door-locking controls should be present.

Articles to be sterilised should be enclosed or wrapped in containers (**e.g.,** paper, cardboard tubes, or aluminium container) with sufficient strength and integrity to provide good post-sterilisation protection against contamination. The container is designed in such a way that heat penetration is encouraged in order to shorten the heating-up stage. For this purpose narrow containers with dull, non-reflecting surfaces are used.

In a hot air oven, heating is done by radiation and convection therefore they should be arranged carefully within the chamber to avoid obscuring in centrally placed articles from wall radiation or impending air flow. The variations in temperature within the chamber should not exceed ±5°C of the recorded temperature. In case of

articles with poor heat-conducting properties, the heating-up time (which may be as long as 4 hours) can be reduced by pre-heating the oven before loading it. Following the sterilisation, the temperature of oven chamber is normally allowed to drop up to 40°C before removal of sterilised articles. To accelerate the process, forced cooling with filtered air is also used.

Merits

1) The installation and operating costs of dry heat cabinet is less.
2) Heat can easily penetrate the materials.
3) Dry heat sterilisation is non-toxic and does not harm the environment.
4) It is non-corrosive to metals and sharp instruments.

Demerits

1) It is a time-consuming method due to slow rate of heat penetration and microbial killing.
2) Most of the materials cannot withstand high temperatures.

Applications

1) Practically, the use of dry heat sterilisation is quite limited, and largely used for items used in hospitals.
2) It is mainly used for metal surgical instruments (where its good penetrability and non-corrosive nature are of benefit), glassware, thermostable powders, and non-aqueous thermostable liquids.
3) The most common use of dry heat sterilisation is for the sterilisation of glass bottles which are to be filled aseptically; and here the attraction of the process is that it not only achieves an adequate sterility assurance level, but may also destroy bacterial endotoxins (products of gram-negative bacteria, also known as pyrogens, that cause fever when injected into the body).
4) For the purposes of depyrogenation of glass, temperature of approximately 250°C is used.
5) This method is suitable for thermolabile and non-aqueous materials which cannot withstand steam sterilisation because of its deleterious effects or penetration failure to glass or metal objects (like glassware, powders, oils, and some oil-based injectable pharmaceuticals).
6) Forceps, scissors, scalpels, and swabs can also be sterilised by this method.
7) The method is also used for sterilising pharmaceutical products like liquid paraffin, dusting powder, fats, and grease.

6.1.3.3. Moist Heat Sterilisation

Moist heat sterilisation utilises **steam** in the temperature range of **121-134°C**. Steam under pressure generates high temperature which is required for sterilisation. Saturated steam (steam in thermal equilibrium with water from which it is derived) is an effective sterilising agent. Steam for sterilisation can be either wet saturated (containing entrained water droplets) or dry saturated (without entrained water droplets).

Principle

The most reliable method for moist heat sterilisation is using moist heat under pressure in the form of saturated steam. Moist heat destroys the microbes by causing coagulation of cellular proteins. The wet proteins when heated, release free –SH groups and form smaller peptide chains; these chains further form complexes which differs from the original protein molecules.

Procedures

Moist heat sterilisation is used at different temperatures as given below:

1) **At a Temperature below 100°C**

i) **Pasteurisation of Milk:** This process is performed by two methods, first is **High Temperature Short Time** (HTST) or **flash method** (72°C for 20 seconds); and second is **holder method** (63°C for 30 minutes). After heating in both the methods, the quick cooling up to 13°C or lower is performed. Almost all non-sporing microbes, **e.g.,** *Mycobacteria*, *Brucellae*, and *Salmonellae* are killed, except *Coxiella burnetii* which being relatively heat resistant may survive in holder method.

ii) **Inspissation:** For this process, a special instrument inspissator is used. In this method, some serum or egg media (such as Lowenstein-Jensen's and Loeffler's serum) are rendered sterile by heating at 80-85°C temperature for half an hour daily for 3 consecutive days.

iii) **Vaccine Bath:** In this method, the bacterial vaccines are sterilised in special vaccine baths at 60°C for an hour. Body fluids and serum are sterilised by heating for an hour at 56°C in a water bath continuously for many days.

iv) **Low Temperature Steam Formaldehyde (LTSF) Sterilisation:** This method is used for those items that cannot withstand 100°C temperature. In this method of sterilisation, steam at sub-atmospheric pressure and at 75°C temperature with formaldehyde vapour is used. For testing the biological control efficacy of LTSF sterilisers, *Bacillus stearothermophilus* has been used.

2) **At a Temperature of 100°C**

i) **Boiling:** It is the most common and easy method. Boiling for 10-30 minutes may kill most of the vegetative forms but many spores withstand boiling for a considerable time. Boiling of glass syringes and rubber stoppers is performed when better methods of sterilisation are not available. But for the sterilisation of surgical instruments, boiling is not a reliable method.

ii) **Tyndallisation or Fractional Sterilisation:** In this method, steaming at 100°C for 20 minutes for 3 successive days is done. The principle of tyndallisation can be stated as first exposure which kills all the vegetative forms, and in the intervals between the heating, the remaining spores germinate into vegetative forms which are killed on subsequent heating. For tyndallisation process, Koch's or Arnold's steam sterilisers are used. This method is mainly used for the sterilisation of egg, serum, or sugar containing media which are damaged at higher temperature of autoclave.

iii) **Steam Steriliser at 100°C for 90 Minutes:** For this purpose, usually Koch's or Arnold's steam steriliser is used to sterilise those media that decomposes at high temperature of autoclave. In this, the item is placed on a perforated tray and exposed to steam for 90 minutes at atmospheric pressure. Almost all the vegetative forms are destroyed except thermophiles.

3) **At a Temperature above 100°C (Under Pressure):** Boiling occurs when the vapour pressure of the given substance reaches its atmospheric pressure. Therefore, on increasing the atmospheric pressure, the vapour pressure also increases, which results in increased boiling temperature. Normally, water boils at 100°C but when pressure inside a closed vessel increases, the temperature at which water boils also increases. This is also the **principle** of pressure cooker and autoclave shown in **figure 6.2**.

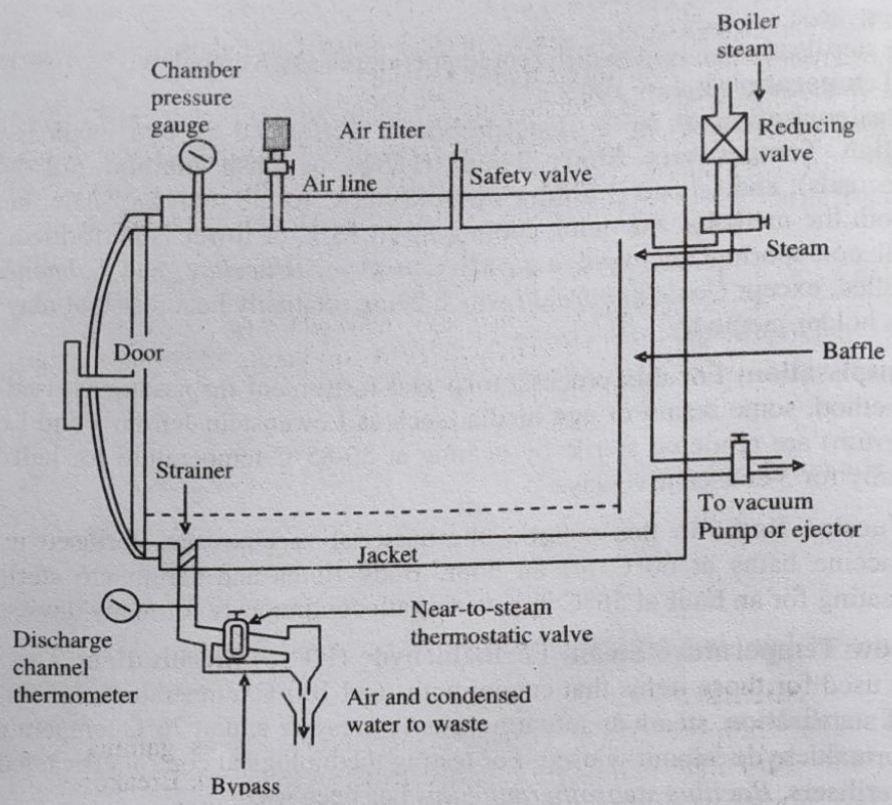


Figure 6.2: Autoclave

An **autoclave's mechanism of action** relies on the material to be sterilised. The **sterilisation cycle** comprises of the following **stages**:

i) Loading and packing the material in an autoclave,
ii) Raising its temperature and pressure,
iii) Holding the load at this temperature and pressure for a definite time interval, and
iv) Cooling and unloading the material from the autoclave.

Table 6.1 enlists the material holding time within an autoclave based on different temperature and pressure conditions:

Table 6.1: Autoclaving Conditions

Temperature (°C)	Corresponding Normal Pressure in Excess of Atmospheric Pressure (lb/in^2)	Recommended Minimum Holding Time (Minutes)
115-116	10	30
121-123	15	15
126-129	20	10
134-138	32	3

Merits

1) Under pressure the penetration power of steam is increased, that destroys the microbes with more efficiency than the dry heat. So lower temperature and less exposure time are required.
2) This method is useful in case of bulk material.
3) Dry saturated steam can be supplied for sterilising porous materials. Thus, this method can be used for wide range of substances.
4) High heat content rapidly transfers heat.
5) Large number of injections, ophthalmic solutions, irrigants, dialysis fluids, etc., can be sterilised by this method.

6) Moist heat effectively sterilises surgical dressings and materials due to its rapid penetration power into porous materials.
7) The method is used for sterilising plastic containers and other special dosage forms.
8) Sharp instruments can be sterilised more suitably with moist heat rather than with dry heat.
9) The entire sterilisation process can be accurately controlled and monitored.
10) The materials sterilised with moist heat contain no toxic contaminants.

Demerits

1) Autoclaving is not suitable for anhydrous materials, **e.g.,** powders and oils.
2) This method is also not used to sterilise thermolabile materials or substances that cannot withstand heating at 115°C and above.
3) Pyrogens cannot be destroyed by this method.

Applications

Moist heat sterilising agent is the most effective biocidal agent. It is used in pharmaceutical industries for sterilising surgical dressings, sheets, surgical and diagnostic equipment, containers, closures, aqueous injections, ophthalmic preparations, irrigation fluids, etc.

6.1.3.4. Radiation Sterilisation

For sterilisation purpose, many radiations are used such as gamma rays, UV light radiation, and particulate radiation (**e.g.,** accelerated electrons). Breakdown of microbial DNA is the main target of these radiations. UV light causes excitation, while the gamma rays and electrons cause ionisation and free radical production.

Principle

Ionising and **non-ionising radiations** are the two common types of radiation used for sterilisation. Ionising radiation uses radiation of short wavelength and high intensity to destroy the microbes; **for example,** gamma or X-rays react with cellular DNA and thus damages it. Non-ionising radiation uses radiation of longer wavelength and lower intensity, thus they fail to penetrate the substances effectively and are limited to surface sterilisation only; **for example**, UV light is used in various industries.

Procedures

Ionising and non-ionising radiation detailed below are used as radiation sterilising techniques:

1) **Ionising Radiation:** In this method, X-rays, gamma rays, and high-speed electrons are used. They have strong penetrating power, therefore, have strong sterility power. They are suitable for sterilising heat-sensitive pharmaceuticals, **e.g.,** antibodies, swabs, plastic, disposable items, hormones, and vitamins. Ionising radiation penetrates deep into the objects and kills the vegetative cells, bacterial endospores of both prokaryotic and eukaryotic microorganisms; however these radiations are ineffective against viruses.

   This method is also known as **cold sterilisation** as there is no notable increase of temperature. In this method many pathogens like *Staphylococcus aureus*, *Escherichia coli*, *Campylobacter jejuni*, etc., are killed. The **World Health Organisation** and **Food and Drug Administration** have approved this method safe for food irradiation. For the ionisation process high-speed electron, accelerator, gamma rays steriliser, and X-rays steriliser are generally used:

   i) **Electron Accelerator:** In this method, high energy electron beam are generated due to the acceleration of electrons from a hot filament down an evacuated tube under high potential difference. After this, additional energy is imparted to this

beam in a pulsed manner by a synchronised travelling microwave. **Two types** of electron accelerator apparatus are available:

a) **Electrostatic Accelerator:** It produces electrons with maximum energies of 5MeV.

b) **Microwave Linear Accelerator:** It produces electrons with maximum energies of 10MeV.

High energy electrons have better penetration power, but there is a risk of induced radiation. Articles to be sterilised are arranged on a horizontal conveyor belt and are irradiated from one or both the sides.

ii) **Gamma Ray Steriliser:** Cobalt-60 isotope is used as a source of gamma rays. Cobalt-60 is held as pellets packed in metal rods, and each rod is carefully arranged within the source of 20KCi of activity. Articles to be sterilised are passed through the irradiation chamber on which a conveyor belt moves around the raised source.

iii) **X-Ray Steriliser:** Use of X-rays is not very common because they also impart harmful effects on human and other animals. This method of sterilisation is mainly used in laboratories and operation theatres, before running any task. In this method, low intensity X-rays are used for the sterilisation process.

2) **Non-Ionising Radiation:** In this method, the material is not ionised. This technique uses the following methods:

i) **Infrared Radiations:** These radiations are used for bulk and rapid sterilisation of disposable catheters and syringes.

ii) **Ultraviolet (UV) Radiations:** These radiations with the wavelength of 240-280nm are lethal and have some bactericidal activity. UV radiations denature the bacterial protein and also disrupt the replication of bacterial DNA.

This method is generally used for the sterilisation of closed areas, inoculation hoods, laminar flow, operation theatres, and in microbiology laboratory. UV radiation is effective in vegetative bacteria but bacterial spores are resistant to these radiations. These radiations cannot pass or penetrate the dirt films, water, glass, and other substances effectively. Prolonged exposure of UV radiation to skin and eyes results in burning and damaging them; therefore, UV lamps should be switched off after regular intervals and also other precautions should be taken.

Merits

1) This method can be used to sterilise thermolabile materials as they do not get degraded during sterilisation.
2) It does not leave any chemical residue.
3) It involves administration of precise dosage and uniform dosage distribution.
4) The media can be immediately availed after sterilisation.

Demerits

1) It is costlier than heat sterilisation.
2) It uses highly specialised equipment.
3) The penetration power of UV is very poor.
4) UV-light sterilisation is not a reliable method as DNA may get repaired under some favourable conditions.

Applications

1) Sterilisation through radiation, by using the high energy gamma rays or accelerated electrons is proved to be a very useful method for industrial sterilisation of heat sensitive products.

2) Radiation sterilisation is usually performed to articles in dry conditions, including sutures, surgical instruments, prostheses, unit dose ointments, plastic syringes, and other dry pharmaceutical products.
3) Despite of poor penetrability, with much lower energy, the UV light finds uses in the sterilisation of air, for the treatment of manufacturing grade water, and for surface sterilisation of aseptic work areas.
4) Ionising radiation is suitable for sterilising the heat-sensitive pharmaceuticals, **e.g.,** antibodies, swabs, plastic, disposable items, hormones, and vitamins.
5) Cobalt-60 is the most commonly used source of gamma radiation, which is used to sterilise animal feeds, antibiotics, hormones, sutures, catheters, metal foils, and plastic disposables (syringes, etc.).
6) This method has also been used to sterilise and pasteurise meat and other food items.
7) Non-ionising radiation is used for the sterilisation of closed areas, inoculation hoods, laminar flow, operation theatres, and in microbiology laboratory.

6.1.3.5. Ultrasonic and Sonic Vibration

These are high-frequency sound waves and are beyond the sensitivity of human ear. They may disrupt microbial cells. Sonication transmits vibrations through a water-filled chamber (sonicator) used to induce pressure changes and create intense points of turbulence. This point of turbulence creates the stress and bursting of cells in the vicinity. The process of sonication can also forcefully dislodge foreign matter from objects. Heat generated by the sonic waves (up to 80°C) also appears to contribute to antimicrobial action.

Gram-negative rods are more sensitive microorganisms to ultra-sonic vibrations, whereas the gram-positive cocci, fungal spores, and bacterial spores are resistant to them. These ultrasonic devices are used for clearing debris and saliva from instruments used in dental clinics and also for clearing dental restrorations. However, most sonic machines are not reliable for regular use in disinfection or sterilisation.

6.1.4. Chemical Methods

Chemical sterilisation is a reliable method, but has its challenges which include that the sterilant may react with the polymer being sterilised, the residual chemicals may exert toxic effect on the product, and operator safety associated with the exposure to a sterilant.

Chemical methods of sterilisation include:
1) Sterilisation by heating with bactericide, and
2) Gaseous sterilisation.

6.1.4.1. Sterilisation by Heating with Bactericide

In this method, microbes are killed by heat and by using bactericide. It is used for sterilising injection fluids in the final containers in boiling water at 98-100°C for 30 minutes. This method can be used for sterilising eye drops using **0.01% benzalkonium chloride** or **thiomersal**. This method is official in I.P. and B.P. In case of injections, **0.2% chlorocresol** and **0.002% phenyl mercury nitrate** or **phenyl mercuric acetate** bactericides can be used which are non-toxic in the given percentage, compatible, and stable at the temperature used.

This method is not suitable for sterilising intrathecal, intracisternal, and peridural injections because bactericides cannot permeate through them. Similarly, intravenous injection having >15ml volume cannot be sterilised by this method.

Principle
This method effectively destroys the microbes, since heat along with bactericidal agents is used. In the presence of bactericidal agents, less temperature is required for destroying the microbes. Also less concentration of bactericidal agents are required since their efficiency increases in the presence of heat.

Procedure
This method is preferred for sterilising aqueous solutions of thermolabile materials which can withstand normal autoclaving temperature.

Condition
Temperature: 98-100°C boiling water

Time: 30 minutes

Bactericides: Must be compatible with the product, container, and closure.

For example, in case of injections

Chlorocresol	0.2% (w/v)
Phenylmercuric Nitrate (PMN)	0.002% (w/v)
Phenylmercuric Acetate (PMA)	0.002% (w/v)

For example, in case of eye drops

Thiomersal	0.01% (w/v)
Chlorhexidine Acetate	0.01% (w/v)
PMA and PMN	0.002% (w/v)
Benzalkonium Chloride (BAC)	0.01% (w/v)

Merits and Applications
1) This method is used for sterilising injection fluids in the final container (i.e., terminal sterilisation).
2) It is also suitable for eye drops.

Demerits
1) It is not suitable for sterilising intrathecal, intra-atrial, intracisternal, intra-peritoneal, peridural, and any other route of injections which access to the cerebrospinal fluid, intra-cardiac, and intraocular injections.
2) Parenteral fluids having >15ml volume are not sterilised with a bactericide because even if bactericide concentration in the solution is less, the total amount of it penetrating the system should be taken into account due to large volume. **For example,** saline solutions are not sterilised using bactericides.

6.1.4.2. Gaseous Sterilisation

Chemically active gases (**e.g.,** ethylene oxide and formaldehyde) possess broad spectrum biocidal activity, therefore are used in the sterilisation of reusable surgical instruments. This method is also preferred for certain medical diagnostic and electrical equipment, and the surface sterilisation of powder.

Principle
Few gases are highly reactive and have biocidal activity, **e.g.,** formaldehyde, (methanol, HCHO) and ethylene oxide $(CH_2)_2O$ possess biocidal activity. It is believed that gases kill the microorganisms by **alkylation** of sulphydryl, amino, hydroxyl, and carboxyl groups on proteins and amino groups of nucleic acids. The concentration used for the gases (weight of gas per unit chamber volume) are usually in the range of 800-1200mg/L for ethylene oxide and 15-100mg/L for formaldehyde with operating temperatures of 45-63°C and 70-75°C, respectively.

Procedure

Gases used for sterilisation may produce acute toxicity including irritation of the skin, conjunctiva, and nasal mucosa. Both of these gases being alkylating agents are potentially mutagenic and carcinogenic. In gas sterilisation techniques, following sterilisers are used:

1) **Ethylene Oxide Steriliser:** Ethylene oxide gas has been used widely to sterilise heat-sensitive devices. Ethylene oxide steriliser is a chamber with capacity of 100-300 litres and surrounded with a jacket of water. Removal of air from steriliser is done by humidification and evacuation. Firstly, conditioning of the load is done by passing sub-atmospheric pressure steam, then evacuation is repeated, and finally pre-heated ethylene oxide vapour is passed. After treatment, the gases are evacuated either directly to the outside atmosphere or through a special exhaust system. The time for aeration is needed at the end of the cycle to eliminate the gas and to make this method slower.

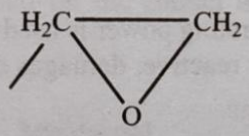
2) **Low Temperature Steam Formaldehyde (LTSF) Steriliser:** It is operated with the atmospheric pressure steam. Firstly the previous air present in the chamber is removed by evacuation and then steam is introduced into the chamber.

Gases Used

Following gases are used commonly for sterilisation:

1) **Ethylene Oxide:** This gas readily penetrates plastics, paperboards, and powders, and dissipates from the materials when exposed to air. However, ethylene oxide limitedly penetrates and diffuses in liquids.

   Ethylene oxide possesses antimicrobial activity due to the alkylating power of sulphydryl, imino, carboxyl, and hydroxyl groups of proteins and other essential cell constituents of microbes. Its sterilising efficiency is affected by its concentration, load temperature, moisture, exposure time, and the conditions and accessibility to microbes.



Method

The material to be sterilised is placed in an autoclave previously heated to 55°C, and at vacuum of 27 inches of Hg. Moisture is introduced to provide a relative humidity of 50-60% for about 60 minutes. Ethylene oxide is then introduced and the pressure required to give its desired concentration is maintained throughout the exposure period of 6-24 hours. Gas is then exhausted and a vacuum of approximately 25 inches of Hg is drawn. Finally, the air filtered is introduced in the chamber till atmospheric pressure is attained.

Applications

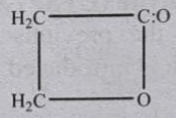
It is used for **sterilising medical and biological preparations**, dry food and cereals in burlap bags, catgut, enzymes (**e.g.,** pancreatin and papain), parts of plastic equipment and bottle closures, antibiotics, plaster bandages, culture media, hospital bedding, eggs, spices, foodstuff, heavy equipment, soil, and final shipping containers, thus easing the handling of sterilised products.

2) **Ozone:** This gas has a pleasant odour, but is irritant and toxic. It possesses bactericidal activity due to its high reactivity with all types of organic substances (aldehydes, fats, amino acids, proteins, and enzymes). Mostly it is used as a protoplasmic oxidant, and its efficiency is governed by the presence of organic matter, humidity, and temperature.

Ozone is used for **disinfecting water**, and for **preserving foods** from spoilage during bulk storage.

3) **Formaldehyde:** This gas is a bactericidal agent having poor penetrating power and polymerises to paraformaldehyde. It is irritant and has a pungent odour. It rapidly kills all bacteria including spores. It is used for **fumigation of rooms** and **hospital blankets**. In gaseous and solution form, it is effectively used as a **virucidal agent**. It rapidly inactivates proteins and organic matter.

4) **β-Propiolactone (BPL):** It is a heterocyclic ring compound. Under conditions of maximum effectiveness, its vapour's disinfecting activity is around 25 times more than that of formaldehyde, about 4000 times more than that of ethylene oxide, and about 50,000 times more than that of methyl bromide. BPL is highly **bactericidal** and used in 5mg/L or less concentrations. Its penetrating power is low and a relative humidity of about 75% is necessary.



At the present time it is used for **decontaminating rooms**, **chambers**, and **buildings**.

It is a highly **virucidal** agent, but irritant and carcinogenic to animals; thus should be handled carefully.

Merits

1) The method of gaseous sterilisation is used for thermolabile materials as it can be carried out at room temperature or slightly above.
2) It does not damage the moisture-sensitive materials and equipment as it requires a low humidity.
3) Ethylene oxide gas having higher penetrating power is used for pre-packaged articles.
4) Ethylene oxide despite of being highly reactive, damages only a few materials.

Demerits

1) Ethylene oxide gas cannot be used in emergency cases, as it is slow acting and its long exposure and desorption periods are necessary.
2) The running cost for ethylene oxide gas is high.
3) It is inflammable, thus is mixed with inert gases (carbon dioxide and fluorinated hydrocarbons).
4) Formaldehyde has low penetrating power, thus cannot be used for polymeric packaging.
5) It polymerises to inactive forms on the surface of low exposure temperature.
6) It cannot be used to sterilise apparatus of narrow lumen.

Applications

1) Ethylene oxide can penetrate paper, plastics, and rubber; thus is used for sterilising disposable syringes, hypodermic needles, pre-packaged materials, etc.
2) Formaldehyde is used for sterilising dressing packs.

6.1.5. Mechanical/Filtration Methods

The mechanical method of sterilisation involves filtration which does not destroy the microbes but removes them. This method can prevent the passage of both viable and non-viable particles, thus is used for clarification as well as sterilisation of both liquids and gases.

Principle

This method mainly involves **sterilisation by filtration** process. Filtration is a general method employed for the sterility testing of liquids. This is useful for large volumes. Filtration sterilisation method is used to reduce the microbial population in solutions of thermolabile material. For this purpose, many types of filters are used. In this method, filters remove the contaminating microorganisms from solutions, rather destroying them directly. This method is also used for sterility testing of liquids, and for large volume solutions, antibiotic solutions, sera, carbohydrate solutions, and eye drops.

The efficiency of filters depends on their pore size, wall thickness, filtration rate, positive or negative pressures, and nature of liquid to be filtered. Filters do not kill microorganisms but remove them.

Procedures

Filters used for sterilisation are of the following types:

1) **Depth Filters:** These are made up of granular or fibrous materials that have been bonded into a thick layer filled with twisting channels of small diameter. By the application of vacuum, this layer absorbs microorganism present in solution; thus the microbial cells are removed by physical screening or entrapment, and also by adsorption to the surface of the filter material.

   Depth filters are further divided into the following:

   i) **Asbestos Filter (Seitz Filter):** These are disposable and single-use discs that are available in various grades of pores (from 0.01-5 microns). These filters are made up of asbestos such as magnesium silicate or magnesium trisilicate. The filter is supported on a perforated metal disc within a metal funnel (**figure 6.3**), and then fitted on sterile flask through a silicone rubber bung. Fluid for sterilisation is placed on the funnel and the flask is connected to the exhaust pump.

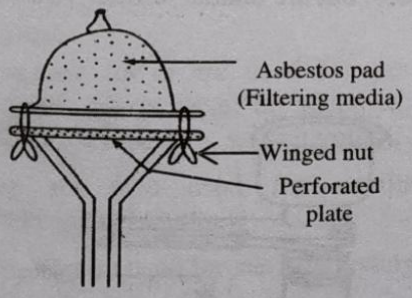


Figure 6.3: Seitz Filter

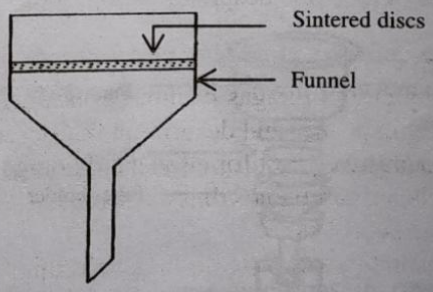


Figure 6.4: Sintered Glass Filter

   Asbestos filters have very high adsorbing capacity and tend to alkalinise the filtered fluid. However, the use of these filters is restricted due to the carcinogenic potential of asbestos.

   ii) **Sintered Glass Filters (Fritted Glass Filter/Morton Filters):** These are made up of finely powdered borosilicate glass particles in a ball mill, and packed into disc moulds, and heated until proper adhesion takes place between the granules. The sintered discs are finally fused in funnels of suitable size and shape (**figure 6.4**). Their adsorbing properties are low and they are available in different pore sizes; but for filtration sterilisation, a number or grade 5 or 3 must be used. The adsorptive property of sintered glass filters is low, and therefore they can be cleaned easily.

iii) **Candle Filters:** These are made up of unglazed porcelain (**e.g.,** Chamberlain filters) or diatomaceous earth or kieselguhr (**e.g.,** Berkefeld filters). These filters are available in different grades of porosity and have been used extensively for the purification of water for both drinking and industrial purpose. These are depth filters with thick cellular walls and are available in the shape of cylindrical candles (**figure 6.5**). They are available in various pore sizes.

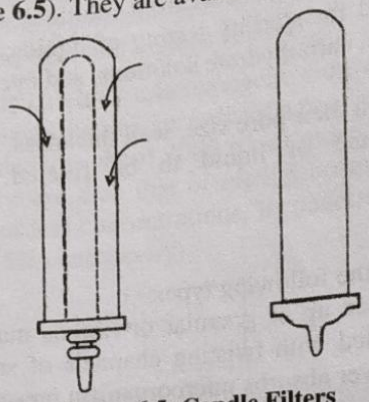
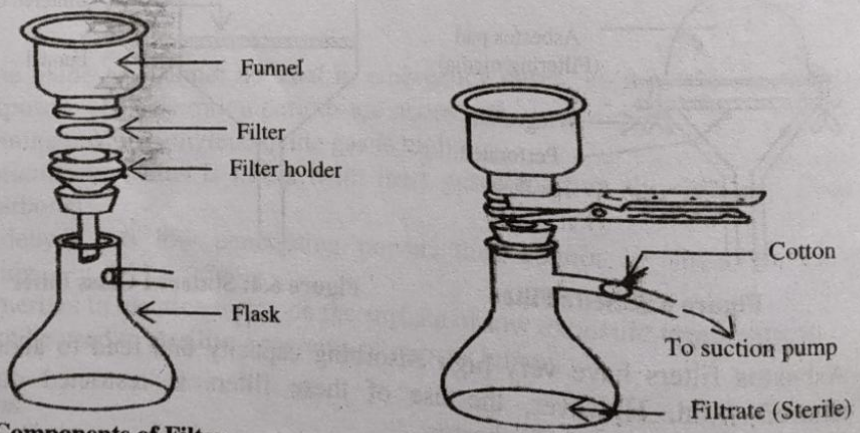


Figure 6.5: Candle Filters

2) **Membrane Filter (Millipore/Ultra Filter):** Nowadays membrane-filters are widely used, and have replaced depth filters. These filters are made up of cellulose acetate, cellulose nitrate, polycarbonate, polyvinylidene fluoride, or other synthetic materials. They are 150μm thick and contain millions of microscopic pores ranging from 0.01-10μm in diameter.

These are circular in shape, having 0.1mm thickness and are available in wide variety of pore sizes (0.015 -12μm). Membranes with pores of about 0.2μm are used commonly, because the pore size is smaller than the size of bacteria. These filters remove most of the vegetative cells of microbes, but are unable to filter viruses.



(a) Components of Filter **(b) Membrane Filtration Assembly**

Figure 6.6: Membrane Filter

During the filtration process, the filter membrane is held in special holders and often preceded by depth filters (made of glass fibres) to remove larger particles that might clog the membrane filter. Membrane filters are supported on a rigid base of perforated metal, plastic, or coarse sintered glass. After that the solution is forced or pushed through the filter using vacuum or pressure from a syringe, peristaltic pump, or nitrogen gas bottle, and collected in previously sterilised containers.

3) **HEPA (High Efficiency Particulate Air) Filters:** These are used to remove microorganisms from air, and their efficiency is up to 99.97% for particles >0.3μm in diameter. These are commonly used in operation theatres and in wards for burn patients to supply an air with reduced air-borne infections.

In microbiological and pharmaceutical laboratories, HEPA filters are used to provide sterile working areas. For filtration process, the air is first allowed to pass through prefilters to remove larger particles and then is passed through HEPA filters. The performance of HEPA filter is monitored by pressure differential and airflow rate measurements.

Merits

1) This method is used for sterilising thermolabile solutions without damage.
2) It removes all microbes, whether dead or alive.
3) It yields a much clearer final solution.

Demerits

1) It demands skilled operators having a full knowledge of aseptic technique.
2) It cannot be used for sterilising suspensions and oils.
3) The filtered solution should undergo a sterility test prior to use.
4) Filters may damage suddenly or gradually on use.
5) They may shed fibres and make the product alkaline.
6) They may absorb the drugs.
7) They may allow the entry of unsterile air in case of any unnoticed leakage during use.
8) They do not filter out the viruses.

Applications

1) Membrane filters are used for sterilising thermolabile materials (media, special nutrients) which might be added to media, enzymes, vaccines, and pharmaceutical products (drugs, sera, and vitamins).
2) They are also used for sterilising beverages, intravenous solutions, and bacteriological media.
3) They are also used for sterility testing.
4) HEPA filters are used to lower the numbers of air-borne microbes in operation theatres and rooms for burn patients.
5) Some filters are attached to syringes for quickly forcing out the materials.
6) In manufacturing of beer, sometimes filtration is preferred instead of pasteurisation.
7) Filters having such pore sizes should be selected that passage of any infectious agent into the product is restricted during the sterilisation process.
8) Filters are also used for large volume solutions, eye drops, antibiotic solutions, sera, and carbohydrate solutions.
9) They are used for separating bacteriophages and bacterial toxins from bacteria and for isolating microbes scanty in fluids.
10) In industries, sterilising grade filters are formed as part of the venting systems on fermentors, centrifuges, autoclaves, and freeze driers.

6.1.6. Merits, Demerits, and Applications of Different Methods of Sterilisation

Merits, demerits, and applications of various sterilisation methods can be summarised as in **table 6.2**:

Table 6.2: Merits, Demerits and Applications of Different Methods of Sterilisation

Methods	Mechanisms	Merits	Demerits	Applications
Heat Sterilisation	Destroys bacterial endotoxins.	Most widely used and reliable method of sterilisation, involving destruction of enzymes and other essential cell constituents.	Can be applied only to thermostable products.	Dry heat is applicable for sterilising glasswares and metal surgical instruments. Moist heat is the most dependable method for decontamination of laboratory waste and sterilisation of laboratory glassware, media, and reagents.
Gaseous Sterilisation	Alkylation.	Penetrating ability of gases.	Gases being alkylating agents are potentially mutagenic and carcinogenic.	Ethylene oxide gas has been used widely to process heat-sensitive devices.
Radiation Sterilisation	Ionisation of nucleic acids.	A useful method for industrial sterilisation of heat sensitive products.	Undesirable changes occur in irradiated products,an example is aqueous solution where radiolysis of water occurs.	Applied to articles in dry state; including surgical instruments, sutures, prostheses, unit dose ointments, and plastics.
Filtration Sterilisation	Does not destroy but removes the microorganisms.	Used for both clarification and sterilisation of liquids and gases as it is capable of preventing the passage of both viable and non-viable particles.	Does not differentiate between viable and non-viable particles.	This method is sterilising grade filters, are used in the treatment of heat sensitive injections and ophthalmic solutions, biological products, air and other gases for supply to aseptic areas.

6.1.7. Evaluation of Efficiency of Sterilisation Methods

Sterile products are free of microbes, pyrogens, and particulates. They also possess high standards of purity and quality. Therefore, the sterilisation methods used during the manufacturing of sterile products should be evaluated and subjected to complex validation procedures.

The final fate of sterility relies on official sterility tests. A manufacturing procedure is said to be validated if it has proved to do what it is meant for.

The data collection and its evaluation, beginning from the development phase up to the production phase of sterile products gives the evaluation proof. Evaluation of processing includes equipment, process, personnel, materials, etc.

The **aims of the evaluation of sterilisation process** are:

1) To build a sterile product,
2) To perform a maximum probability level,
3) To establish specification and performance characteristics,
4) To provide greater assurance of support for the result,
5) To provide specific methodology, process, and equipment,
6) To test the final product by validated analytical methods, and
7) To verify, calibrate, and maintain the equipment used.

The sterilisation methods are evaluated to ensure yielding the best quality product. The process control and finished product testing do not sufficiently assure the product quality. When a specific part of the total product tested passes the sterility test, it does not indicate that the complete product is sterile.

Sterilisation methods are evaluated to provide a high degree of assurance, indicating that a particular process will reliably form a product that will meet its predetermined specifications and quality assurance.

This proves that any procedure, process, equipment, material activity, or system leads to the expected result and produce quality product. This evaluation concept has been used to encompass various activities from analytical methods used for quality control of drug substance and drug products.

Material equipment is evaluated by a validation protocol carrying the tests to be performed, testing frequency, and results expected that is the acceptance criteria.

6.1.8. Sterility Indicators

Sterility indicators for various sterilisation methods are discussed below:

1) **Dry Heat Sterilisation**

   i) **Physical Indicators:** To monitor the process of dry heat sterilisation, a temperature record chart of every sterilisation cycle is made. In the chart, the batch details should be mentioned and also compared against a master temperature records. Temperature is considered as the coolest part of the loaded steriliser; further information on heat distribution and penetration within the steriliser can be gained with the use of thermocouple placed at selected site in the chamber or injected into test packs or bottles.

   ii) **Chemical Indicators:** These indicators are based on the ability of heat to alter the physical and chemical properties of given chemical substances. Chemical changes should take place only when satisfactory condition for sterilisation prevails, that confirm the successful completion of sterilisation cycle. Chemical indicators usually show a change in colour, or undergo melting.

   iii) **Biological Indicators:** Generally, the preparations of standardised bacterial spores are in the form of suspension in water or a culture media, or of spores dried on paper or plastic carriers, kept in sterilisers function as the biological indicators.

   The aqueous suspension or spores are transferred under aseptic conditions to an appropriate nutrient medium. Once the process of sterilisation is completed, it is incubated and frequently examined for the growth. *Clostridium* species is usually used as an indicator for dry heat sterilisation.

   Table 6.3 enlists the indicators used for dry heat sterilisation:

Table 6.3: Dry Heat Sterilisation

Indicators	Sterilisation Methods	Principle	Devices	Parameters Monitored
Physical	Dry heat	Temperature recording charts	Temperature recording charts	Temperature
Chemical	Dry heat	1) Temperature-sensitive coloured solution	Browne's tube	Temperature and time
		2) Temperature-sensitive chemical	Temperature-sensitive white wax concealing a black marked	Temperature
Biological	Dry heat	Temperature-sensitive microbes	*Bacillus subtilis*	D-value

2) **Moist Heat Sterilisation**

i) **Physical Indicators:** To monitor the process of moist heat sterilisation, a temperature record chart of every sterilisation cycle is made. In the chart, the batch details should be mentioned and also compared against master temperature records. Temperature is considered the coolest part of the loaded steriliser; further information on heat distribution and penetration within steriliser can be gained with the use of thermocouple placed at selected site in the chamber or injected into test packs or bottles.

ii) **Chemical Indicators:** These indicators are based on the ability of heat to alter the physical and chemical properties of given chemical substances. Chemical changes should take place only when satisfactory condition for sterilisation prevails, that confirms the successful completion of sterilisation cycle. Chemical indicators usually show a change in colour, or undergo melting.

iii) **Biological Indicators:** To monitor moist heat sterilisation, spores of *B. stearothermophilus* in sealed ampoules of culture medium are used. They can undergo incubation directly at temperatures of 55°C, thus eliminating the need for aseptic transfer.

Table 6.4 enlists the indicators used for moist heat sterilisation:

Table 6.4: Moist Heat Sterilisation

Indicators	Sterilisation Methods	Principle	Devices	Parameters Monitored
Physical	Moist heat	Temperature recording charts	Temperature recording charts	Temperature
Chemical	Moist heat	1) Temperature-sensitive coloured solution	Browne's tube	Temperature and time
		2) Steam-sensitive chemical	Device which is impregnated into a carrier material.	Saturated steam
Biological	Moist heat	Temperature-sensitive microbes	*Bacillus stearothermophilus*	D-value

3) **Gaseous Sterilisation**

i) **Physical Indicators:** Rise in pressure does not influence the concentration measurement of gas. The concentration of gas is measured often in reference to the weight of the used gas.

ii) **Chemical Indicators:** Royach Sacket, an indicator paper (soaked with reactive chemical) is used as a chemical indicator for gaseous sterilisation. A distinct change in its colour is seen when it reacts. For the coolest part of a steriliser that is accessible, chemical indicators prove to be valuable in monitoring the prevailing condition.

iii) **Biological Indicators:** These indicators are generally packed inside dummy packs, set at strategic sites in the steriliser, similar to the way in chemical indicators. They can also be positioned in tubular helix device, alternatively for gaseous sterilisation. Usually, *B. subtilis var. niger* and *B. subtilis var. golbigii* bacterial species are used for gaseous sterilisation.

One major **drawback of biological indicators** is the lengthy incubation period, which is necessary in order to obtain satisfactory results.

Table 6.5 enlists the indicators used for gaseous sterilisation:

Table 6.5: Gaseous Sterilisation

Indicators	Sterilisation Methods	Principle	Devices	Parameters Monitored
Physical	Gaseous	Temperature recording charts	Temperature recording charts.	Temperature
Chemical	Gaseous	1) Reactive chemical	Indicator paper impregnated with reactive chemical.	Gas concentration, temperature, and time
		2) Capillary principle	Based on same migration along wick principle.	Gas concentration, temperature, and time
		3) Temperature-sensitive chemical	A temperature-sensitive white wax concealing a black marked.	Temperature
Biological	Gaseous	Temperature-sensitive microbes	*Bacillus subtilis*	D-value

4) **Radiation Sterilisation**

i) **Physical Indicators:** A plastic or perspex dosimeter that gradually darkens in proportion to the radiation it absorbs is used for accurate measure of radiation sterilisation. It gives an accurate measurement of the radiation dose. As a physical indicator for the process of radiation sterilisation, it is considered to be the best practice presently available.

ii) **Chemical Indicators:** Chemical dosimeters that have been acidified using ceric ammonium sulphate or ceric sulphate solution are used as chemical indicators. A change in the applied density of dose is the response it shows to irradiation. They accurately measure relation dose and are considered best.

iii) **Biological Indicator:** These are standardised bacterial spore preparations that are used generally in the form of suspension in water or culture medium of spore dried on paper or plastic carriers. They are kept in sterilisers.

When the process of sterilisation is completed, aqueous suspension or spores are transferred under aseptic conditions to an appropriate nutrient medium, then incubated, and frequently examined for the growth. *Clostridium* species is usually used as an indicator for dry heat sterilisation.

Table 6.6 enlists the indicators used for radiation sterilisation:

Table 6.6: Radiation Sterilisation

Indicators	Sterilisation Methods	Principle	Devices	Parameters Monitored
Physical	Radiation	Recording charts	Recording charts	Radiation dose
Chemical	Radiation	1) Radio chromic chemicals	Plastic device impregnated with radio-sensitive chemicals which undergo colour changes at relative low radiation doses.	Only indicate exposure to radiation
		2) Dosimeter device	Acidified ferric ammonium sulphate solutions responds to irradiation by dose related changes in their optical density.	Accurately measures radiation doses
Biological	Radiation	Radiation-sensitive microbes	*Bacillus pumilus*	D-value

5) **Filtration Sterilisation**

i) **Physical Indicators:** Filters used for sterilisation are exposed to a bubble point pressure test. To determine the pore size of a filter, and to check the integrity of certain type of filters, this technique is employed. Principle of the test involves subjecting the dampened filter to an increasing pressure difference of air or nitrogen gas, in its assembled unit.

The pressure difference recorded when the first bubble of gas breaks away from the filter is related to maximum pore size. On slowly increasing the pressure of the gas, the bubble generally erupts over the entire surface. Here, the relation between the pressure difference and the mean pore size is seen. A damage or faulty filter signifies the pressure difference obtained below the expected value.

ii) **Biological Indicators:** Filtration sterilisation requires a different approach from biological monitoring; the test effectively measures the ability of a filter to produce a sterile filtrate from a culture of suitable small gram-negative, rod-shape bacterium (*S. marcesence*). *B. diminuta* used as a biological indicator having a dimension 0.5µm and 0.3µm respectively has been used for filters of 0.45µm and 0.22µm.

The extent of the passage of organism through membrane filter is enhanced by increasing the filtration pressure. Thus, successful sterile filtration depends markedly on the given critical condition. Such test is used as the part of filter manufacture characterisation and quality assurance process. They are not employed as a test of filter performance in use.

Table 6.7 enlists the indicators used for filtration sterilisation:

Table 6.7: Filtration Sterilisation

Indicators	Sterilisation Methods	Principle	Devices	Parameters Monitored
Physical	Filtration sterilisation	Forcibly passing of solution through the membrane	Bubble point pressure test	Pressure
Biological	Filtration sterilisation	Retention of bacteria	*P. diminuta*	Size of microorganism

6.2. EQUIPMENT EMPLOYED IN LARGE-SCALE STERILISATION/INDUSTRIAL STERILISATION

6.2.1. Introduction

Sterilisation process differentiates the biochemical and chemical processes from each other. An engineer (or a biochemical engineer specifically) provides a 'contamination-free' environment during the entire process of sterilisation.

In the ancient times, most of the processes which have performed well in terms of technical feasibility at laboratory-scale faced the challenges of contamination when the scale of operation increased.

A traditional **example** is of **penicillin fermentation** during which the engineer had to face a technical and operational problem, which was designing and operating a deep aerobic fermenter under contamination-free environment with pure culture (this is because the environment inside the fermenter was favourable for the growth of contaminants).

This has led in the new era of biochemical engineering as a distinct discipline in engineering and technology field. Even mechanical fabrication of fermenter should be performed by the engineers who are aware of the design and fabrication of fermenters; otherwise a mechanical engineer (versatile in fabrication of chemical equipment) fails to understand that asepsis is essential for biochemical engineering operations, and not for chemical equipment.

A biochemical process of producing metabolic products by the action of a microbe or a group of microbes on a substrate, in the presence of nutrients in the medium is termed **fermentation**, which involves:

1) A microbe,
2) A medium,
3) A fermenter,
4) Nutrients/other additives, and
5) Air in case of aerobic processes.

A sterile environment should be maintained for all the above, or else large amount of contaminants will develop resulting in a zero yield or a product contaminated with toxicants. Attack by a foreign organism (or contaminant) may result in the following **problems in the process of fermentation**:

1) The invaded foreign organisms contaminate the final product.
2) These organisms will utilise the medium for supporting their growth.
3) A contaminated product is obtained instead of the desired product; this mainly occurs in continuous fermentation processes.
4) The contaminated product interferes with the isolation of desired product.
5) In aerobic fermentations (**e.g.,** fermentation of antibiotics), an unsterile air spoils the fermentation product.

Methods to Avoid Contamination in a Fermentation Process

Given below are the methods by which contamination in a fermentation process can be avoided:

1) Medium sterilisation,
2) Using a pure inoculum,
3) Fermenter sterilisation,
4) Sterilisation of pipes, valves, bends, etc., in contact with the fermentation process,
5) Sterilisation of materials to be added to the fermenter,
6) Air sterilisation,
7) Disinfection of the fermenter and contact parts using a non-toxic disinfectant,
8) Maintenance of aseptic conditions in the fermenter during fermentation, and
9) Maintenance of optimum or desired pH which restricts the growth of contaminants or undesired organisms, thus preventing the formation of undesired products.

The following points should be observed to ensure sterility during fermentation:

1) Sterility of culture media, and
2) Sterility of incoming and outgoing air.

6.2.2. Sterilisation of Culture Media

Nutrient media contain different vegetative cells and spores derived from the constituents of culture medium, water, and the vessel. These cells and spores should be removed by sterilisation before inoculation. Although numerous large-scale installations are available for sterilisation, the commonly used mechanism is heat.

6.2.2.1. Heat Sterilisation

Nutrient media is sterilised widely using heat. Given below are the **factors** influencing heat sterilisation process:

1) Number and type of microorganisms present,
2) Culture media composition,
3) pH value, and
4) Size of the suspended particles.

For the removal of vegetative cells from the culture media, low temperature is required; but for destroying spores, temperature of 121°C is required. *Bacillus stearothermophilus* spores are the most heat resistant, thus are used as assay organisms for testing various procedures used for equipment sterilisation. **Table 6.8** enlists the sterilisation time and temperature specified for various organisms:

Table 6.8: Sterilisation Time and Temperature of Various Groups of Organisms

Cells	Sterilisation Time (min)	Sterilisation Temperature (°C)
Vegetative cells	5-10	60
Fungus spores/yeast Spores	15	80
Bacterial spores; general	5	121
Spores of *Bacillus Stearotbermopbilus*	15	121

Radiation which is sometimes used in food industries, are however not used in industrial fermentation. A number of different chemical disinfectants are available; however they cannot be used for sterilising nutrient media as inhibition of the fermentation organism could occur from the residual chemical.

6.2.2.2. Mechanical Removal of Organisms

Centrifugation, ion exchange adsorption, adsorption to activated carbon, and filtration are some of the mechanical methods of removing microorganisms from culture media. Among these methods, the most commonly used one is **filtration**. **Filter sterilisation** is used for components of thermolabile nutrient solutions which would denature by steam sterilisation process used in industrial fermentation. **Deep** or **plate filters** are sometimes used for the filtration of complex nutrient solutions. The process of **filtration** has the following **disadvantages**:

1) The filter material adsorbs certain components of the nutrient solution, and
2) Filtration utilises high pressure (up to 5 bar), which is industrially undesirable.

6.2.2.3. Batch Sterilisation

Currently, nutrient media are mostly sterilised in batches in bioreactors at 121°C. The sterilisation time depends on the nature and size of medium. Along with the nutrient media, the fittings, valves, and electrodes of the fermenter should also be sterilised. Thus, the actual sterilisation time is longer than that calculated and need to be empirically determined for the specific nutrient media in the fermenter.

A sterilisation method involves injecting steam into the fermenter mantle or interior coils red sterilisation. Another method involves injecting steam directly into the nutrient solution as a result of which the condensate accumulates in the fermenter. Thus, the liquid volume increases during sterilisation and reaches 121°C (sterilisation temperature) in 2-3 seconds, depending on steam conduction and entrance size. After reaching the desired temperature, 20-60 minutes more are required for killing the microorganisms, followed by 1 hour more for cooling.

A **disadvantage** of heat sterilisation is that during the heating, sterilisation, and cooling phases, the nutrient solutions are severely altered along with the destruction of microorganisms. Caramelisation and Maillard reactions discolour and change the pH value. Degradation of vitamins and deterioration in the quality of culture medium also occurs. The extent to which fermentation is affected depends on the organism as well as the process.

6.2.2.4. Continuous Sterilisation

In the process of continuous sterilisation, continuous inflow and outflow of material is maintained. Therefore, this method is best used when the operation capacity is high. Given below are the **advantages** of continuous sterilisation over batch sterilisation:

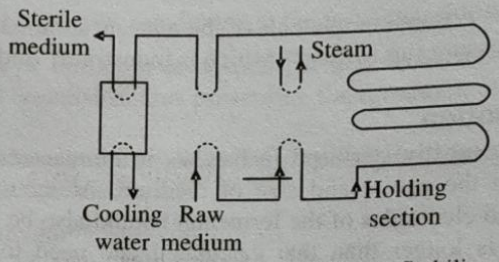
1) Its capacity is comparatively higher,
2) The medium quality is maintained in a better way,
3) The system is controlled automatically, thus human operational error is avoided,
4) Its running costs are comparatively lower,
5) The sterilisation times are comparatively shorter,
6) The scaling-up of the process is comparatively easier,
7) The holding capacities of steam is less, and
8) It is widely used for HTST method of sterilisation.

If the nutrient medium has to undergo the HTST method for avoiding protein denaturation or enzyme destruction, the commercial scale operations in batch sterilisation cannot quickly heat and cool large volumes of medium in a short time. Thus, HTST method can only be carried out successfully in continuous sterilisation.

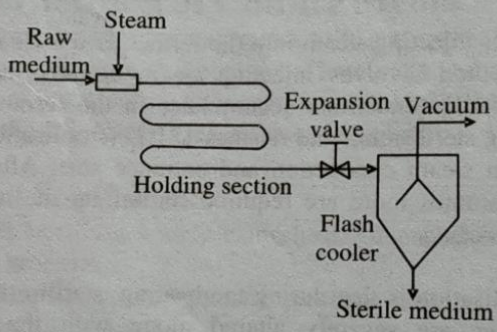
The major **disadvantage** of continuous sterilisation is its initial capital investment as it demands the use of aseptic transfer system while transferring the sterile broth in the sterile vessel.

There are **two common systems** of continuous sterilisation:

1) Continuous plate heat exchange sterilisation, and
2) Continuous steam injection and flash cooling sterilisation.



(a) Continuous Plate Heat Exchange Stabiliser



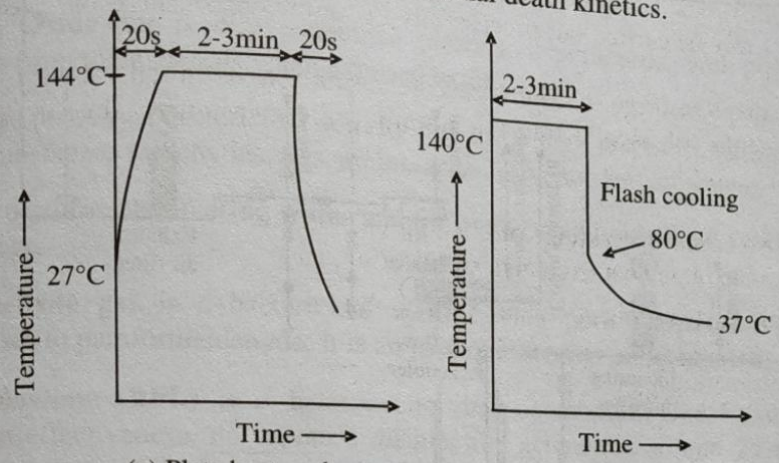
(b) Continuous Steam Injection Flash Cooling Steriliser

Figure 6.7: Continuous Sterilisation Methods

In continuous plate heat exchange sterilisation system **[figure 6.7 (a)]**, the heat exchanger pre-heats the incoming unsterile medium with the outgoing sterile medium. Then, the incoming medium is heated via steam in a heat exchanger. The heated medium is then passed through the holding section. The residence time in the holding section depends on the holding time requirement, flow rate of the heated medium, and the length of holding section. The time-temperature profile is shown in **figure 6.8 (a)**. Heating and cooling times are considered before and after the holding time

In continuous steam injection and flash cooling sterilisation system **[figure 6.7 (b)]**, steam is directly and continuously injected to the medium; thus the heating time and the heating section are negligible in the time-temperature profile **[figure 6.8 (b)]**. The holding time depends on the length of holding section, and sterilisation occurs during the holding period only. Then, under the influence of pressure, the steam and sterilised medium are passed through the expansion valve to reach the vacuum chamber where steam is removed. Further, the sterile medium passes into the pre-heat exchanger zone where it gives out some heat to be absorbed by the incoming unsterile medium through

the heat exchanger. In the last step, the sterile medium passes through the cooling zone; hence, it has a considerable cooling zone **[figure 6.8 (b)]**. Since the temperature is <100°C, it may be neglected for the purpose of thermal death kinetics.



(a) Plate heat exchanger (b) Steam injection type

Figure 6.8: Time-Temperature Profiles of Continuous Sterilisation

Table 6.9 enlists the advantages and disadvantages of the steam injection system:

Table 6.9: Advantages and Disadvantages of Continuous Steam Injection Sterilisation System

Advantages	Disadvantages
1) Better sterilisation of media containing solids.	1) Since the steam and the medium are in direct contact, the steam should be clean and free of anti-corrosive agents.
2) Low capital investment.	2) Foaming may occur during heating and cooling.
3) Cleaning and maintenance is easy.	
4) Shorter heating and cooling periods.	
5) Very high steam efficiency as steam is directly injected into the medium.	

6.2.3. Sterilisation of Fermentation Air

Industrial fermentation processes are mostly operated under vigorous aeration, maintaining the supply of sterilised air to the fermenter. **Air filters** should be attached to air input and exhaust ports for preventing the contamination of fermentation by air-borne microorganisms or of environment by aerosols generated within the fermenter. These air filters made up of glass fibre, mineral fibres, Poly Tetra Fluoro Ethylene (PTFE), or Polyvinyl Chloride (PYC) can trap the contaminants. They should be steam sterilisable and easily changeable.

In conditions where pathogenic organisms are being grown, fermenter exhaust should also be treated with **dry heat sterilisation** (incineration). This is an additional safety method used for sterilising gases including filtration, gas injection (ozone), gas scrubbing, radiation (UV), and heat. Out of these methods, filtration and heat are used on an industrial scale. Earlier sterilisation of air was carried out by passing it over electrically heated elements; however this method became out-dated due to the high electricity cost. This was replaced with **filtration using glass wool filters**. This method traps particles with a combined effect of inertial effects, blocking effect, diffusion, gravity separation, and electrostatic attraction.

Glass wool filters had a **disadvantage** of shrinkage and solidification during steam sterilisation; thus, were replaced with glass fibre filter cartridges.

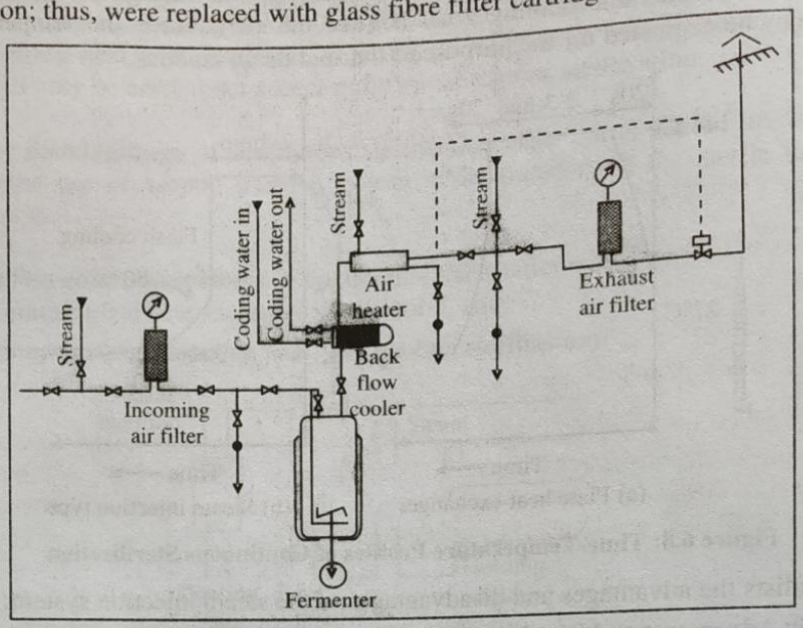


Figure 6.9: Installation of an Air Filter System in a Fermenter

6.3. SUMMARY

The details given in chapter can be summarised as follows:

1) Sterilisation is the process of **effective killing** or **removal of transmissible agents** or **microorganisms** (**e.g.,** bacteria, fungi, virus, and prions), from any surface, food, equipment, medications, or biological culture medium.
2) **Heat sterilisation** is the most reliable and widely used method of sterilisation, in which enzymes and other essential cell constituents are destroyed.
3) **Moist heat sterilisation** utilises **steam** in the temperature range of **121-134°C**. Steam under pressure generates high temperature which is required for sterilisation.
4) **Sunlight** possesses germicidal activity due to the combined effect of UV and heat rays, thus effectively kills bacteria in water and other surfaces of earth in tropical countries.
5) **Pasteurisation of milk** is performed by two methods, first is **High Temperature Short Time** (HTST) or **flash method** (72°C for 20 seconds); and second is **holder method** (63°C for 30 minutes).
6) In **radiation sterilisation**, breakdown of microbial DNA is the main target.
7) For sterilisation purpose, many radiations are used such as gamma rays, UV light radiation, and particulate radiation (**e.g.,** accelerated electrons).
8) Sonication transmits vibrations through a water-filled chamber (sonicator) used to induce pressure changes and create intense points of turbulence.
9) In the method of **sterilisation by heating with bactericide**, microbes are killed by heat and by using bactericide.

10) Chemically active gases (**e.g.,** ethylene oxide and formaldehyde) possess broad spectrum biocidal activity, therefore used in the sterilisation of reusable surgical instruments.

11) **Ethylene Oxide** gas readily penetrates plastics, paperboards, and powders, and dissipates from the materials when exposed to air.

12) **Ozone** gas possesses bactericidal activity due to its high reactivity with all types of organic substances (aldehydes, fats, amino acids, proteins, and enzymes).

13) Ozone is used for **disinfecting water** and for **preserving foods** from spoilage during bulk storage.

14) **Formaldehyde** gas is a bactericidal agent having poor penetrating power and polymerises to paraformaldehyde. It is an irritant and has a pungent odour.

15) **β-Propiolactone (BPL)** is a heterocyclic ring compound. Under conditions of maximum effectiveness, its vapour's disinfecting activity is around 25 times more than that of formaldehyde, about 4000 times more than that of ethylene oxide, and about 50,000 times more than that of methyl bromide.

16) The **mechanical method** of sterilisation involves filtration which does not destroy the microbes but removes them.

17) **Depth filters** are made up of granular or fibrous materials that have been bonded into a thick layer filled with twisting channels of small diameter.

18) **Membrane filters (millipore/ultra filter)** are made up of cellulose acetate, cellulose nitrate, polycarbonate, polyvinylidene fluoride, or other synthetic materials.

19) **HEPA (High Efficiency Particulate Air)** filters are used to remove microorganisms from air, and their efficiency is up to 99.97% for particles >0.3μm in diameter. These are commonly used in operation theatres and in wards for burn patients to supply an air with reduced airborne infections.

6.4. EXERCISE

6.4.1. True or False

1) The dry heat sterilisation usually employs higher temperatures ranging from 160-180°C and requires exposures time of upto 2 hours.
2) In incineration process, the objects are not burnt in direct flame.
3) Pasteurisation of milk is performed by two methods, first is High Temperature Short Time (HTST) or flash method (72°C for 20 seconds); and second is holder method (63°C for 30 minutes).
4) The mechanical method of sterilisation involves filtration which does not destroy the microbes but removes them.
5) Ionising radiation uses radiation of longer wavelength and low intensity to destroy the microbes.
6) Sonication transmits vibrations through a water-filled chamber (sonicator) used to induce pressure changes and create intense points of turbulence.
7) HEPA filters are made up of cellulose acetate, cellulose nitrate, polycarbonate, polyvinylidene fluoride, or other synthetic materials.

6.4.2. Fill in the Blanks

8) _____ utilises steam in the temperature range of 121-134°C.
9) HEPA filters are used to remove microorganisms from air, and their efficiency is up to _____ for particles _____ in diameter.
10) The sterilisation by heating with bactericide method used to sterilise ___ using 0.01% benzalkonium chloride or thiomersal.
11) Ionisation radiation method is also known as _____ as there is no notable increase of temperature.
12) The flash method of milk pasteurisation is also known as _____.
13) _____ are made up of granular or fibrous materials that have been bonded into a thick layer filled with twisting channels of small diameter.

Answers:

1) True 2) False 3) True 4) True 5) False
6) True 7) False 8) Moist heat sterilisation 9) 99.97% and >0.3µm
10) Eye drops 11) Cold sterilisation 12) High Temperature Short Time
13) Depth filters

6.4.3. Very Short Answer Type Questions

1) What is sterilisation?
2) Enlist various methods of sterilisation.
3) Give the importance of sterilisation.
4) Write the aim of the evaluation of sterilisation process.
5) Draw a clear diagram of autoclave with naming.
6) What is fermentation?
7) Give examples of gases used in sterilisation process.
8) Write chemicals used in sterilisation.

6.4.4. Short Answer Type Questions

1) Write note on following sterilisation methods:
   i) Sunlight
   ii) Ultrasonic
   iii) Radiation
2) Write principle, procedure merits, demerits and applications of dry heat sterilisation.
3) Write a note on evaluation of efficiency of sterilisation methods
4) Give principle, procedure, merits, demerits and applications of mechanical method of sterilisation.
5) Write a note on gaseous sterilisation.
6) Write a note on sterilisation method involving heating with bactericide.

6.4.5. Long Answer Type Questions

1) Give principle, procedure, merits, demerits and applications of chemical or physica method of sterilisation.
2) Write a detail note on sterility indicator.
3) Write a note on large-scale sterilisation.
4) Write a note on moist heat sterilisation.

CHAPTER 7

Study of Fungi and Virus

7.1. FUNGI

7.1.1. Introduction

The word **mycology** comes from the Greek word *mykes* which means **mushroom**. Mycology is a branch dealing with the **study of fungi**. Fungi (singular fungus) are achlorophyllous, thalloid, heterotrophic, eukaryotic microorganisms that take organic compounds as nutrition.

The body of fungi is known as **thallus** which is generally multicellular, branched, and filamentous, but may be single-celled also, **e.g.,** yeasts. Few members are dimorphic, i.e., they possess two distinct physical forms in their life cycle. The cell wall of fungi is made up of chitin and cellulose that encloses the fungal cell.

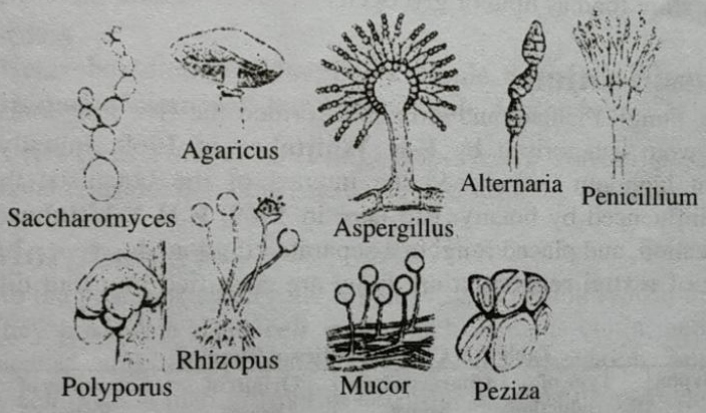


Figure 7.1: Some Fungi

It was observed in the early 19th century that fungi possessed the capability of invading plant and animal tissues. **Bassi** was the first one who reported the infection in an animal caused by a fungus. In 1835, he studied the muscardine disease of silkworm and documented that it is a fungal infection caused by *Beauveria bassiana*. ***Les Teignes***, a book published in 1910 carried a comprehensive study of dermatophytic fungi and was written by **Raymond Sabouraud**, who has been designated with the title '**Father of Medical Mycology**'.

Fungi are natural saprophytes; hence they work as natural decomposers in the ecological chain by decomposing the complex organic materials. They can easily grow on textiles, timbers, and other materials. Fungi have many uses; few (mushroom, mycoprotein) of them are used in the food industry (**e.g.**, ripening of cheese, bread-making), while some are involved in the contamination and degradation of food.

Fungi are also used in the production of antibiotics, but at the same time they cause many diseases also. Fungi are also parasitic and are found to be growing on the animal or plant host.

7.1.2. Properties

Fungi are present widely in the ecosystem and possess a few specific characters:

1) Fungi may be unicellular or multicellular but they have eukaryotic cell (i.e., they are eukaryotic microorganisms). They have membrane-bound cell organelles, **e.g.,** nuclei, mitochondria, golgi apparatus, endoplasmic reticulum, lysosomes, etc. They multiply by mitosis.
2) Ergosterol (a sterol) is found in the cell membranes of fungi. Eukaryotic ribosome, i.e., 80S ribosomes are found in the fungal cells.
3) A rigid cell wall made up of chitin is present in fungi; thus they are non-motile.
4) They lack chlorophyll, therefore, do not perform photosynthesis, and are grouped under chemoheterotrophs. Thus, they need organic compounds as a source of carbon as well as energy.
5) Since fungi obtain their own nutrients by the process of absorption from the environment around them, they are categorised as osmotrophs.
6) They are either parasites (living in or on another organism, i.e., host) or saprophytes (living on dead organic matter).
7) They need oxygen and water, and are not obligate anaerobes.
8) Asexual and/or sexual (by production of spores) type of reproduction is seen in them.
9) They show movement either non-reproductively by elongation of the hyphal tip, or reproductively by a process of budding.
10) They usually store food as lipid or glycogen.

7.1.3. Classification

Monera, Protista, Fungi, Plantae, and Animalia formed the five kingdoms in which all living creatures were categorised by **R.H Whittaker** in **1969**. Initially, fungi were included in plant kingdom and gained the interest of the botanists; therefore, their classification is influenced by botany. But later in 1969, **R.H Whittaker** gave the five kingdom classification, and placed fungi in a separate kingdom.

1) On the basis of **sexual reproduction**, fungi are classified into four different classes **(table 7.1)**:

Table 7.1: Characteristics of Fungi

Taxonomic Class of Fungi	Hypha	Type of Reproduction	Characteristic Spore	Origin of Spore	Examples of Fungi	Pathogenicity
Phycomycetes	Asptate	Asexually	Sporangiosphore	Sporangiophore	Nuisance fungi including general *Absidia*, *Muclor*, and *Rhizopus*	Very rate Mucormycosis
		Sexually	Zygospore or oospore	Fusion of nuclei		
Ascomycetes	Septate	Asexually	Blastospore	Budding	*Allescheria*	Rare
			Conidium	Conidiophore	*Aspergillus*	Maduromcosis
					Piedraia	Aspergillosis
		Sexually	Ascospore	Ascus	*Saccharomyces* (perfect yeast)	Black Piedra
Basidiomycetes	Septate	Sexually	Basidio-spore	Basidium	Mushrooms, smuts, and rusts	Rare Mushroom poisoning
Deuteromycetes (Fungi Imperfecti)	Septate	Asexually	Thallospore	Thallus (hypha)	Most saprophytes and pathogens encountered in medical mycology (Imperfect mold and yeast)	Most mycoses encountered in medical mycology
			Conidium	Conidiophore		

2) **Martin** divided Eumycetes (true fungi) into the following classes:
   i) **Class (A) Phycomycetes:** Aquatic or terrestrial, aseptate, coenocytic mycelium, having fully developed sexual organs.

      Sub-Class
      a) **Trichomycetidae:** Mycelium absent, **e.g.,** Synchytrium.
      b) **Oomycetidae:** Mycelium present, oospore is formed, **e.g.,** Albugo (= Cystopus).
      c) **Zygomycetidae:** Mycelium present, zygospore is formed, **e.g.,** Mucor, Rhizopus.

   ii) **Class (B) Ascomycetes:** Terrestrial, septate mycelium, sexual degeneration, and ascus is formed. Divided into two sub-classes:
      a) **Hamiascomycetidae:** In these asci are naked, no ascocarp is formed, **e.g.,** Yeast, Taphrina.
      b) **Euascomycetidae:** Asci is not naked but present in ascocarps, **e.g.,** Eurotium. Euascomycetes are classified into three series:
         - Plectomycetes,
         - Pyrenomycetes, and
         - Discomycetes.

   iii) **Class (C) Basidiomycetes:** Terrestrial, septate mycelium, sexual degeneration, and basidium present.

      Sub-Class
      a) **Heterobasidiomycetidae: For example,** Smuts and rusts.
      b) **Homobasidiomycetidae: For example,** Mushrooms.

   iv) **Class (D) Deuteromycetes (Fungi Imperfecti):** Terrestrial, septate mycelium, no sexual reproduction, and asexual reproduction by conidia.

7.1.4. Morphology

Fungi belong to the group of eukaryotic organisms and exhibit typical morphology of the eukaryotes. They possess a rigid **cell wall** which contains chitin; and are covered by mannans, glucans, and other polysaccharides in association with polypeptides. Exceptionally, cellulose is also present in cell wall of some lower fungi. Polysaccharide capsules are present in some fungal groups like *Cryptococcus* and the yeast form of *Histoplasma capsulatum*. The presence of polysaccharide capsules help to avoid the phenomenon of phagocytosis.

On the inner side of the cell wall, a bi-layered plasma membrane (or cell membrane) remains present. Fungal plasma membrane shows similarity with mammalian plasma membrane, except that fungal plasma membrane has ergosterol in place of cholesterol present in mammalian plasma membrane. **Cell organelle** like nucleus, mitochondria, Golgi apparatus, ribosomes, endoplasmic reticulum, lysosomes, and microtubules are present in fungal cytoplasm. The **nuclear membrane** in fungal cells exhibits a unique property as it does not break down and persists throughout the metaphase of mitosis in contrast to plant and animal cells, where it gets dissolved and forms again. Paired chromosomes are present in the nucleus.

Fungi show different morphological characters, few of them have more than one type of morphology during their life cycle:

1) Yeast,
2) Yeast-like fungi,
3) Moulds or filamentous fungi, and
4) Dimorphic fungi.

7.1.4.1. Yeasts

Yeasts are unicellular organisms and the shape of yeast cell ranges from spherical to ellipsoidal (**figure 7.2**). Reproduction in yeast occurs by the process of budding, resulting in the formation of **blastoconidia** (blastospore). Sometimes, after budding, the buds fail to detach from the parent cell, and undergoes elongation, resulting in the formation of a chain of elongated hyphae-like filaments known as **pseudohyphae**, **e.g.,** *Candia albicans*. The fungal cell is also capable in forming true hyphae in the form of germ tube.

The only difference between psueudohyphae and germ tube is that at the point of budding, a constriction is seen in psueudohyphae, which is absent in germ tube.



Yeast cell

Figure 7.2: True Yeast

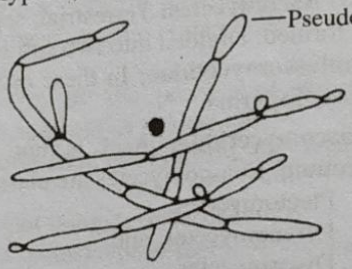


Figure 7.3: Yeast-Like Fungi

A characteristic polysaccharide capsule is produced by some yeast, **e.g.,** *Cryptococcus* and *Blastomyces dermatitidis*. Under the microscope, the India ink stain or Nigrosin (negative staining methods) stain helps to visualise the capsule. Few yeasts show characteristic pigmentation, **e.g.,** pink colour of carotenoid in colonies of *Rhodotorula sp.* Fungi that are dark in colour (when colonies are formed) are referred to as **dematiaceous fungi**, **e.g.,** *Phaeoannellomyces werneckii* and *Piedraia hortae*. The colour of the colonies produced by them ranges from brown to olive colour.

7.1.4.2. Yeast-Like Fungi

They are similar to yeast but have pseudohyphae (**figure 7.3**). They produce yeast-like daughter cells by the process of budding from the pseudohyphae, collectively called **mycelium**, **e.g.,** *Candida albicans*. The yeast cells are often predominated in cultures.

7.1.4.3. Moulds or Filamentous Fungi

The thallus of mould is known as **hyphae**, which are tube-like cylindrical structures. Hyphae elongate by apical (tip) elongation, and are responsible for the filamentous nature of mould. Hyphae in a mass are collectively called **mycelium**. The hyphae may be branched or unbranched, and may be septate or aseptate. Generally, cross walls known as **septa** are present which divide hyphae into multiple cells. Small pores are found on septa through which the cytoplasm is present in a continuous form in the hyphae, thus, all moulds are septate. Therefore, all hyphal fungi are **coenocytic**, i.e., multinucleate, except the zygomycetes (Rhizopus, Mucor). It is believed that non-septate hyphae are more primitive because when the hyphal strand is damaged, the entire strand dies. However, in case of septate hyphae, the pores present between neighbouring compartments can be plugged, and hence the whole hyphal strand is prevented from being killed even when a septate hyphal strand has been damaged.

Mycelium may exhibit the following **three forms**, when grown in a culture medium (preferably agar) (**figure 7.4**):

1) **Vegetative Mycelium:** This mycelium penetrates into the surface of the medium and absorb the nutrients. They do not produce spores.

2) **Aerial Mycelium:** The growth of this mycelium goes beyond that of the surface of agar. They show asexual reproduction.

3) **Fertile Mycelium:** This mycelium grows above the surface of agar and bears reproductive structures like sporangia or conidia.

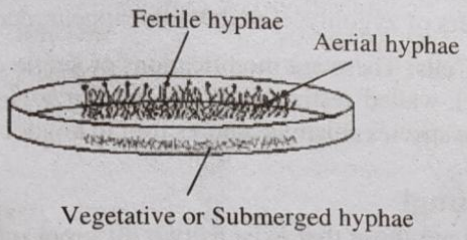


Figure 7.4: Fungal Growth in Agar Medium

The structural unit of mould is hypha, which collectively form mycelium. Mycelium gives texture, colour, and topography to the fungal colony. Presence of melanin pigment in the cell walls of fungi, imparts grey, black, or olive colour to their colonies and these fungi are known as **dematiaceous** or **phaeoid** fungi, **e.g.,** species of genus *Fonsecaea*, *Bipolaris*, *Wangiella*, *Exophiala*, *Phialophora*, and *Cladosporium*. Hyphae that lack pigment in its cell wall are termed **hyaline**. Hyphae are identified by some specialised appearances and structures; some of them are given here (**figure 7.5**):

1) **Spiral Hyphae:** These appear in the form of spiral coils, **e.g.,** *Trichophyton mentagrophytes.*

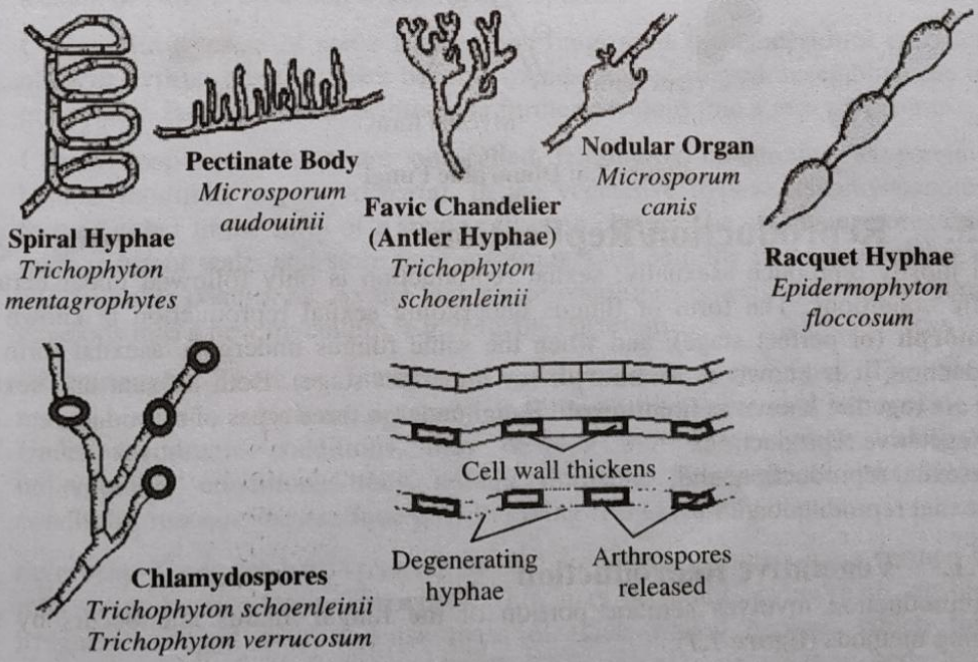


Figure 7.5: Types of Hyphae

2) **Pectinate Body:** These are short, unilateral projections arising from the hyphae, and appear like a broken comb, **e.g.,** *Microsporum audouinii.*

3) **Favic Chandelier (Antler Hyphae):** These are a group of hyphal tips which collectively resemble an antler of a deer or a chandelier, **e.g.,** *Trichophyton schoenleinii* and *Trichophyton violaceum.*

4) **Nodular Organ:** These are recognised as nodular enlargement in the mycelium, and consist of closely twisted hyphae, **e.g.,** *Microsporum canis* and *Trichophyton mentagrophytes.*

5) **Racquet Hyphae:** Each segment of these hyphae shows consistent enlargement of one end while the other end remains thin, **e.g.,** *Trichophyton mentagrophytes* and *Epidermophyton floccosum*.

6) **Rhizoides:** These are root-like structures developed on some portion of vegetative hyphae, **e.g.,** few members of zygomycetes show the appearance of rhizoides.

7) **Thick-Walled Resting Cells:** These are modifications of single cell in the hyphae that transform itself into thick-walled resting cell, **e.g.,** in *Trichophyton schoenleinii* and *Trichophyton verrucosum* species chlamydospores (or chlamydoconidia) are formed.

7.1.4.4. Dimorphic Fungi

Dimorphic fungi (**figure 7.6**) are those that exist as two different morphological forms at two different environmental settings. They characteristically grow as **moulds** in their natural habitat and *in vitro* at room temperature; and as **yeasts** in culture, usually at 37°C, **e.g.,** *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, and *Coccidioides immitis*.

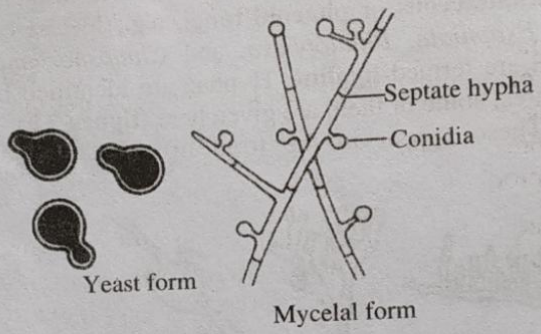


Figure 7.6: Dimorphic Fungi

7.1.5. Reproduction/Replication

Fungi mostly reproduce asexually; sexual reproduction is only followed under certain specific conditions. The form of fungus undergoing sexual reproduction is known as **teleomorph** (or perfect stage), and when the same fungus undergoes asexual form of reproduction, it is known as **anamorph** (or imperfect stage). Both asexual and sexual forms are together known as **holomorph**. Fungi undergo three types of reproduction:

1) Vegetative reproduction,
2) Asexual reproduction, and
3) Sexual reproduction.

7.1.5.1. Vegetative Reproduction

This reproduction involves somatic portion of the fungal thallus and occurs by the following methods (**figure 7.7**):

1) **Fragmentation:** In this method, the **mycelium** either accidentally or by mechanical injuries **splits into fragments** or breaks into hyphae segments. Under favourable conditions, each segment develops into a new mycelium by cellular division and apical growth.

2) **Fission:** This method occurs in unicellular fungi (**e.g.,** yeast whose single cell multiplies by this method). In this method, the **parent cell** undergoes **elongation** and transverse division to form **two daughter cells**. The process starts with the division of parent cell nucleus, followed by the division of cytoplasm, and cell wall formation; thus the parent cell divides into two daughter cells (each living as a new parent cell).

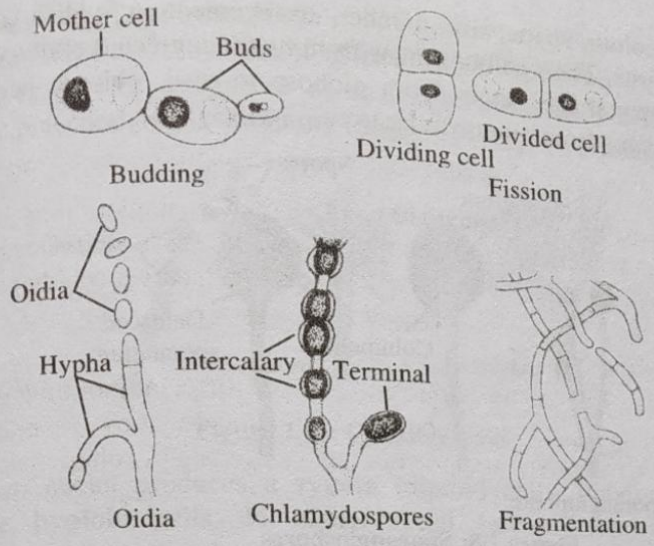


Figure 7.7: Different Types of Vegetative Reproduction in Fungi

3) **Budding:** This method occurs in budding yeast. In budding, the **parent cell develops** a small outgrowth (called **bud**), which **enlarges** and **separates** from the parent cell by **cross-wall formation**. This end then develops into new yeast. In some cases, the bud before detaching from the parent cell may develop a new bud and thus a chain of buds is produced which finally separates.

4) **Oidia:** The **hyphae** of some filamentous fungi **split into individual cells** (called **oidia** or **arthrospores**), which become round- or oval-shaped, resembling the beads of a rosary. Each oidium or arthrospore further develops into a new mycelium.

5) **Chlamydospores:** These are **one-celled fragments** functioning as perennating bodies (modified vegetative parts). In the vegetative hyphae, chlamydospores are formed either in the form of a single cell or in chains. The chlamydospores develop thick resistant walls and store food materials, thus help the fungus to survive under unfavourable conditions. As the favourable conditions reoccur, each chlamydospore develops into a new mycelium, **e.g.,** Mucor, Fusarium.

6) **Rhizomorphs:** These are interwoven hyphae of some higher fungi. They are fine, root-like strands, which are generally dark brown, and serve as a perennating body. Under favourable conditions, they develop into new mycelia; while under unfavourable conditions, they remain dormant. However, as the favourable conditions reoccur, they resume growth giving rise to fruiting bodies.

7) **Sclerotia:** These are hard covering or rind around the compact mass formed by the interwoven hyphae (as in Claviceps). They may be round-, cylindrical-, cushion- or irregularly-shaped with a dense mass of thick walled hyphae, which forms a compact, pseudoparenchymatous tissue. They serve as perennating body and as a means of vegetative propagation. Under unfavourable conditions, sclerotia remain dormant; while as the favourable conditions reoccur they develop into new mycelia, **e.g.,** Claviceps (ergot).

7.1.5.2. Asexual Reproduction

The fungi undergo asexual reproduction more commonly than the sexual reproduction. Asexual reproduction usually repeats several times in a season, and occurs by the formation of **spores** (special reproductive cells); by the process called **sporulation**. The spores are also called **mitospores** as they are formed as a result of mitosis in the parent cell. Each spore grows into a new mycelium.

The spores vary in colour, shape, size, number, arrangement on hyphae, and the manner in which they are borne. Their colour ranges from hyaline, green, yellow, orange, red, or brown to black. Their shape ranges from globose to oval, oblong, needle-shaped to helical. Their size ranges from minute to large.

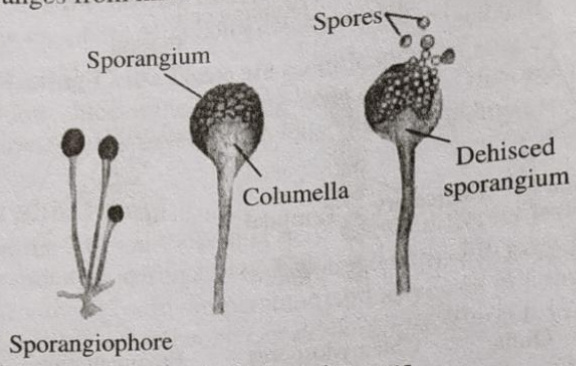


Figure 7.8: Sporangiospores

Fungi may produce the following three types of spores:

1) **Sporangiospores:** These are produced within sac-like structures called **sporangium** (**figure 7.8**). The branched hyphae bearing a sporangium are called **sporangiophores**, which may be motile or non-motile. The non-motile ones are called **aplanospores**, which are present in terrestrial species like Mucor and Rhizopus.

2) **Zoospores:** In aquatic fungi (**e.g.**, Pythium of *Oomycota* division), motile biflagellate sporangiospores called **zoospores** (**figure 7.9**) are produced within **zoosporangium**. Zoospores are motile and lack cell wall. After a swarming period they secrete a wall and germinate to form a germ tube. Unlike zoospores, the aplanospores possess a definite spore wall and are dispersed by wind and insects.

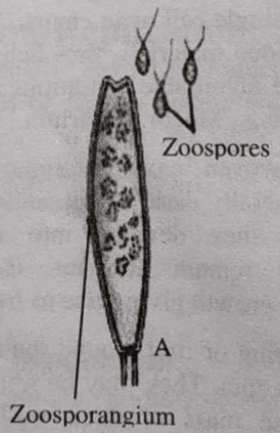


Figure 7.9: Zoospores

3) **Conidiophores:** Conidia are non-motile, deciduous mitospores formed as single separate cells (**figure 7.10**). They develop directly either on the mycelium or on morphologically differentiated hyphae called **conidiophores**, which may be simple or branched, septate or aseptate. Conidia are developed singly (**e.g.,** Phytophthora) or in chains at the tips of conidiophores (**e.g.,** Aspergillus) or at the tips of their branches (**e.g.,** Penicillium). The conidiophores often arise singly and are scattered in the mycelium; while sometimes they arise within fruiting bodies. Based on their appearance, they are also called **synnema**, **sporodochia**, **acervuli** (saucer-shaped), **pycnidia** (flask-shaped, globular), or **pustules**.

7.1.5.3. Sexual Reproduction

Sexual reproduction occurs by **fusion of two nuclei** from two individuals of opposite mating types (generally designated as male and female). Nuclei fusion known as **karyogamy** is preceded by **plasmogamy** (fusion of two protoplasts).

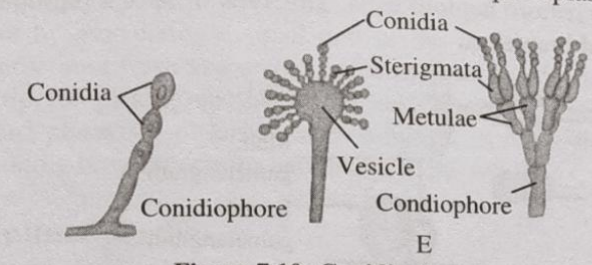


Figure 7.10: Conidiophores

Fusion of two nuclei produces a **zygote** (diploid cell). Finally, **meiosis** occurs for restoring the **hyploid cells**. In higher fungi (ascomycetes and basidiomycetes), plasmogamy is not immediately followed by karyogamy; but the pair of nuclei undergoes synchronous division to produce a **dicaryophase**. Ultimately, fusion of two nuclei occurs to produce a **diploid nucleus** in few cells before production of **ascospores** and **basidiospores**. This diploid nucleus then immediately undergoes meiosis to produce **haploid nuclei** which pass into the ascospores and basidiospores.

Given below are the various ways by which plasmogamy and karyogamy occur in different taxonomic groups of fungi:

1) **Planogametic Copulation:** In this method of sexual reproduction, the morphologically similar (**isogamy**) or dissimilar (**anisogamy**) male and female gametes undergo fusion (**figure 7.11**). This widely occurs in lower fungi (**e.g.,** chytrids). One or both the gametes may be motile or non-motile (**aplanogamy**).

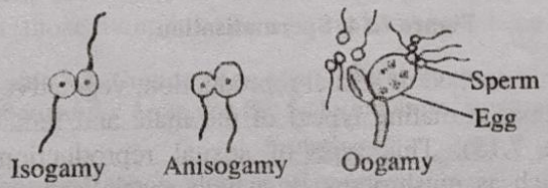


Figure 7.11: Planogametic Copulation

2) **Gametangial Contact:** In this method of sexual reproduction, the **male and female gametangia** (structures in which gametes are formed) come in **contact** forming a pore at the contact point (**figure 7.12**). Through this pore the **male gametes pass into the female gametangium** to fertilise the eggs or oospheres and **produce diploid oospores**. This type of sexual reproduction occurs in many phycomycetes (**e.g.,** saprolegnia).

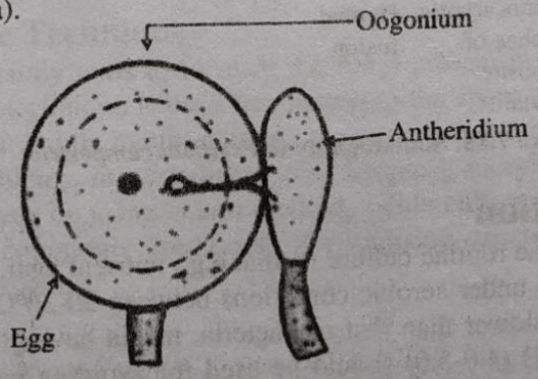


Figure 7.12: Gametangial Contact

) **Gametangial Copulation:** In this method of sexual reproduction, the **male and female gametangia fuse** with each other; thus losing their separate identity and producing a **zygospore** (**figure 7.13**). In this zygospore, the male and female nuclei pair with each other and some of these pairs fuses to form diploid nuclei which undergo meiosis to restore haploid cells. This type of sexual reproduction occurs in **zygomycetes** (**e.g.,** Mucor).

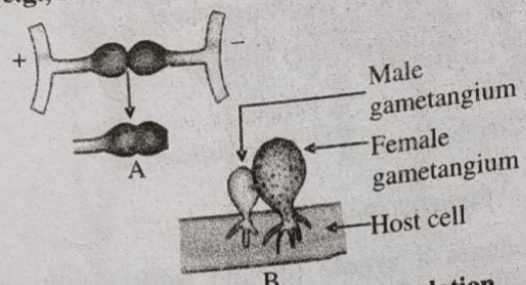


Figure 7.13: Gametangial Copulation

4) **Spermatisation:** In this method of sexual reproduction (**figure 7.14**), the uninucleate non-motile **male gametes** (called **spermatia** produced in spermogonia or pycnidia) are **carried by the insects** to the receptive **female hyphae** for **plasmogamy**. Thus, the **male nucleus** passes into the **female receptive hypha** and fuse with a female nucleus. This type of sexual reproduction occurs in some **basidiomycetes** (**e.g.,** rust-fungus Puccinia).

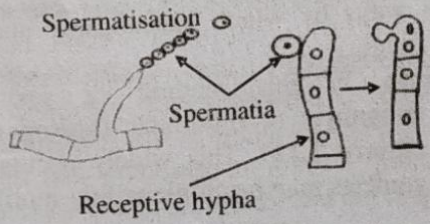


Figure 7.14: Spermatisation

5) **Somatogamy:** In this method of sexual reproduction, vegetative hyphae (originating from mycelia of opposite mating types) of the male and female nuclei fuses with each other (**figure 7.15**). This type of sexual reproduction occurs in **higher basidiomycetes** (such as mushrooms) in which specialised sex organs are totally absent.

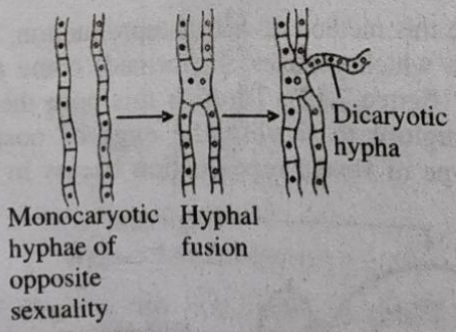


Figure 7.15: Somatogamy in *Pselliota campestris*

7.1.6. Cultivation

Fungi mostly grow on the routine culture media, **e.g.,** nutrient agar, potato dextrose agar, Czapek's dox agar, etc., under aerobic conditions between 20-30°C. Since the growth of fungi is comparatively slower than that of bacteria, media having high concentration of sugar or salt and low pH (4.0-5.0) should be used for avoiding bacterial overgrowth or contamination. High sugar concentrations and low pH favour fungal growth but inhibit

bacterial growth. The commonly used medium for fungal growth is SDA-Sabouraud's Dextrose Agar medium (formed by **Sabouraud**) containing dextrose and peptone as its principle ingredients.

Any part of fungi (like spores, mycelia, etc.) can be used for regenerating fungal colonies. It is carried out by inoculating a small part of the parent colony using a pre-sterilised inoculation needle on a fresh and sterile medium. The needle should be stiff and should have a sharp tip for picking-up the mycelium. Since fungi grow slowly, well-grown colonies on petri plates are observed after 3-5days. In some cases, bacterial growth is prevented by adding broad spectrum antibiotics to the medium.

7.1.6.1. Culture Media

Similar to bacteria, fungi also do not ingest nutrient but only absorb them. Absorption occurs under the influence of enzymes secreted into the environment; these enzymes break down the large organic molecules into smaller ones which easily pass into the cell. All fungi are heterotrophs. Most of the fungi can be grown in laboratories in an uncomplicated mixture of a sugar, an inorganic or a simple organic nitrogen source, and a few minerals. Some of them require vitamins also for their growth, while others can grow only on complex media containing organic compounds derived from peptone and meat extract.

The culture media used for growth of fungi have a higher sugar concentration (4%) and a lower pH (3.8-5.6) as compared to the media used for growing bacteria (whose pH ranges from 6.5-7.5). This is observed especially when bacteria frequently contaminate meat and milk, while fungi grow on acidic citrus fruits, bakery products, jams, and jellies. A chemically undefined, general-purpose medium for cultivating many saprophytic fungi (those living on dead organic matter) is given in **table 7.2**. The combination of high glucose concentration (4%) and low pH (5.6) favours fungal growth and inhibits bacterial growth. Some parasitic fungi (those living in or on a living host) have been cultivated *in vitro*.

Table 7.2: Composition of a General-Purpose Medium, Sabouraud's Agar, for the Isolation and Growth of Fungi

Ingredients	Functions	Amount
Peptone	Source of carbon, nitrogen, elements	10gm
Glucose	Carbon and energy source; high concentration favours growth of fungi but inhibits growth of bacteria	40gm
Agar	Solidifying agent	15gm
Water	Solvent	1000ml
pH	Low pH suppresses bacterial growth but enhances fungal growth	5.6

7.1.6.2. Culture Technique

Laboratory culture mainly aims to identify the fungi present in a sample. A commonly employed taxonomic technique is used for observing the spore structures produced by a mould. Preparation of a slide mount disrupts the structure of fungus under examination, thereby causing problems in visualising the sample's morphological features. This problem can be resolved by using *in situ* methods (**slide culture** and **cellophane culture** techniques being the commonly used ones) of visualising structures without causing any disruption in fungal growth.

Slide culture technique depends on the hyphae and sporophores attached on the coverslip or microscope slide; and thus this technique is not suitable for examining all the fungi. A slide culture is prepared by placing a sterile glass slide on a bent glass rod in a

petri dish. This glass rod raises the slide above a sterile, moist pad, used for keeping the culture hydrated. About 1cm^2 block is cut from a solid growth medium, supporting the fungal growth. The block is then transferred to the centre of the slide under aseptic conditions. Each edge of the block is inoculated with the fungus, covered with a sterile coverslip, and then incubated. After sufficient growth is observed in the culture, the coverslip is removed and a drop of alcohol is placed on the coverslip to remove any air from the fungal material. This preparation is stained with a drop of lactophenol cotton blue, coverslip is placed inverted over a clean slide, and then the culture is examined microscopically.

An **advantage** of the **slide culture method** is that from a single culture two preparations can be made. The second preparation is made by removing the agar block from the supporting microscope slide, and treating it with alcohol, staining as done previously, and covering with a clean coverslip.

Cellophane culture technique works similarly. About 1.5cm^2 small pieces of sterile cellophane are placed on the solid growth medium surface, and inoculated with the fungus to be examined. The growth of fungi on cellophane support is generally good as they obtain their nutrients by diffusing through it. When sufficient fungal growth is observed, the cellophane piece is removed from the medium, placed on a slide, added with alcohol and lactophenol cotton blue, and mounted with a clean coverslip. A small inoculum should be used for cellophane preparations, as it is very difficult to prepare large colonies in this way. A mixture of mycelia and spores is considered ideal for initiating the culture.

Agar cultures can be produced conventionally using some aquatic fungi. However spores are formed only in natural waters; thus for initiating spore formation in these fungi, natural water should be added in the culture. This is done using **bait** (a mixture of small seeds). Cress, onion, or hemp seeds are sterilised by autoclaving, and their seed coat is removed using sterile forceps.

These sterilised seeds along with the fungus to be examined are added to a sterile mixture of coarsely filtered pond water and distilled water. The fungus grows on the bait, and is easily recovered from the culture for further examination when the spore structures have time for their formation.

Fungi from natural environments can be cultured with the aid of baiting techniques. **For example,** by burying hair or tennis balls in soil, fungi which can attack keratin substrates can be cultivated; leather, foodstuffs, and other natural substrates can be used as bait to attract specific fungi. Using leather and sterilised dung for cultivating fungi in laboratories has become a common practice nowadays.

7.1.7. Importance of Fungi

Fungi inhabit almost every ecosystem present on the earth. They affect human and other organisms by the following different ways:

1) **Decomposition:** They are natural decomposers. They obtain their food and nutrients from other living as well as non-living entities through the process of decomposition. They are also involved in carbon recycling.

2) **Participate in Biosynthetic Processes:** They actively participate in various biosynthetic processes which are used in the production of many useful products, **e.g.,** they are actively involved in fermentation process and are used in the industrial production of alcohol, fats, citric, oxalic and gluconic acids.

3) **Used in Pharmaceutical Industry:** They are the source of many antibiotics, **e.g.,** Penicillin and alkaloid ergot is produced by *Claviceps purpurea* (which is a medically important alkaloid that helps in inducing uterine contractions, control bleeding, and treating migraine).

4) **In Biological Researches:** They are used as research organisms in various biochemical and genetic studies, **e.g.,** *Neurospora crassa*.

5) **Genetic Engineering:** Fungi of some genus are used in genetic engineering for the development of different disease preventing methods and to increase their usefulness in daily life, **e.g.,** *Saccharomyces cerviciae* is extensively used in recombinant DNA technology.

6) **Edible Fungi:** Many of them are used as food supplement, **e.g.,** mushrooms, and yeasts provide nutritional supplements such as vitamins and co-factors, *Penicillium* is used to flavour Roquefort and Camembert cheese.

7) **In Control of Insects and Larvae:** They are used to control bacteria, insects, and other harmful organisms, **e.g.,** fungi (*Leptolegnia caudate* and *Aphanomyces laevis*) are used to trap mosquito larvae in paddy fields, and thus help in malaria control.

7.2. VIRUSES

7.2.1. Introduction

The Latin word, 'virus' means a **slimy**, **toxic fluid-like living organism** responsible for many diseases in humans, plants, and animals. Viruses are a **connecting link** between the non-living and living, as they possess both non-living and living characters. Virus is so small that it is not visible directly even under the light microscope. Since, they do not have any cellular structure, they replicate inside the cells of other organisms called host. They are non-living outside the host cell and can survive only inside the host cell. Therefore, they come under the category of microbes having obligate intracellular parasitic nature. No cell organelles are found in viruses, leading them to be dependent on a host cell, and to carry all the functions important for life.

The **first virus**, i.e., **Tobacco Mosaic Virus** was discovered by **Martinus Beijerinck** in 1898, since then millions of viruses have been discovered but only around 5000 of them have been studied. They are present in every ecosystem, so they are the most abundant biological entity. The viruses are studied under a specific branch of microbiology, known as **virology**.

The **largest known virus** is the **mimivirus** that has a capsid of 400nm diameter and protein filaments protruding out of the surface that measures upto 100nm. The probable shape of mimivirus is icosahedral, since under the electron microscope the capsid appears to be hexagonal.

7.2.2. Properties

Viruses have the following specific properties:

1) They are ultramicroscopic infectious particles of 20-350nm diameter.
2) They are nucleoproteins.
3) They contain either DNA or RNA (but not both), which is either single or double stranded.
4) The DNA or RNA has a protein coat to more complicated structures.

5) They are highly pathogenic (as they cause disease in plants, animals, and bacteria).
6) They lack protoplasm.
7) They grow, and undergo multiplication and mutation within living host cells.
8) They infect the host by nucleic acids.
9) The genome has four genes, one for the protein present in coat, two for replicase enzyme, and the last one for a protein which allows the virus to spread from one plant cell to another.
10) They easily spread from one organism to another.
11) They are resistant to antibiotics.
12) They can be crystallised.
13) They are obligate intracellular parasites, i.e., they reproduce and develop on host cells of animals, plants, or bacteria. Outside the host cells, viruses exist as individual particles called **virions**.

Based on the above mentioned properties, it cannot be proved that whether viruses are living or non-living; since they possess the properties of both:

1) **Characters of Non-Living Beings**
   i) They lack protoplasm.
   ii) They lack enzyme system.
   iii) They do not respire.
   iv) They can be crystallised.
   v) They cannot be cultured outside the cells even by using culture media.

2) **Characters of Living Beings**
   i) They can undergo replication within the living cells.
   ii) Nucleic acids present in them synthesise protein for their coat, although they utilise host's ribosomes for protein synthesis.
   iii) Gene mutations in nucleic acids are similar to that in chromosomes of the living organisms.
   iv) They cause disease.

Based on the above mentioned characters, scientists stated that viruses are a connecting link between living and non-living. In other words, they are the transitional form of life, lying on the border line between the living and non-living. Viruses lack many characters related to life, thus are referred as **particles**.

7.2.3. Classification

Viruses are classified by the following classification systems:

1) Baltimore classification,
2) Holmes classification, and
3) LHT system of virus classification.

7.2.3.1. Baltimore Classification

Baltimore classification system of viruses is named after a biologist, **David Baltimore**. As per this system (first defined in **1971**), viruses are classified into **7 groups** (designated by Roman numerals) depending on the combination of their nucleic acid (DNA or RNA), strandedness (single-stranded or double-stranded), sense, and replication method.

These 7 groups discriminate viruses on the basis of their replication method and genome type. Other classifications are determined by the disease caused by the virus or its morphology; but neither of these characters are satisfactory as there are different viruses causing the same disease or having similar morphology.

The structure of viruses is microscopically determined with much difficulty. Classification of virus based on their genome indicates that those in a particular category will behave similarly, offering some indication of how to proceed with further research.

Viruses can be categorised in one of the seven following groups:

Group	Type of Genetic Material	Examples
Group I	dsDNA viruses	Adenoviruses, Herpesviruses, Poxviruses
Group II	ssDNA viruses	Parvoviruses
Group III	dsRNA viruses	Reoviruses
Group IV	(+)ssRNA viruses	Picornaviruses, Togaviruses
Group V	(−)ssRNA viruses	Orthomyxoviruses, Rhabdoviruses
Group VI	ssRNA-RT viruses	Retroviruses
Group VII	dsDNA-RT viruses	Hepadnaviruses

DNA Viruses

1) **Group I**: Viruses of this group have double-stranded DNA, and synthesise mRNA by transcription from the DNA genome template.

2) **Group II**: Viruses of this group have single-stranded DNA, and during replication they first convert their single-stranded DNA genome into double-stranded DNA, which is then used as a template for mRNA transcription.

DNA viruses are classified in the following families as shown in **table 7.3**:

Table 7.3: DNA Viruses

Virus Family	Examples (Common Names)	Virion Naked/Enveloped	Capsid Symmetry	Nucleic Acid Type	Group
Adenoviridae	Adenovirus, infectious canine hepatitis virus	Naked	Icosahedral	ds	I
Papillomaviridae	Papillomavirus	Naked	Icosahedral	ds circular	I
Parvoviridae	Parvovirus B19, canine parvovirus	Naked	Icosahedral	ss	II
Herpesviridae	Herpes simplex virus, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus	Enveloped	Icosahedral	ds	I
Poxviridae	Smallpox virus, cow pox virus, sheep pox virus, orf virus, monkey pox virus, vaccinia virus	Complex coats	Complex	ds	I
Hepadnaviridae	Hepatitis B virus	Enveloped	Icosahedral	circular, partially ds	VII
Polyomaviridae	Polyoma virus; JC virus (progressive multifocal leukoencephalopathy)	Naked	Icosahedral	ds circular	I
Circoviridae	Transfusion transmitted virus	Naked	Icosahedral	ss circular	II

RNA Viruses

1) **Group III:** Viruses of this group have double-stranded RNA genomes. These viruses have segmented genomes and synthesise mRNA by transcription from their double-stranded RNA template.

2) **Group IV:** Viruses of this group have positive-sense single-stranded RNA genomes. They utilise the genomic RNA directly as mRNA.

3) **Group V:** Viruses of this group have negative-sense single-stranded RNA genomes. They synthesise mRNA by transcription from their RNA genome template.

RNA viruses are classified in the following families as shown in **table 7.4**:

Table 7.4: RNA Viruses

Virus Family	Examples (Common Names)	Virion Naked/Enveloped	Capsid Symmetry	Nucleic Acid Type	Group
Reoviridae	Reovirus, Rotavirus	Naked	Icosahedral	ds	III
Picornaviridae	Enterovirus, Rhinovirus, Hepatovirus, Cardiovirus, Aphthovirus, Poliovirus, Parechovirus, Erbovirus, Kobuvirus, Teschovirus, Coxsackie	Naked	Icosahedral	ss	IV
Caliciviridae	Norwalk virus, Hepatitis E virus	Naked	Icosahedral	ss	IV
Togaviridae	Rubella virus	Enveloped	Icosahedral	ss	IV
Arenaviridae	Lymphocytic choriomeningitis virus	Enveloped	Complex	ss(-)	V
Flaviviridae	Dengue virus, Hepatitis C virus, Yellow fever virus	Enveloped	Icosahedral	ss	IV
Orthomyxoviridae	Influenza virus A, Influenza virus B, Influenza virus C, Isavirus, Thogotovirus	Enveloped	Helical	ss(-)	V
Paramyxoviridae	Measles virus, Mumps virus, Respiratory syncytial virus, Rinderpest virus, Canine distemper virus	Enveloped	Helical	ss(-)	V
Bunyaviridae	California encephalitis virus, Hantavirus	Enveloped	Helical	ss(-)	V
Rhabdoviridae	Rabies virus	Enveloped	Helical	ss(-)	V
Filoviridae	Ebola virus, Marburg virus	Enveloped	Helical	ss(-)	V
Coronaviridae	Corona virus	Enveloped	Helical	ss	IV
Astroviridae	Astrovirus	Naked	Icosahedral	ss	IV
Bornaviridae	Borna disease virus	Enveloped	Helical	ss(-)	V

7.2.3.2. Holmes Classification

Holmes (1948) used Carolus Linnaeus's system of binomial nomenclature for classifying viruses into the following **three groups** under the order **Virales**:

1) **Group I:** Phaginae (attacks bacteria),
2) **Group II:** Phytophaginae (attacks plants), and
3) **Group III:** Zoophaginae (attacks animals).

7.2.3.3. LHT System of Virus Classification

The LHT system classifies viruses based on their chemical and physical characters (like presence of DNA or RNA), symmetry (helical or icosahedral or complex), presence of envelope, capsid diameter, and number of capsomers.

This classification system was approved by the Provisional Committee on Nomenclature of Virus (PNVC) of the International Association of Microbiological Societies in 1962. It is as follows:

Phylum Vira
- **Subphylum Deoxyvira** (DNA viruses)
  - **Class Deoxybinala** (dual symmetry)
    - **Order** Urovirales
      - **Family** Phagoviridae
  - **Class Deoxyhelica** (Helical symmetry)
    - **Order** Chitovirales
      - **Family** Poxviridae
  - **Class Deoxycubica** (cubical symmetry)
    - **Order** Peplovirales
      - **Family** Herpesviridae (162 capsomeres)
    - **Order** Haplovirales (no envelope)
      - **Family** Iridoviridae (812 capsomeres)
      - **Family** Adenoviridae (252 capsomeres)
      - **Family** Papiloviridae (72 capsomeres)
      - **Family** Paroviridae (32 capsomeres)
      - **Family** Microviridae (12 capsomeres)
- **Subphylum Ribovira** (RNA viruses)
  - **Class Ribocubica**
    - **Order** Togovirales
      - **Family** Arboviridae
    - **Order** Lymovirales
      - **Family** Napoviridae
      - **Family** Reoviridae
  - **Class Ribohelica**
    - **Order** Sagovirales
      - **Family** Stomataviridae
      - **Family** Paramyxoviridae
      - **Family** Myxoviridae
    - **Order** Rhabdovirales
      - **Suborder** Flexiviridales
        - **Family** Mesoviridae
        - **Family** Peptoviridae

Suborder Rigidovirales
Family Pachyviridae
Family Protoviridae
Family Polichoviridae

7.2.4. Morphology

The morphological characteristics of viruses can possibly be determined by electron microscope. The dimensions of virion range from 20-300nm (1nm = 1/1000/Å), thus representing the smallest and simplest form of infective agents. Most viruses are detectible only by an electron microscope as their size is less than 150nm.

Either DNA or RNA particle is present (but never both) in the same viral particle. The viral genome consists of DNA or RNA that is either double-stranded or single-stranded.

But the amount of nucleic acid differs in every group of virus, as small viruses (**e.g.,** parvoviruses and picomaviruses) contain about three or four genes respectively); while large viruses (**e.g.,** the herpesviruses and poxviruses) have several hundred genes.

The structure of double-stranded or single-stranded DNA may be either linear or circular (e.g., herpesvirus has linear dsDNA); while simian vacuolating virus 40 (SV 40), found in monkey's kidney cells, has circular dsDNA.

In contrast, the RNA in animal viruses exists only as linear double-stranded or single-stranded molecules. However, some plant viruses appear to have circular dsRNA as genome.

The morphology of viruses can be studied on the basis of their:
1) Size,
2) Structure,
3) Symmetry, and
4) Shape.

7.2.4.1. Size

The extracellular infectious virus particle is termed a **virion**. Viruses are so small (**figure 7.16**) that they cannot be viewed even under the light microscope. Some large-sized viruses (like the poxvirus) can be observed under the light microscope only when stained.

The size of viruses ranges between 20-300nm. **Poxviruses** are one of the **largest** viruses, whereas **parvoviruses** are one of the **smallest**.

Earlier the size of virus particles was determined by passing them through **collodion membrane filters** of graded porosity. The average pore diameter of the finest filter that allowed the virion to pass through, gave an estimate of its size. Another method available for determination of virus size was **ultracentrifugation method**.

In this method, the sedimentation rate of the virus in the ultracentrifuge was used for calculating the virion size using **Stoke's law**. The third and most common method of determining the virus size was **electron microscope**. This method is used to study both the size as well as the shape of virions.



Figure 7.16: Comparative Shapes and Sizes of Viruses

7.2.4.2. Structure

The virion consists of a nucleic acid enclosed within a protein coat called **capsid**. The capsid enclosing the nucleic acid is called the **nucleocapsid**. The nucleic acid is protected by the capsid from harmful environmental agents. The morphological units of capsid are termed **capsomers** (**figure 7.17**) present in large number. The chemical units of capsid are symmetrically arranged **polypeptide molecules**, which form a shell around the nucleic acid.

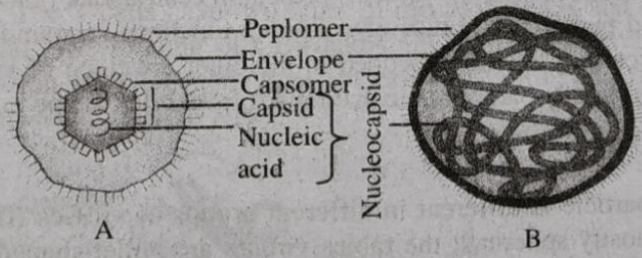


Figure 7.17: Virus Particles A. Enveloped Virus with Icosahedral Symmetry B. Virus with Helical Symmetry

Virions are either **enveloped** or **non-enveloped**. The envelope is derived from the host cell membrane, when the host cell releases the virus by budding. The envelope surface may have projecting spikes of protein sub-units, called the **peplomers**. Two types of peplomers are present in influenza virus, i.e., **haemagglutinin** (a triangular spike) and **neuraminidase** (mushroom-shaped). Envelopes are sensitive to lipid solvents and are responsible for chemical, antigenic, and biological properties of viruses.

7.2.4.3. Symmetry

Based on morphology of proteinaceous coat, viruses may be of the following types (**figure 7.17**):

1) **Icosahedral (or Cubical):** Most of the animal viruses are icosahedral or almost spherical in shape, having icosahedral symmetry. Normally, the icosahedron shape is formed by the closed shell of identical sub-units. Minimum 12 identical capsomeres, each composed of 5 identical sub-units, are required for the formation of icosahedral shape. But few viruses (**e.g.,** rotavirus) appear spherical having more than 12 capsomere but they retain icosahedral symmetry.

2) **Helical:** Helical viruses are made up of a single type capsomere that is piled in a spiral pattern around the central axis forming a helical structure, **e.g.,** tobacco mosaic virus. This helical structure may have either central cavity or a hollow tube. Such helical arrangements lead to the formation of rod-shaped or filamentous virions, which may be either short and highly rigid, or long and highly flexible.

   Commonly, the single-stranded RNA (ssRNA) is the genetic material; but in few viruses, the single-stranded DNA (ssDNA) may be present as the genetic material. These strands are bound to the protein helix by the formation of bond between the positively charged proteins and negatively charged nucleic acid. The **length of a helical capsid is related to the length of the nucleic acid** contained within it, and the diameter is dependent on the size and arrangement of capsomeres.

3) **Polyhedral (or Spherical or Complex):** Viruses with polyhedral or spherical appearance have a capsid whose basic framework is that of an icosahedron, i.e., its surface is made up of 20 triangular facets or 12 vertices. These triangular facets come together to form 12 vertices and each facet is an equilateral triangle.

   The polyhedral heads of few bacteriophages are greater in length than in width, giving a distorted icosahedral shape, and have a tail attached to it. The 'complex viruses' have capsid which is a mixture of both helical and icosahedral, with some additional structures, such as protein tails or complex outer wall. There are bacteriophages (**e.g.,** Enterobacteria phage T4) having complex structure with icosahedral head, which is bound to helical tail. This helical tail consists of a hexagonal base plate and protruding protein tail fibres. The tail of the virus acts as a molecular syringe that is attached to host cell, and injects the vital genome into it.

   Another **example** of morphologically rare, complex viruses is **poxvirus**. The genome of this virus is associated with the proteins present in central disk (known as **nucleoid**). A membrane and two lateral bodies of unknown functions surround the nucleoid. A thick layer of protein is found embedded in the outer envelope of virus. The overall shape of virion is slightly pleomorphic that ranges from brick-shape to ovoid-shape.

7.2.4.4. Shape

The shape of virus particle is different in different groups of viruses (**figure 7.16**). The animal viruses are mostly spherical; the rabies viruses are bullet-shaped; poxviruses are brick-shaped; while others are regular and pleomorphic in shape.

7.2.5. Reproduction/Replication

The complete lifecycle of an animal virus takes 20-60 hours for completion. The replication of a virus can be understood by the following stages (**figure 7.18**):

1) Adsorption and entry of the virus into the host cell,
2) Reverse transcription process,
3) Integration,

4) Transcription,
5) Translation,
6) Assembly of the progeny virus particles, and
7) Release of virus particles from the cell and maturation.

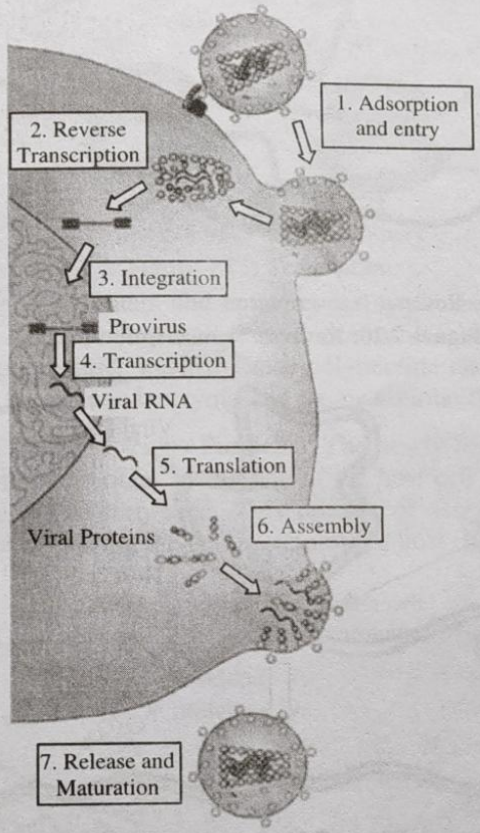


Figure 7.18: Replication in Virus

The detailed life-cycle of virus has been discussed taking an example of HIV (Human Immunodeficiency Virus):

1) **Adsorption and entry of the Virus into the Host Cell:** The envelope proteins present on the outer membrane of HIV binds to the receptors present on the target cell surface. The T-cells have two types of receptors (CD4 and CCR5) on which the HIV can bind. The binding of CD4 and CCR5 with the envelope protein of HIV, results in fusion of outer membranes of cell and virion, and finally, viral contents enter in the host cell (**figure 7.19**).

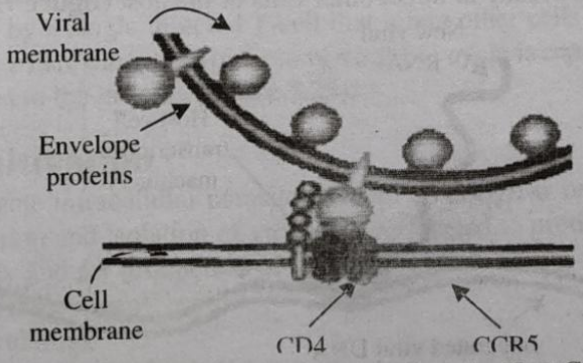


Figure 7.19: Entry of the Virus into the Host Cell

2) **Reverse Transcription Process:** RNA is the main genetic material present in a virus; and in HIV-1 virus, double stranded RNA is present. The formation of double stranded DNA molecule is initiated by reverse transcriptase enzyme, the sequence for this DNA strand is copied from the RNA strand already present in the virus (**figure 7.20**).

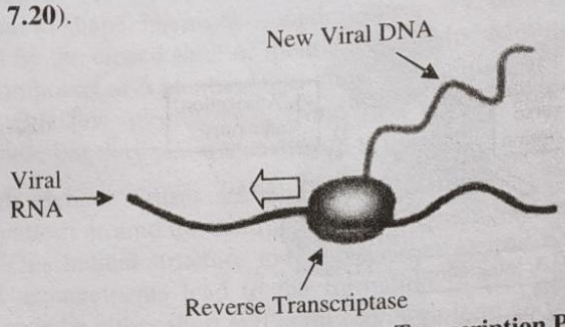


Figure 7.20: Reverse Transcription Process

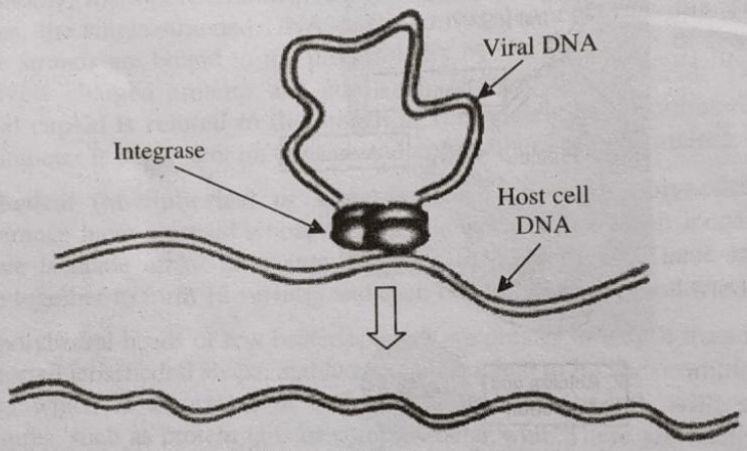


Figure 7.21: Integration

3) **Integration:** The nucleus of host (T-cell) cell is attacked by the viral DNA, and it gets integrated with the help of integrase enzyme. After the integration of viral DNA with the host cell DNA, the host cell remains infected for its lifetime. This integrated viral DNA is called as **provirus** (**figure 7.21**).

4) **Transcription:** Now, the DNA present in provirus acts as a template for the formation of new viral RNA by transcription (the process for formation of RNA from DNA). The machinery of the host cell is used for creating new RNA molecules of the virus. After the formation, these newly formed RNA molecules leave the host cell's nucleus and are ready to infect other cells of the host (**figure 7.22**).

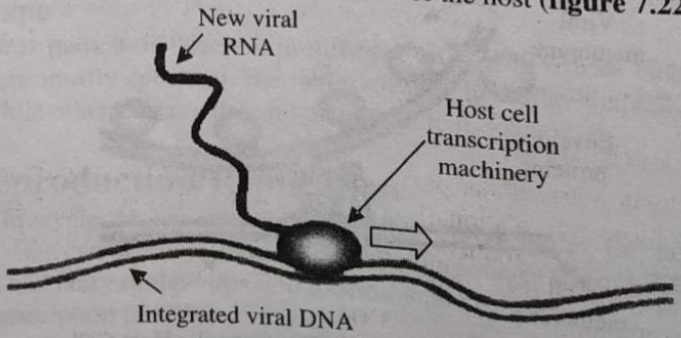


Figure 7.22: Transcription

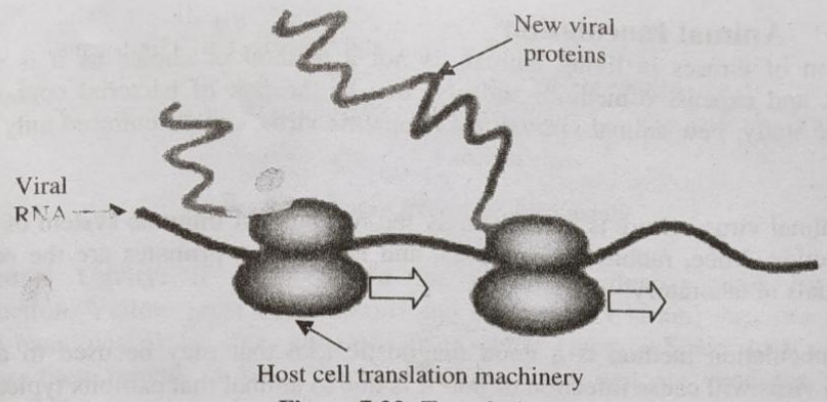


Figure 7.23: Translation

5) **Translation:** The viral protein and enzymes are synthesised as per the code carried by the viral RNA. The translation of these codes result in the formation of long chain of amino acids (polypeptide chain). These polypeptide chains further fold and form new components (protein and enzymes) of the new virus (**figure 7.23**).

6) **Assembly of the Progeny Virus Particles:** The newly formed components of virus start moving towards the outer membrane of the host cell and gets accumulated and assembled in a bud-like formation. This process of assembling new virion (virus) components is done by using the cell protein of the host (**figure 7.24**).

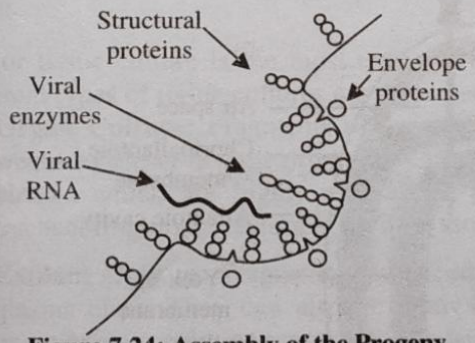


Figure 7.24: Assembly of the Progeny Virus Particles

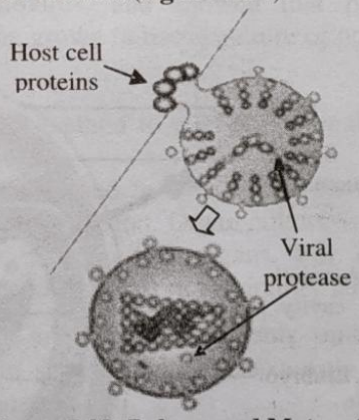


Figure 7.25: Release and Maturation

7) **Release and Maturation:** The proteins of host cell cuts-off the bud-like viral structure from the outer membrane of the cell, and the newly formed viruses are released out of the cell. During the process of assembling and the release of viral particles, protease (a type of viral enzyme) performs the cutting of HIV polypeptide chains at various positions; this is termed as **maturation**. A number of HIV particles are released by a single infected T-cell that infect other cells present in various parts of the body, where the whole process of viral life cycle is repeated. The infected cells are destroyed in the process (**figure 7.25**).

7.2.6. Cultivation

Viruses are obligate intracellular parasites, which do not grow on culture media used for bacteria. Cultivation and isolation of viruses is performed to produce them in the quantity required for study and for production of vaccines. Cultivation of viruses is carried mainly by the following three means:

1) Animal inoculation,
2) Embryonated hen's egg inoculation, and
3) Tissue culture.

7.2.6.1. Animal Inoculation

Cultivation of viruses in living animals is not a method of choice as it is a labour-intensive, and expensive method; and also due to the risk of bacterial contamination, during the study. Few animal viruses, e.g., hepatitis virus, can be cultured only in living animals.

Living animal virus culture is used to study the response of immune system of host to a viral infection. Mice, rabbits, guinea pigs, and non-human primates are the commonly used animals in laboratory.

Animal inoculation method is a good diagnostic tool that may be used to determine whether a virus will cause infection or not. It is due to animal that exhibits typical disease symptoms, and sections of infected tissue can be examined microscopically for the evidence of infection.

7.2.6.2. Embryonated Hen's Egg Inoculation

Use of fertilised, embryonated chicken eggs is one of the cost-effective and convenient methods of cultivation for many animal viruses. A needle or syringe is put through a hole drilled in the shell to introduce the virus aseptically into the embryonated chicken eggs. The whole is sealed with paraffin and the eggs are incubated for 2-3 days at 36°C and viruses are allowed to multiply.

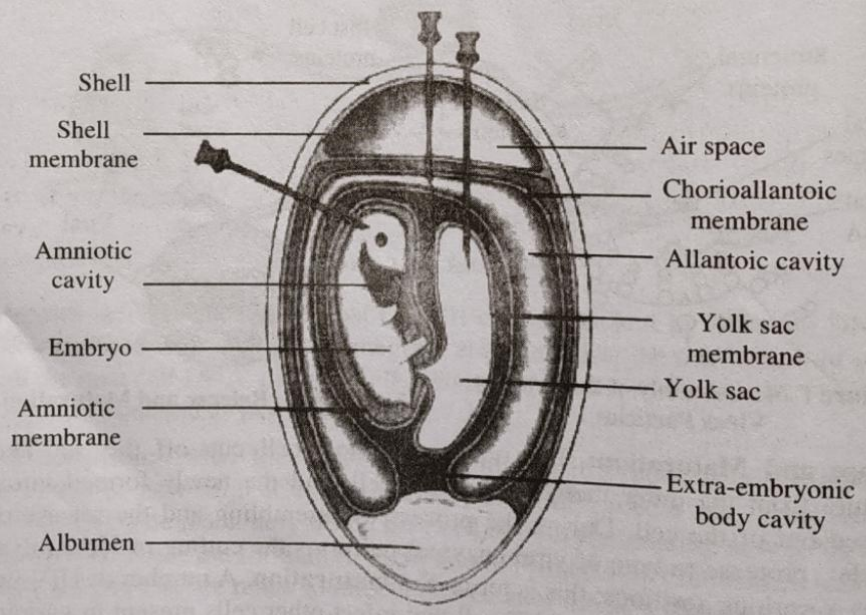


Figure 7.26: Embryonated Hen's Egg (10 to 12 Days Old). The hypodermic needles show the routes of inoculation of the yolk sac, Allantoic cavity, and embryo (Head).

Any one of the given routes (**figure 7.26**) such as Chorioallantoic Membrane (CAM), allantoic cavity, amniotic sac, and yolk sac are used for inoculating the 7-12 days old embryonated hen's eggs, followed by incubation for 2-9 days:

1) **Chorioallantoic Membrane (CAM):** It is inoculated for growing poxviruses as it produces visible lesions (pocks), each of which is derived from a single virion. By counting the number of pocks, the number of viruses present in the inoculum can be estimated. The morphological features of pocks produced by different viruses are different (**figure 7.27**).

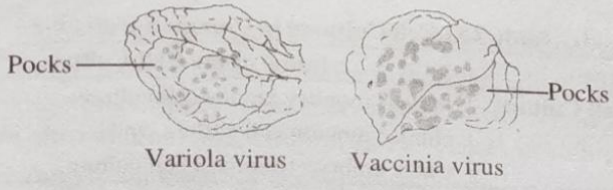


Figure 7.27: Pocks Produced by Variola Virus and Vaccinia Virus

2) **Allantoic Cavity:** It is inoculated for growing influenza virus for vaccine production. Yellow fever (17D strain) and rabies (flury strain) vaccines are some other examples of vaccines obtained from chick embryo. Since duck's eggs are bigger, they provide a better yield of rabies virus and are used for preparing inactivated non-neural rabies vaccine.

3) **Amniotic Sac:** It is inoculated for isolation of influenza virus.

4) **Yolk Sac Inoculation:** It is inoculated for cultivation of some viruses and bacteria (chlamydia and rickettsiae).

7.2.6.3. Tissue Culture

Steinhardt and **colleagues** in **1913** introduced the first application of tissue culture in virology. They maintained the vaccinia virus in fragments of rabbit cornea. Later in **1949**, **Enders**, **Weller**, and **Robbins** cultivated poliovirus, and showed that poliovirus (considered a strictly neurotropic virus) can also be grown in tissue culture of non-neural origin.

Cell or tissue culture is the most extensively used method to cultivate animal viruses. Different types of tissue cultures are discussed below:

1) **Organ Culture:** Fragments of organs are maintained *in vitro* for a few days and weeks to preserve their original architecture and function. Organ culture is used for viruses which are highly specialised parasites of certain organs. **For example**, tracheal ring organ culture is used for isolating coronavirus (a respiratory pathogen).

2) **Explant Culture:** Fragments of minced tissue are grown as 'explants' embedded in plasma clots. They can also be cultivated in suspensions. **For example**, adenoid tissue explant cultures are used for isolating adenovirus.

3) **Cell Culture:** Normally, this method is used for growing viruses. Tissues are broken down into cell components by enzymatic action and mechanical shaking. The cells are then washed, counted, and suspended in a growth medium, containing amino acids, glucose, vitamins, salts, a buffer, and antibiotics (to prevent contamination by bacteria). This suspension is dispensed in bottles, tubes, and petridishes, on the surface of which the cells adhere and divide within a week to form a confluent monolayer.

   Cell culture is further **sub-divided into three types** based on their origin, chromosomal characters, and the number of generations through which they can be maintained (**table 7.5**):

   i) **Primary Cell Culture:** In this culture, cells freshly obtained from the animal or human organs are cultured. These cells can grow limitedly in culture (mostly up to 5-10 divisions). This culture is used for isolating viruses and cultivating them for production of vaccines. **For example**, monkey kidney cell, human amnion cell, and chick embryo cell cultures.

Table 7.5: Cell Cultures in Common Use

Type	Name of the Cell Culture
Primary Cell Culture	Rhesus monkey kidney cell culture, Human amnion cell culture, and Chick embryo fibroblast cell culture.
Diploid Cell Strain	WI-38 (Human embryonic lung cell strain), and HL-8 (Rhesus embryo cell strain).
Continuous Cell Line	HeLa (Human carcinoma of cervix cell line), HEP-2 (Human epithelioma of larynx cell line), Vero (Vervet monkey kidney cell line), McCoy (Human synovial carcinoma cell line), BHK-21 (Baby Hamster kidney cell line), and KB (Human carcinoma of nasopharynx cell line).

ii) **Diploid Cell Strain:** A diploid cell of a single type contains the same number of chromosomes as the parent cells. It can be sub-cultured for a limited number; after about 50 serial sub-cultures it undergoes '**senescence**' and loses its strain. Diploid cell is susceptible to various human viruses if developed from human fibroblasts. It is used for isolating some fastidious pathogens. It is also used for producing viral vaccines. **For example**, rabies vaccine is produced in WI-38 human embryonic lung cell strain.

iii) **Continuous Cell Line:** This cell of a single type is derived from cancerous tissue, and thus can grow faster *in vitro*. It contains haploid chromosomes. Since continuous cells can be serially cultivated indefinitely, it is termed continuous cell line, which can be maintained by serial sub-culturing or storing at –70°C temperature to be used further. **For example,** HeLa, HEp-2, and KB cell lines have been used in the virus laboratories since many years. Some cell lines are now used for vaccine production, **e.g.,** Vero cell line is used for producing rabies vaccine.

7.3. SUMMARY

The details given in chapter can be summarised as follows:

1) **Mycology** is a branch dealing with the study of fungi.
2) **Fungi** (singular fungus) are achlorophyllous, thalloid, heterotrophic, eukaryotic microorganisms that take organic compounds as nutrition.
3) The body of fungi is known as **thallus** which is generally multicellular, branched, and filamentous, but may be single-celled also, **e.g.,** yeasts.
4) Fungi possess a rigid **cell wall** which contains chitin; and is covered by the mannans, glucans, and other polysaccharides in association with polypeptides.
5) **Yeasts** are unicellular organisms and the shape of yeast cell ranges from spherical to ellipsoidal.
6) **Reproduction** in yeast occurs by the process of budding, resulting in the formation of **blastoconidia** (blastospore).
7) **Yeast-like fungi** are similar to yeast but have pseudohyphae. They produce yeast-like daughter cells by the process of budding from the pseudohyphae, collectively called **mycelium**, **e.g.,** *Candida albicans*.
8) The thallus of mould is known as **hyphae**, which are tube-like cylindrical structures.
9) **Hyphae** in a mass are collectively called **mycelium**.

) **Septa** are cross walls which divide hyphae into a multiple cells.

) **Dimorphic fungi** exist as two different morphological forms at two different environmental settings. They characteristically grow as **moulds** in their natural habitat and *in vitro* at room temperature; and as **yeasts** in culture, usually at 37°C.

2) The form of fungus undergoing sexual reproduction is known as **teleomorph** (or perfect stage), and when the same fungus undergoes asexual form of reproduction, it is known as **anamorph** (or imperfect stage). Both asexual and sexual forms are together known as **holomorph**.

3) **Vegetative reproduction** involves somatic portion of the fungal thallus and occurs by the following methods:
   i) **Fragmentation** may result either from accidental cutting of the mycelium into fragments or bits or by mechanical injuries or otherwise, the mycelium splits into segments of hyphae.
   ii) **Fission** occurs in unicellular fungi (**e.g.,** yeast), in which single cell multiplies by fission method. In this method, the **parent cell** undergoes elongation and transverse division to form **two daughter cells**.
   iii) **Budding** occurs in budding yeast, in which the **parent cell develops** a small outgrowth called **bud**.
   iv) **Oidia** are formed when hyphae of some filamentous fungi split into individual cells.
   v) **Chlamydospores** are **one-celled fragments**, which function as perennating bodies.
   vi) In higher fungi, many hyphae interweave to form rope-like structure known as **rhizomorphs**.
   vii) **Sclerotia** are formed when the hyphae interweave to form a compact mass and get surrounded by a hard covering or rind.

14) Fungi may produce **asexually** via following three types of spores:
   i) Sporangiospores,
   ii) Zoospores, and
   iii) Conidiophores.

15) **Sexual reproduction** occurs by **fusion of two nuclei** from two individuals of opposite mating types (generally designated as male and female). Nuclei fusion known as **karyogamy** is preceded by **plasmogamy** (fusion of two protoplasts).

16) Viruses are a **connecting link** between the non-living and living, as they possess both non-living and living characters.

17) **Poxviruses** are one of the **largest** viruses and **parvoviruses** are one of the **smallest**.

18) The extracellular infectious virus particle is termed a **virion**. The virion consists of a nucleic acid enclosed within a protein coat called **capsid**.

19) The capsid within the enclosed nucleic acid is called the **nucleocapsid**.

20) The morphological units of capsid are termed **capsomers** present in large number. The capsid consists of symmetrically arranged **polypeptide molecules**, which form a shell around the nucleic acid.

21) Based on morphology of proteinaceous coat, viruses may be of the following types:
   i) Icosahedral (or cubical),
   ii) Helical, and
   iii) Polyhedral (or spherical or complex).

22) The replication of a virus can be understood by the following stages :
   i) Adsorption and entry of the virus into the host cell,
   ii) Reverse transcription process,
   iii) Integration,
   iv) Transcription,
   v) Translation,
   vi) Assembly of the progeny virus particles, and
   vii) Release and maturation of virus particles.

23) Cultivation of viruses is carried mainly by the following three means:
   viii) Animal inoculation,
   ix) Embryonated hen's egg inoculation, and
   x) Tissue culture.

7.4. EXERCISE

7.4.1. True or False

1) Fungi are natural saprophytes; hence they work as natural decomposers in the ecological chain by decomposing the complex organic materials.
2) Dimorphic fungi are those that exist as four different genetic forms at two different environmental settings.
3) Conidia are either directly formed on the mycelium or on morphologically differentiated hyphae called conidiophores.
4) Fusion of nuclei is known as plasmogamy.
5) Viruses are a connecting link between the plants and animals, as they possess characters of both.
6) The largest known virus is the mimivirus.
7) The virion consists of a nucleic acid enclosed within a protein coat called capsid which protects nucleic acids from harmful environmental agents.
8) The intracellular infectious virus particle is termed a virion.

7.4.2. Fill in the Blanks

9) The thallus of mould is known as_____, which are tube-like cylindrical structures.
10) Reproduction in yeast occurs by the process of budding, resulting in the formation of _____.
11) Zoospores are produced within sporangium known as_____.
12) The body of fungi is known as _____ which is generally multicellular, branched, and filamentous, but may be single-celled.
13) _____ are produced within sac-like structures called sporangium.
14) This integrated viral DNA is called as_____.
15) Earlier the size of virus particles was determined by passing them through _____ of graded porosity.
16) The morphological units of capsid are termed _____ present in large number.
17) The capsid within the enclosed nucleic acid is called the_____.

Answers:

1) True 2) False 3) True 4) False 5) False
6) True 7) True 8) False 9) Hyphae 10) Blastoconidia
11) Zoosporangium 12) Thallus 13) Sporangiospores
14) Provirus 15) Collodion membrane filters 16) Capsomers 17) Nucleocapsid

7.4.3. Very Short Answer Type Questions

1) Write properties of fungi.
2) Define following terms:
   i) Mycelium
   ii) Dematiaceous fungi
   iii) Pseudohyphae
   iv) Moulds
3) What are sclerotia?
4) Define following types spores formed in fungi
   i) Zoospores
   ii) Conidiophores
   iii) Sporangiospores
   iv) Chlamydospores
5) Define plasmogamy and karyogamy.
6) Viruses are connecting link between living and non-living. Comment.
7) Give properties of viruses.
8) Enlist steps of reproduction of virus.
9) Define following terms:
   i) Capsid
   ii) Nucleocapsid
   iii) Capsomers
10) Define viruses and virions.

7.4.4. Short Answer Type Questions

1) Give the Baltimore system of classification of viruses.
2) Write a note on asexual reproduction in fungi.
3) What are the cultural requirements for the cultivation of fungi?
4) Write a note on sexual reproduction in fungi.
5) Give a short note on importance of fungi in present era.
6) Write a short on yeasts.
7) What are filamentous fungi?
8) Give a note on embryonated egg inoculation method.

7.4.5. Long Answer Type Questions

1) Give the classification of fungi.
2) Write a note on morphology of viruses.
3) Write a detail note on cultivation of fungi.
4) Classify viruses according to LHT classification and give the structure of TMV.
5) Give a detail note on cultivation of viruses.

CHAPTER 8

Disinfectants and Antispetics

8.1. DISINFECTANTS AND ANTISEPTICS

8.1.1. Introduction

Disinfection is a process of destruction or elimination of microbes, and decreasing their numbers in such a low level, that they become unable to impart any harmful effect. The process of disinfection is more effective in killing or removing vegetative cells, than endospores, because vegetative cells are less resistant as compared to endospores (as they are heat-resistant). The antimicrobial agents that are applied on the surface of non-living or inanimate objects (**e.g.,** working area, dishes, bench, etc.) are termed as **disinfectants**, whereas the term **antiseptics** is used for those chemical agents that are used on animate or living bodies (**e.g.,** human body tissue, plant tissue, etc.). Generally, disinfectants are bactericidal, though they may sometimes be bacteriostatic.

Antiseptics are **specific type of disinfectants** that are used in the reduction of number of viable organisms on the skin or other exposed parts of the living body.

An **ideal antiseptic** or **disinfectant** should have the following **properties**:

1) It should have a broad spectrum of activity.
2) It should be capable of eradicating microbes in a specified time period.
3) It should be active in the presence of organic matter also.
4) It should be effective enough to make contact and show proper wettablility.
5) It should be active at all pH.
6) It should be stable.
7) It should have long shelf-life.
8) It should have a high penetration power and rapid action.
9) It should be non-toxic, non-allergenic, non-irritative, and non-corrosive.
10) It should not leave non-volatile residue or stain.
11) Its efficacy should not drop on dilution.
12) It should be inexpensive and easily available.
13) It should have a pleasant odour.

8.1.2. Classification

Disinfectants are classified on the basis of the following factors:

1) **Based on Consistency:** On the basis of consistency or state of matter, disinfectants are of two types:
   i) Liquid (**e.g.,** alcohols, phenols), and
   ii) Gaseous (**e.g.,** formaldehyde vapour).

2) **Based on Spectrum of Activity:** On the basis of spectrum of activity, disinfectants are of three types:
   i) **High Level Disinfectants:** These disinfectants are used against certain types of endoscopes, cystoscopes, and surgical instruments with plastic components which cannot withstand sterilisation procedures such as autoclaving. **Examples** of high level disinfectants include glutaraldehyde, hydrogen peroxide, peracetic acid, and chlorine compounds.

ii) **Intermediate Level Disinfectants:** These disinfectants may not be effective against bacterial spores; so are used for instruments (**e.g.,** laryngoscopes, fiberoptic endoscopes) where contamination with spores and other highly resistant organisms is unlikely. **Examples** of these disinfectants include alcohols, iodophores, and phenolic compounds.

iii) **Low Level Disinfectants:** These disinfectants are used against items which come in contact with the patients, but they do not penetrate the tissues. Stethoscopes, electrocardiogram, electrodes, etc., are examples of such items. **Examples** of these disinfectants include quaternary ammonium compounds, and phenolic disinfectants.

3) **Based on Mechanism of Action:** On the basis of mechanism of action, disinfectants are classified into:
   i) Disinfectants **acting on plasma membrane** (**e.g.,** alcohol, detergent),
   ii) Disinfectants **denaturing cellular proteins** (**e.g.,** alcohol, phenol),
   iii) Disinfectants responsible for **oxidation of essential sulphydryl groups of enzymes** (**e.g.,** H_2O_2, halogens),
   iv) Disinfectants responsible for **alkylation of amino-**, **carboxyl-**, and **hydroxyl group** (**e.g.,** formaldehyde), and
   v) Disinfectants **damaging nucleic acid** (**e.g.,** formaldehyde).

8.1.3. Mode of Action

Many substances act as disinfectants like alcohols (also have antiseptic property), bleaches, hydrogen peroxide, and iodine. Different disinfectants have different mechanism of action. An ideal disinfectant would offer complete sterilisation, without harming other life forms. Along with these essential properties, an ideal disinfectant should be inexpensive and non-corrosive. However, such ideal disinfectants do not exist and many disinfectants generally show partial sterilisation. The bacterial spores, some viruses, and bacteria are highly resistant to many disinfectants.

Mode of action and applications of the following disinfectants are discussed below:

1) Alcohols,
2) Phenols and phenolic compounds,
3) Aldehydes,
4) Halogens,
5) Heavy metals,
6) Oxidising agents,
7) Surface active agents, and
8) Dyes.

8.1.3.1. Alcohols

Alcohols show broad spectrum antimicrobial activity. Ethyl alcohol, methyl alcohol, and isopropyl alcohol are most frequently used.

Mode of Action

Alcohols act by dehydrating the cells, by disrupting bacterial cell membranes, and by coagulating the protein content of microorganisms. The ethyl alcohol or ethanol (CH_3CH_2OH) solutions of concentration between 70 and 90% are very effective against the vegetative forms of microorganisms. But ethyl alcohol does not completely sterilise an object since it does not affect bacterial endospores; **for example,** it is reported that the endospores of *Bacillus anthracis* (causative agent of anthrax) can survive in alcohol for 20 years.

In alcohols, the bactericidal property increases with the increase in the length of carbon chain. Also alcohols with carbon chains longer than propyl alcohol and isopropyl alcohol are slightly water-soluble (even less than ethyl alcohol); therefore, they are not used as disinfectants.

Applications

1) **Antiseptic Action:** A solution of **ethyl alcohol** (70%) and **isopropyl alcohol** (90%) work as a skin antiseptic. This solution is used as a disinfectant for clinical oral thermometers and other surgical instruments.
2) **Cleaning Agent:** Wipes of alcohol are used to clean skin before taking blood samples.
3) **Isopropyl Alcohol:** It is preferred over ethanol for disinfecting surfaces.
4) **Methyl Alcohol:** It kills fungal spores, therefore is best suited for disinfecting inoculation hoods.

8.1.3.2. Phenols and Phenolic Compounds

Phenols (or carbolic acids) are derived from coal tar and are used as disinfectants. It was first time used by **Lister**. It has a corrosive property and may produce harmful effects in sensitive people. **Examples** of phenolic compounds used as disinfectants include 5% phenol, 1-5% cresol, 5% lysol (saponified cresol), hexachlorophene, chlorhexidine, and chloroxylenol (Dettol).

Mode of Action

Phenols act by disrupting the cell membranes, precipitating the proteins, and by inactivating the microbial enzymes. They alter the selective permeability of cytoplasmic membrane of microbial cell, thereby the vital intracellular substances leak out. Depending on their concentration to be used, they may be either bacteriostatic or bactericidal.

Applications

1) **Phenol** functions as bactericidal, fungicidal, and mycobactericidal, but is ineffective against spores and viruses.
2) The **corrosive phenolic** is used to disinfect the ward floors, discarded jars in laboratories, and bedpans.
3) **Cresol** (lysol) is a soap solution of phenol-like compounds (*o*-phenyl-phenol, *o*-benzyl-*p*-chlorophenol, xylenols). It is useful in disinfecting the inanimate objects like floors, walls, table surfaces, and contaminated hospital items (like rectal thermometers, excreta, secretions from infected patients, etc.).
4) **Chlorhexidine** in isopropanol solution is used for skin disinfection, and its aqueous solution is used for wound irrigation. It is also used as an antiseptic hand wash.
5) **Chlorhexidine gluconate** solution (20%) is used as a general skin disinfectant and also as a preoperative hand and skin disinfectant. Chlorhexidine gluconate solution with quaternary ammonium compounds (**e.g.,** cetrimide) show stronger and broader antimicrobial effects (**e.g.,** Savlon).
6) **Chloroxylenols** are topically suitable due to less irritant property. These compounds are more effective against gram-positive bacteria than against gram-negative bacteria.
7) **Hexachlorophene** is a chlorinated diphenyl preparation and is relatively less irritant. It is highly effective against gram-positive bacteria and is less effective against gram-negative bacteria, mycobacteria, fungi, and viruses.

8) **Triclosan** is organic phenyl ether and is very effective against gram-positive bacteria and to some extent against gram-negative bacteria. It is also effective against fungi and viruses.

9) **Hexylresorcinol** is a complex chlorinated phenolic compound. It is incorporated into mouthwashes, topical antiseptics, and throat lozenges. It is also used as dusting powders and creams.

8.1.3.3. Aldehydes

Formaldehyde works as a bactericidal, sporicidal, and virucidal agent. It is used in aqueous as well as gaseous form. For general purposes a **10% aqueous formalin solution** is used.

Glutaraldehyde is highly effective against bacteria (**e.g.,** *M. tuberculosis*), fungi, and viruses (like HIV). Its toxicity and irritancy to skin and eyes is comparatively less than formaldehyde. Generally, it is used as a 2% buffered solution.

Mode of Action

Aldehydes cause alkylation of amino-, carboxyl-, or hydroxyl groups, and damage nucleic acids. They are effective against all microorganisms and spores.

Applications

1) A **40% formaldehyde** solution (formalin) is used to disinfect surface. It is used to fumigate rooms, chambers, operation theatres, biological safety cabinets, wards, sick rooms, etc.
2) It is used as a preservative for histological examination of tissues.
3) It is used for sterilising bacterial vaccines.
4) It is used for preparing toxoid from toxin.
5) Fumigation process is performed by boiling formalin, heating paraformaldehyde, or treating formalin with potassium permanganate.
6) It is also used for sterilising bedding, furniture, and books.
7) **10% Formalin** solution with 0.5% tetraborate is used for sterilising clean metal instruments.
8) **2% Gluteraldehyde** solution is used for sterilising thermometers, cystoscopes, bronchoscopes, centrifuges, anasethetic equipment, etc. Gluteraldehyde action requires an exposure of at least 3 hours at alkaline pH.
9) It is also used for sterilising plastic endotracheal tube, face masks, corrugated rubber anaesthetic tubes, and metal instruments.
10) **2% Formaldehyde** solution is used at 40°C for 20 minutes for disinfecting wool; and **0.25% formaldehyde** solution is used at 60°C for 6 hours for disinfecting animal hair and bristles.

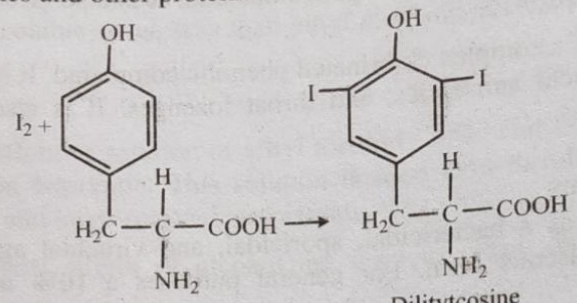
8.1.3.4. Halogens

Halogens being strong oxidising agents are highly reactive and destructive towards vital cellular components of microbes. Members of the halogen family (especially iodine, chlorine, and to some extent bromine) are used as key components in various antimicrobial chemicals. Iodine is the oldest microbicidal agent with highest efficacy.

Mode of Action

Halogens act as oxidising agents and damage the essential sulfydryl groups of enzymes by oxidation reaction. **Iodine** is a strong oxidising agent and can destroy essential

microbial metabolic compounds by oxidation. Iodine combines with tyrosine amino acid to inactivate enzymes and other proteins:



Iodine Tytcosine (active) Dilitytcosine (inactive)

On adding free chlorine to water, hypochlorous acid (HClO) is formed, which is responsible for the antimicrobial activity of chlorine and its compounds:

$$Cl_2 + H_2O \longrightarrow \underset{\text{Hydrochloric acid}}{HCl} + \underset{\text{Hypochlorous acid}}{HClO}$$

In water, **hypochlorites** and **chloramines** hydrolyses into hypochlorous acid, which further gives nascent oxygen [O]:

$$HClO \longrightarrow HCl + \underset{\text{Nascent oxygen}}{[O]}$$

Nascent oxygen being a strong oxidising agent damages the vital cellular substances of microbes. Chlorine combines directly with the cellular proteins of microbes and block their biological activity.

Applications

1) **Iodine** acts as a germicidal agent, highly effective against all types of bacteria.
2) It also acts as a sporicidal, fungicidal, virucidal, and amoebicidal.
3) **Tincture of iodine** made of 2% iodine in 70% alcohol acts as an antiseptic.
4) Iodine with neutral carrier polymers (**e.g.,** polyvinyl pyrrolidone) forms iodophores (**e.g.,** povidone-iodine), which allow slow release and reduce the irritation of antiseptic. Iodophores diluted with 50% alcohol are used for preparing hand washes.
5) **10% Povidone iodine** in undiluted form is used as a pre- and post-operative skin disinfectant.
6) **Iodine preparations** are mainly used as skin disinfectants.
7) Iodine is also used for disinfecting small quantities of water and for sanitising food utensils.
8) Sometimes, **iodine vapour** is used for disinfecting air.
9) **Liquefied chlorine gas** is preferred for disinfecting drinking water and swimming pool water. It is also used for treating effluents from sewage-treatment plants.
10) Products having **calcium hypochlorite** are used to sanitise utensils in restaurants and equipment in dairy plants.
11) **1% Sodium hypochlorite** solution is used for personal hygiene and for affordable household disinfection.
12) Higher concentrations (around 5-12%) of sodium hypochlorite are used as household bleaches, disinfectants, and as sanitisers in dairy and food-processing plants.

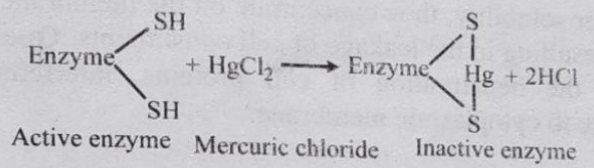
8.1.3.5. Heavy Metals

The term 'heavy metal' is used for denoting mercury, lead, zinc, silver, and copper. In ancient times, water-storage containers of silver and copper were used, as it was noticed that these metal vessels kept water safe to drink. In the early 20th century, **mercuric chloride** ($HgCl_2$) had been widely used as a general disinfectant; but later, it was replaced with other less toxic and corrosive agents.

Other than mercuric chloride, silver nitrate, copper sulphate, and organic mercury salts (mercurochrome, merthiolate) are **examples** of other heavy metal compounds.

Mode of Action

Heavy metals bind to the cellular proteins and inactivate them. **For example,** mercuric chloride combines with sulphydryl (—SH) groups that inactivate enzymes.



Applications

1) Certain organic **mercury-containing compounds** show high antimicrobial activity with low toxicity in comparison to inorganic mercury compounds. **Examples** of such organic compounds are merbromin (Mercurochrome), thimerosal (Merthiolate), and nitromersol (Metaphen), which are used for treating minor cuts, wounds, and skin infections.
2) Earlier a 1% **silver nitrate** ($AgNO_3$) solution was used for disinfecting the eyes of infants at birth to prevent the gonococcal infections.
3) **0.5% Silver nitrate** solution is used in sponges or layers of gauze for preventing infections of burns.
4) **Silver sulphadiazine** is applied topically for preventing colonisation and infection of burned tissues.
5) **Copper salts** are used as fungicides. **For example, copper sulphate ($CuSO_4$)** is used as algicide for disinfecting open water bodies like reservoirs and pools.
6) It also acts as a fungicide and is a constituent of Bordeaux mixture used as a garden spray to protect plants against fungal infections.
7) **Zinc compounds** are fungicidals and are used in formulation of ointments and powders used for treating athlete's foot.

8.1.3.6. Oxidising Agents

Oxidising agents oxidise the cell membrane of microorganisms, resulting in the loss of cellular structure and cell-lysis or death of microbes. The key elements in oxidising agents are chlorine and oxygen. **Hydrogen peroxide** (H_2O_2) is a chief oxidising agent which in 3-6% concentration is effective against almost all organisms. At higher concentration (10-25%), it kills all the organisms including spores.

Mode of Action

Hydrogen peroxide releases nascent oxygen, therefore is used as an antimicrobial agent. It also gives hydroxyl-free radical which denatures proteins and DNA molecules.

Applications

1) At 6% concentration, it is used for decontaminating the instruments and equipment (like ventilators).
2) At 3% concentration, it is used for disinfecting the skin and deodorising wounds and ulcers.
3) Strong solutions of hydrogen peroxide act as sporicidal.

8.1.3.7. Surface Active Agents

Soaps or **detergents** are the examples of surface active agents. Detergents carrying negatively charged long chain hydrocarbons are **anionic detergents** (**e.g.,** soaps and bile salts); and detergents having the fat-soluble part which is made to have a positive charge by combining with a quaternary nitrogen atoms are **cationic detergents** (**e.g.,** cetrimide and benzalkonium chloride). Cationic detergents are also known as **quaternary ammonium compounds** (or quat).

Mode of Action

Surface active agents align themselves at the interfaces between lipid layer of bacterial cell membrane and surrounding aqueous medium. These compounds carry long chain of fat-soluble hydrocarbons and water-soluble charged ions. Under the influence of this dual nature of fat and water solubility, they concentrate on the membrane surfaces and disrupt the membrane, thus resulting in the leakage of cell constituents. **Quaternary ammonium compounds** act by the denaturation of cell proteins, interference with metabolic processes, and damage to cytoplasmic membrane.

Applications

1) They inhibit the growth of vegetative cells, Mycobacteria, and enveloped viruses.
2) Solutions of 1-2% of dilution are widely used as disinfectants for domestic and hospital purposes.
3) The **quaternary ammonium compounds** are germicidal in nature with low toxicity, high water solubility, high stability in solution, and with less corrosive properties; hence preferred as useful antiseptics, disinfectants, and sanitising agents.
4) They are used for disinfecting floors, walls, and other surfaces in hospitals, nursing homes, and other public places.
5) They are also used for sanitising food and beverage utensils in restaurants, surfaces, and equipment in food-processing industry.

8.1.3.8. Dyes

Aniline dyes include crystal violet, malachite green and brilliant green, while **acridine dyes** are acriflavin and aminacrine. Acriflavine is a mixture of proflavine and euflavine; in which only euflavine has effective antimicrobial properties.

Dyes show better antibacterial property for gram-positive bacteria than gram-negative bacteria; and are more bacteriostatic in nature.

Mode of Action

Both aniline and acridine dyes have low bactericidal action, and are bacteriostatic in high dilution. Aniline dyes interrupt the synthesis of peptidoglycan (a component of bacterial cell wall) and acridine dyes interrupt the synthesis of cellular nucleic acids and proteins of bacteria.

Applications

1) They have better antibacterial action for gram-positive bacteria.
2) They are topically applied as antiseptics for treating mild burns.
3) Gentian violet and acriflavine are applied as paint over the skin to treat bacteria infections.
4) Melachite green is incorporated in medium for inhibiting the growth of all bacteria except *Mycobacterium tuberculosis*.

8.1.4. Factors Influencing Disinfection

Extent of antimicrobial action, rate of disinfection, and potency of a disinfectant depends on the following factors:

1) **Concentration of Disinfectant:** Concentration of the disinfectant directly influences the rate of killing microorganisms. The curve obtained between the efficiency and concentration of disinfectant is generally exponential, rather than linear.

2) **Temperature:** Normally, on increasing the temperature the rate of disinfection increases. Temperature coefficient (Q_{10}) is used for quantitative representation of temperature effect on bacterial activity. Thus,

$$\theta^{10} \text{ or } Q_{10} = \frac{\text{Time required to kill at T°}}{\text{Time required to kill at (T+10)°}}$$

$$\theta(T_2 - T_1) = \frac{t_1}{t_2}$$

Where,

T_2 and T_1 = Two temperatures differing by 10°C

t_2 and t_1 = Corresponding lethal times

3) **Time of Exposure:** Each disinfectant takes sufficient time of contact to produce its action and to explain this first-order kinetics. The velocity or rate constant (K) is the measurement of the efficiency of the disinfectant:

$$K = \frac{1}{t} \log \frac{N_0}{N_t}$$

Where, t = Time for the viable count to fall from N_0 to N_t

N_0 = Initial number of microorganisms

N_t = Final number of microorganisms

The survivor/time curve is not constant; and shape of the curve mainly depends on the concentration of the disinfectant (**figure 8.1**):

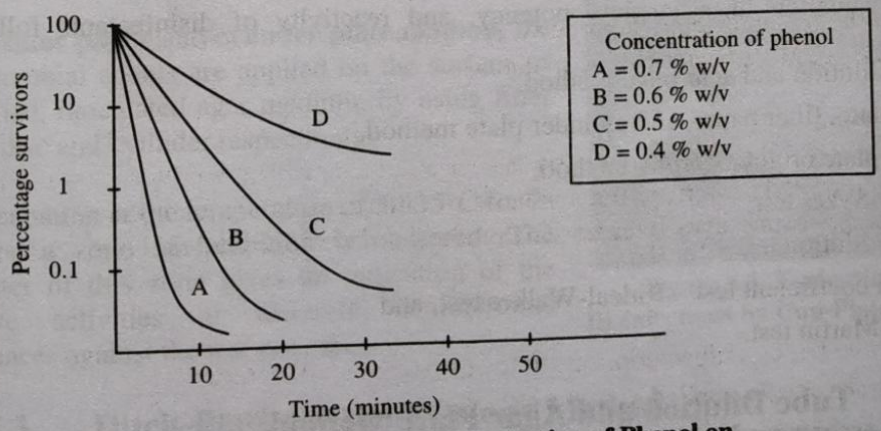


Figure 8.1: Effect of Concentration of Phenol on Survivor/Time Curves of Gram-Negative Organisms

4) **The pH of Environment:** The rate of growth of inoculum, and the potency and ability of the disinfectant to combine with the cell surface during the disinfection process depends on the pH changes.

5) **Surface Tension:** The surface properties influence the contact between the aqueous solutions of disinfectants. These surface properties help in adsorption of surface active disinfectants on the surfaces of cell, and also in spreading and wetting properties of the solution.

6) **Formulation of the Disinfectant:** Formulation of a disinfectant is an important factor as it influences the effective use of disinfectant. **For example,** quaternary ammonium compounds and chlorhexidine are efficient in 70% alcohol than in aqueous solution.

7) **Chemical Structure of Disinfectant:** Any change in the molecular structure of the chemical compound may alter the activity and efficiency of the disinfectant. **For example,** *para*-substitution of an alkyl chain up to 6-carbons in length increases antimicrobial activity, but greater than 6-carbons in length decreases water solubility and disinfectant activity.

8) **Type and Number of Microorganisms Present:** Number and nature of the contaminating microorganisms directly influence the efficiency of disinfection. The presence or absence of bacterial spores directly affects the efficiency of disinfectant, as most of the disinfecting agents are ineffective on bacterial spores and viruses.

9) **Interfering Substances in the Environment:** Presence of many compounds (**e.g.,** body fluid, food residues, blood, milk, pus, or colloidal proteins), even in small amount may decrease the effectiveness of many disinfecting agents.

10) **Synergism, Antagonism, and Potentiation of Disinfectants: Synergism** or **synergistic effects** are often shown by two antimicrobial agents which gives an increased activity. **Antagonism** or **antagonistic effects** result in decreased antimicrobial activity and is used in the elimination of antimicrobial properties of materials (tested for sterility), **e.g.,** sodium thiosulphate, lubrol W+ lecithin, etc. **Potentiation** of a disinfectant leads to enhanced antimicrobial activity, **e.g.,** polysorbate 80.

8.1.5. Evaluation for Bacteriostatic and Bactericidal Actions

For the evaluation of efficiency, potency, and reactivity of disinfectants following techniques are used:

1) Tube dilution and agar plate method,
2) Cup-plate, filter paper, and cylinder plate method,
3) Ditch-plate or giant colony method,
4) Kelsey-Sykes test,
5) In-use dilution test,
6) Phenol coefficient test – Rideal-Walker test, and
7) Chick-Martin test.

8.1.5.1. Tube Dilution and Agar Plate Method

The chemical disinfecting agent is introduced into the agar medium or nutrient broth medium. After that the medium are inoculated with the test microorganisms. The test tubes containing medium and microorganism is incubated at 30-35°C for 2-3 days and then the results are observed in the form of turbidity or colonies. These turbid colonies are recorded and the activity of given disinfectant is compared as shown in the **figure 8.2**:

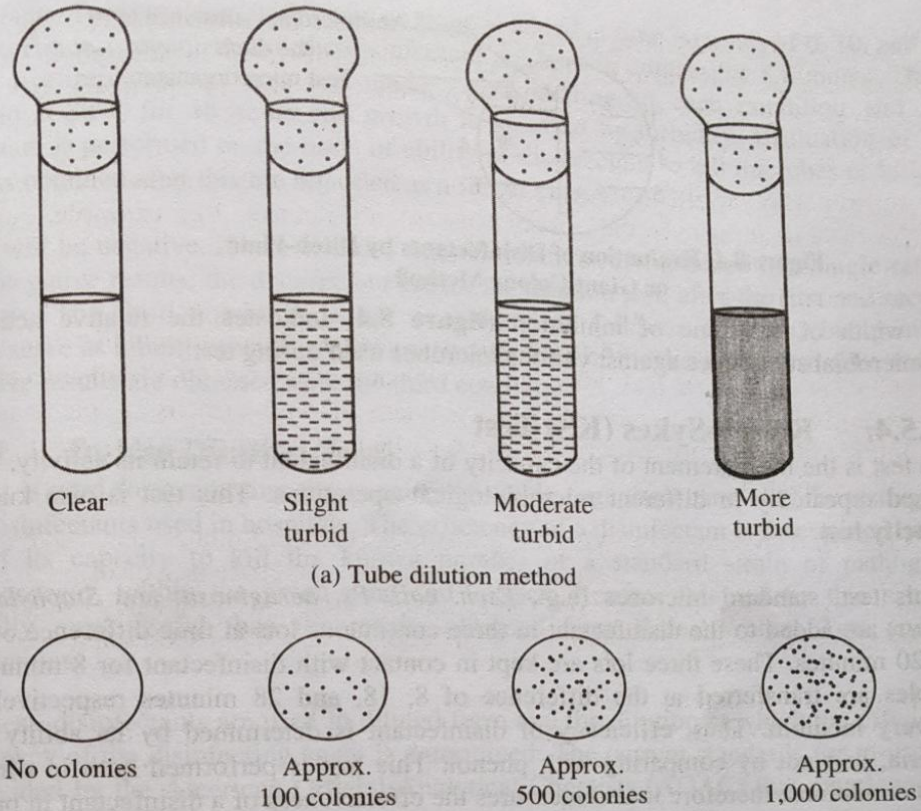


Figure 8.2: Evaluation of Disinfectants by (a) Tube Dilution and (b) Agar Plate Methods

8.1.5.2. Cup-Plate, Filter Paper, and Cylinder Plate Method

In this method, heating is performed for melting the agar, and cooled up to 45°C. The agar medium is inoculated with the test microorganisms and poured into a sterile petri dish. In the **cup-plate method**, when the inoculated agar has been solidified, holes of about 9mm in diameter are made by cutting the medium with sterile cork borer and the antimicrobial agents are directly placed in these holes (**figure 8.3**).

In the **filter paper** and **cylinder plate method**, the antimicrobial agents are applied on the surface of solidified, inoculated agar medium, by using filter paper disc and cylinder respectively.

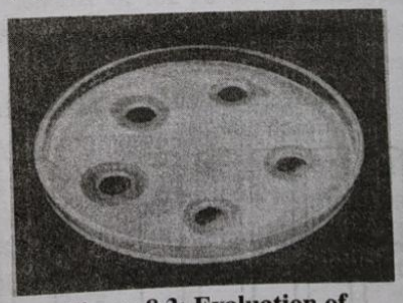


Figure 8.3: Evaluation of Disinfectants by Cup-Plate Method

On incubation at the temperature of 30-35°C for 2-3 days, a zone of inhibition is observed. The diameter of this zone gives an indication of the relative activities of different antimicrobial substances against the test microbes.

8.1.5.3. Ditch-Plate or Giant Colony Method

A ditch is prepared in agar plate and the solution of antimicrobial substances is carefully poured into the ditch. On the agar surface, a loopful of each test microorganism is streaked outwards from the ditch. Microbes resistant to the antimicrobial agent grow on right of the ditch; whereas susceptible microorganisms show a zone of inhibition adjacent to the ditch or centre of plate.

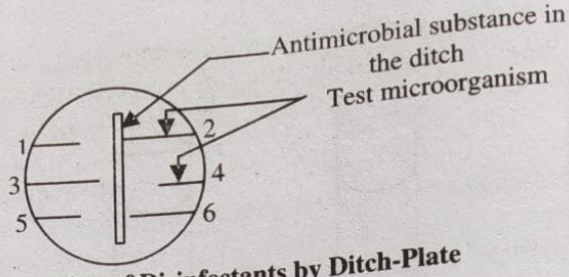


Figure 8.4: Evaluation of Disinfectants by Ditch-Plate or Giant Colony Method

The width of the zone of inhibition (**figure 8.4**) indicates the relative activity of antimicrobial substances against various microbes used during test.

8.1.5.4. Kelsey-Sykes (KS) Test

This test is the measurement of the capacity of a disinfectant to retain its activity, when it is used repeatedly in different microbiological operations. This test is also known as **capacity test**.

In this test, standard microbes (**e.g.,** *Esch. coli, Ps. aeruginosa*, and *Staphylococcus aureus*) are added to the disinfectant in three continuous lots at time difference of 0, 10, and 20 minutes. These three lots are kept in contact with disinfectant for 8 minutes and samples are transferred at the difference of 8, 18, and 28 minutes respectively to a recovery medium. Thus, efficiency of disinfectant is determined by its ability to kill bacteria, and not by comparing with phenol. This test is performed both in **dirty** and **clean** conditions; therefore it also measures the effectiveness of a disinfectant in presence of an organic matter.

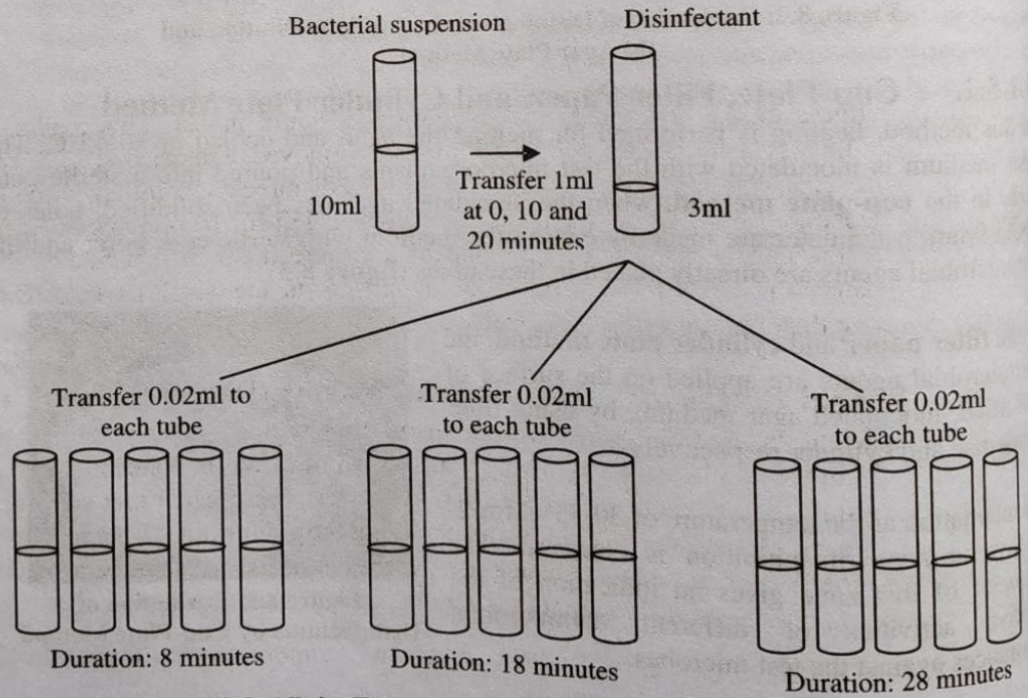


Figure 8.5: All the Tubes are Incubated at 32°C for 48 Hours

The selection of the test organism (*S.aureus*, *P. aeruginosa*, *P. vulgaris*, and *E. coli*) depends on the type of disinfectant used for testing procedure. For carrying the test under 'clean' conditions, the dilutions of the disinfectant are made in hard water; whereas, to run the test under 'dirty' conditions yeast suspension is used for making dilutions of the

disinfectants. Test organism with yeast or alone is added at time intervals of 0, 10, and 20 minutes. The test organism and the disinfectant are kept in contact for 8 minutes. Three sets of five replicate cultures are made, corresponding to each condition and are incubated at 32°C for 48 hours and growth is assessed by turbidity. Evaluation of the disinfectant is performed on the basis of ability of disinfectant to kill microbes or lack of it; results obtained after this are regarded as a fail or pass.

Results will be negative, if two or more negative cultures are obtained in a single set. In case of negative results, the disinfectant passes the dilution test, after the first and second conditions. The third condition is not considered in fail/pass criterion, but positive cultures serve as inbuilt controls. A lower concentration of the disinfectant may be tested, if negative results are obtained after the third condition.

8.1.5.5. In-Use Dilution Test

This test is used for examining the quantity of viable organisms, and also for testing the liquid disinfectants used in hospitals. The efficiency of a disinfectant is determined on the basis of its capacity to kill the known number of a standard strain of pathogenic *Staphylococcus*, within the given time and surface. Results obtained by this test are practically more useful than the results obtained from Rideal-Walker Test and its modifications.

In this test, disinfectants are used in diluted form and the dilution at which the efficiency and potency of the disinfecting agent is determined. The current standards for in-use test are provided by the American Official Analytical Chemists (AOAC) that mentioned the use of **three bacterial strains**:

1) *Staphylococcus aureus*,
2) *Salmonella choleraesuis*, and
3) *Pseudomonas aeruginosa*.

Standardised cultures of the test organisms are grown in liquid media. This media is standardised and metal carrier rings are dipped into it and removed, and dried at 37°C for a short time. After that, the dried cultures are placed into a disinfectant solution, made at a concentration specified by the manufactures. The culture is left for 10 minutes at 20°C. In the next step, the carrier rings are transferred to a culture medium that allows the growth of any surviving organisms. Number of organisms in the resulting culture is calculated showing the effectiveness of the disinfectants.

8.1.5.6. Phenol Coefficient Test – Rideal-Walker (RW) Test

In phenol coefficient or Rideal-Walker test, a suspension with similar quantities of organisms is used in the estimation of efficiency and action of different concentration of phenol and the disinfectant to be tested. A solution of the test disinfectant that sterilises the suspension in a given time is divided by the corresponding dilutions of phenol; this gives the phenol coefficient. However, phenol coefficient is not the measurement of the practical functioning of the test disinfectant in the presence of organic matters.

By Rideal-Walker test (that uses Rideal-Walker broth and *Salmonella typhi*), the phenol coefficient of test disinfectant may be calculated.

By using the testing disinfectant and phenol, different dilutions are prepared and 5ml of each dilution are inoculated with 0.5ml of the 24 hours broth culture of the organisms. All incubated tubes (disinfectant + organisms and phenol + organisms) are kept at the temperature of 17.5°C in a water bath. Sub-cultures of each reaction mixture is prepared and transferred to 5ml sterile broth, at time intervals of 2.5, 5, 7.5, and 10 minutes.

The broth tubes are incubated at the temperature of 37°C for 48-72 hours, and examined for the absence or presence of the growth of microorganisms. A typical example of test result is shown below in **table 8.1**. The Rideal-Walker coefficient of the test disinfectant is calculated, according to the data obtained (shown in **table 8.1**).

Table 8.1: Determination of Rideal-Walker Coefficient

Disinfectant	Dilution	Time Interval for Sub-Culture (Minute)			
		2.5	5	7.5	10
Test disinfectant	1 : 1000	+	–	–	–
	1 : 2000	+	+	–	–
	1 : 3000	+	+	+	–
	1 : 4000	+	+	+	+
Phenol	1 : 80	+	–	–	–
	1 : 100	+	+	–	–
	1 : 120	+	+	+	–
	1 : 140	+	+	+	+

$$\text{R.W.coeffieient} = \frac{\text{Dilution of disinfectant killing in 10 minutes but not in 5 minutes}}{\text{Dilution of phenol killing in 10 minutes but not in 5 minutes}}$$

$$= \frac{2000}{100} = 20$$

Conclusion

1) If the Rideal-Walker or phenol coefficient for a given test disinfectant is 1, the disinfectant has the same effectiveness as phenol.
2) If phenol coefficient of test disinfectant is 20, the disinfectant is 20 times more active than phenol.
3) If phenol coefficient of test disinfectant is less than 1, it is less effective; and if more than 1, it is more effective as compared to phenol.

8.1.5.7. Chick-Martin Test

The phenol coefficient test is performed on laboratory level to study the bactericidal action of compound with respect to phenol. In 1908, **Chick** and **Martin** suggested that the disinfectants are essential to act in the presence of organic matter, so they recommended the use of dried human faeces in the test system. As a substitute to human faeces, **Garrod** suggested to use dried yeast.

In this test, *Salmonella typhi* (test organism) is inoculated into solutions having graded concentrations of test substance (or phenol in case of standard) along with dried yeast. The components are left for 30 minutes at 20°C to interact and lastly duplicate sub-cultures are made into nutrient broth.

These sub-culture tubes are incubated for 48 hours at 37°C. Observations are made regarding the presence or absence of growth of microorganisms. The phenol concentration that prevents growth in both systems is determined and the mean value is calculated. The same value would be obtained for the unknown. For calculating the coefficient, the value obtained for phenol is divided by the value found for the unknown.

8.2. SUMMARY

The details given in chapter can be summarised as follows:

1) **Disinfection** is a process of destruction or elimination of microbes, and decreasing their numbers in such a low level, that they become unable to impart any harmful effect.

2) The chemical agents used in case of lifeless or inanimate objects (**e.g.,** working area, dishes, bench, etc.) are termed as **disinfectants**.

3) The **antiseptics** are chemical agent, used on animate or living bodies (**e.g.,** human body tissue, plant tissue, etc.).

4) Disinfectants are classified on the basis of the following factors:
   i) **On the basis of consistency** or **state of matter**, disinfectants are of two types:
      a) Liquid (**e.g.,** alcohols, phenols), and
      b) Gaseous (**e.g.,** formaldehyde vapour).
   ii) **On the basis of spectrum of activity**, disinfectants are of three types:
      a) High level disinfectants,
      b) Intermediate level disinfectants, and
      c) Low level disinfectants.
   iii) **On the basis of mechanism of action**, disinfectants are of following types:
      a) Disinfectants **acting on plasma membrane** (**e.g.,** alcohol, detergent),
      b) Disinfectants **denaturing cellular proteins** (**e.g.,** alcohol, phenol),
      c) Disinfectants **oxidising essential sulphydryl groups of enzymes** (**e.g.,** H_2O_2, halogens),
      d) Disinfectants **alkylating amino-, carboxyl-, and hydroxyl group** (**e.g.,** formaldehyde), and
      e) Disinfectants **damaging nucleic acid** (**e.g.,** formaldehyde).

5) **Alcohols** (**e.g.,** ethyl alcohol, methyl alcohol, and isopropyl alcohol) are broad spectrum antimicrobial agents. They act by dehydrating the cells, by disrupting bacterial cell membranes, and by coagulating the protein content of microorganisms.

6) **Phenols** (or carbolic acids) are derived from coal tar and are used as disinfectants. They act by disrupting the cell membranes, precipitating the proteins, and by inactivating the microbial enzymes.

7) **Formaldehyde** works as a bactericidal, sporicidal, and virucidal agent.

8) **Aldehydes** cause alkylation of amino-, carboxyl-, or hydroxyl groups, and also damage nucleic acids.

9) **Halogens** are strong oxidising agents, therefore are highly reactive and destructive towards vital microbial cellular components. They oxidise and damage the essential sulfydryl groups of enzymes by oxidation reaction.

10) The term 'heavy metal' is used for metals like mercury, lead, zinc, silver, and copper. **Heavy metals** bind to the cellular protein and inactivate them. **For example,** mercuric chloride combines with sulphydryl (—SH) groups that inactivate enzymes.

11) Oxidising agents oxidise the cell membrane of microorganisms, resulting in the loss of cellular structure and cell-lysis or death of microbes. The key elements in oxidising agents are chlorine and oxygen.

12) **Hydrogen peroxide (H_2O_2)** is a chief oxidising agent which is effective against almost all organisms at 3-6% concentration. At higher concentration (10-25%), it kills all the organisms including spores.

13) Hydrogen peroxide releases nascent oxygen, therefore is used as an antimicrobial agent. It also gives hydroxyl-free radical which denatures proteins and DNA molecules.

14) **Soaps** or **detergents** are the examples of surface active agents.

15) Detergents carrying negatively charged long chain hydrocarbon are **anionic detergents** (**e.g.,** soaps and bile salts).

16) Detergents having the fat-soluble part which is made to have a positive charge by combining with a quaternary nitrogen atoms are **cationic detergents** (**e.g.,** cetrimide and benzalkonium chloride). Cationic detergents are also known as **quaternary ammonium compounds** (or quat).

17) **Aniline dyes** include crystal violet, malachite green and brilliant green, while **acridine dyes** are acriflavin and aminacrine. Acriflavine is a mixture of proflavine and euflavine, in which only euflavine has effective antimicrobial properties.

18) Extent of antimicrobial action and rate of disinfection as well as potency of a disinfectant depends on the following factors:
    i) Concentration of disinfectant,
    ii) Temperature,
    iii) Time of exposure,
    iv) The pH of environment,
    v) Surface tension,
    vi) Formulation of the disinfectant,
    vii) Chemical structure of disinfectant,
    viii) Type and number of microorganisms present,
    ix) Interfering substances in the environment, and
    x) Synergism, antagonism, and potentiation of disinfectants.

19) On increasing the temperature the rate of disinfection increases. **Temperature coefficient (Q_{10})** is used for quantitative representation of effect of temperature on bacterial activity.

20) **Synergistic** effects are shown by two antimicrobial agents which gives an increased activity.

21) **Antagonism** or **antagonistic effects** result in decreased antimicrobial activity and is used in the elimination of antimicrobial properties of materials (tested for sterility), **e.g.,** sodium thiosulphate, lubrol W+ lecithin, etc.

22) **Potentiation** of a disinfectant leads to enhanced antimicrobial activity, **e.g.,** polysorbate 80.

23) For the evaluation of efficiency, potency, and reactivity of disinfectants following techniques are used:
    i) Tube dilution and agar plate method,
    ii) Cup-plate, filter paper, and cylinder plate method,
    iii) Ditch-plate or giant colony method,
    iv) Kelsey-Sykes test,
    v) In-use dilution test,
    vi) Phenol coefficient test – Rideal-Walker test, and
    vii) Chick-Martin test.

8.3. EXERCISE

8.3.1. True or False

1) Heavy metals bind to the cellular protein and activate them.
2) Halogens are strong oxidising agents, therefore are destructive towards vital microbial cellular components.
3) Disinfection is a process of destruction or elimination of microbes, and decreasing their numbers in such a low level, that they become unable to impart any harmful effect.
4) The antiseptics are chemical agents, used on non-living bodies.
5) Alcohols act by dehydrating the cells, by disrupting bacterial cell membranes, and by coagulating the protein content of microorganisms.
6) Synergistic effects are often shown by two antimicrobial agents, giving a decreased activity.
7) Antagonism or antagonistic effects result in decreased antimicrobial activity.

8.3.2. Fill in the Blanks

8) Soaps or detergents are the examples of_____.
9) Hydrogen peroxide (H_2O_2) is an _____, effective against almost all organisms at 3-6% concentration.
10) _____ works as a bactericidal, sporicidal, and virucidal agent.
11) The chemical agents used in case of lifeless or inanimate objects are termed as_____ .
12) Alcohols are _____ antimicrobial agents.
13) _____ is used for quantitative representation of effect of temperature on bacterial activity.
14) _____ of a disinfectant leads to enhanced antimicrobial activity.

Answers:

1) False 2) True 3) True 4) False 5) True
6) False 7) True 8) Surface active agents 9) Oxidising agent
10) Formaldehyde 11) Disinfectants 12) Broad spectrum
13) Temperature coefficient (Q_{10}) 14) Potentiation

8.3.3. Very Short Answer Type Questions

1) Give examples of oxidising agents.
2) What is temperature coefficient?
3) What is Chick-Martin test?
4) Enlist various factors influencing disinfection.
5) Give mode of action of dyes.
6) Give application of halogens as disinfectants.
7) What is the mechanism of heavy metals as disinfectant?

8.3.4. Short Answer Type Questions

1) Write a note on ideal properties of disinfectant.
2) What is Phenol coefficient test?
3) Write a short note on following methods of evaluation:
   i) Tube dilution method
   ii) Filter paper method
4) Give the mode of action and applications of phenols and phenolic compounds.
5) Write mode of action of action of following disinfectants
   i) Surface active agents ii) Alcohols
6) Write a note on factors affecting disinfection.

8.3.5. Long Answer Type Questions

1) What are disinfectants? Classify them.
2) Give a descriptive note on mode of action of various disinfectants.
3) Write a note on evaluation of bacteriostatic and bactericidal actions of disinfectants.

CHAPTER 9

Sterility Testing of Products

9.1. STERILITY TESTING OF PRODUCTS

9.1.1. Introduction

Sterility test evaluates whether or not a sterilised pharmaceutical product is free from contaminating microorganisms. **According to I.P. 1996**, the sterility tests are intended for detecting the presence of viable forms of microorganisms in or on the Pharmacopoeial preparations.

It is essential to strictly follow the important guidelines and vital precautionary measures to achieve maximum accuracy and precision of the entire concept of sterility testing for life-saving secondary pharmaceutical products (drugs). Some of these basic factors, guidelines, and necessary details are given below:

1) Sterility testing is closely related to a statistical process in which a batch portion is randomly sampled; thus, the chance of the particular batch (lot) duly passed for consumption depends on the 'sample' which has passed the sterility test.

2) Sterility testing should be performed under such conditions which are strictly designed to avoid accidental contamination of the product under analysis. These precautionary measures should not adversely affect the microbes that are required to be revealed in the final test results.

3) The working environment where the sterility tests are performed should be monitored at regular intervals by sampling the air and working area surface by performing necessary control tests.

4) Sterility testing is based on the principle that when bacteria are deliberately placed in a specific medium which provides the required nutrition and water, and is also maintained at a favourable temperature (37±2°C), the microbes tend to grow, and their growth is visible in the form of turbidity in the medium (which was originally clear).

5) The extent of probability in detection of viable microorganisms for the sterility tests increases with the actual number present in a given quantity of the preparation under analysis; it varies as per the species of microorganisms present. However, very low contamination levels cannot be detected by random sampling of a batch.

   A **batch** is defined as a homogeneous collection of sealed containers prepared such that the contamination risk is same for each of the units present in it.

6) Random sampling cannot accurately detect contamination if the observed contamination is not uniform throughout the batch. In such a case, compliance with the sterility tests alone cannot assure freedom from microbial contamination; and thus a However, greater assurance of sterility should be originated from reliable manufacturing processes (such as strict compliance with Good Manufacturing Practices).

7) Sterility tests are designed for detecting the presence of microorganisms in the samples under analysis. However, the results depend on the assumption that the contents of each and every container in the batch (tested) should comply with the

tests. Practically each and every container cannot be tested; therefore a sufficient number of containers should be examined to give a suitable degree of confidence in the final test results.

8) It has been observed that no definite sampling plan exists for applying the tests to a specified proportion of discrete units (carefully selected from a batch capable of demonstrating that all the untested units are sterile). Therefore, it is relevant that while determining the number of units to be tested, the manufacturer should have adequate regards to the environment parameters of manufacture, the volume of preparation per container, and other special considerations related to the preparation under analysis. **Table 9.1** records the exact number of items recommended to be tested with respect to the number of items in the batch on the assumption that the preparation has been manufactured under specified parameters designed for preventing contamination.

Table 9.1: Profile of Guidance – Number of Items in a Batch *versus* Minimum Number of Items Recommended to be Tested

Product Variants	Number of Items in a Batch	Minimum Number of Items Recommended to be Tested
1) **Injectable Preparations**	i) Not more than 100 containers. ii) More than 100, but not more than 500 containers. iii) More than 500 containers.	i) Either 10% or 4 containers, whichever is greater. ii) 10 containers. iii) Either 2% or 20 containers, whichever is less.
2) **Ophthalmic and Other Non-Injectable Preparations**	i) Not more than 200 containers. ii) More than 200 containers.	i) Either 5% or 2 containers, whichever is greater. ii) 10 containers.
3) **Surgical Dressings**	i) Not more than 100 packages. ii) More than 100, but not more than 500 packages. iii) More than 500 packages.	i) Either 10% or 4 packages, whichever is greater. ii) 10 packages. iii) Either 2% or 20 package, whichever is less.
4) **Bulk Solids**	i) Less than 4 containers. ii) 4 containers, but not more than 50 containers. iii) More than 50 containers.	i) Each container. ii) Either 20% or 4 containers, whichever is greater. iii) Either 2% or 10 containers, whichever is greater.

9.1.2. Sterility Test for Pharmaceutical Products

The wide-spectrum of pharmaceutical products (both pure as well as dosage forms) may be achieved by adopting any one of the two well-recognised, time-tested, and universally accepted methods:

1) Membrane filtration, and
2) Direct inoculation.

9.1.3. Membrane Filtration

The membrane filtration method is used for avoiding and also overcoming the activity of antibiotics for which practically little inactivating agents exist. Following conditions should be fulfilled for the membrane filtration method:

1) An exceptional skilled and knowledgeable operators, and
2) Rigorous routine usage of positive and negative controls.

The **salient features** of membrane filtration method are:

1) Solution of the product under analysis is filtered via hydrophobic-edged membrane filter which effectively retains the contaminating microbes.
2) The resulting membrane is washed *in situ* to remove 'traces of antibiotic' adhered to the membrane surface.
3) The segregated microorganisms are aseptically transferred to the culture media.

9.1.3.1. Culture Media

For sterility testing following media are preferred:

1) **Fluid Thioglycolate Medium:** This medium is used for clear fluid products (**table 9.2**):

Table 9.2: Fluid Thioglycollate Medium

Ingredients	Quantity (g)
1) L-Cystine	0.5
2) Sodium chloride	2.5
3) Dextrose [$C_6H_{12}O_6 \cdot H_2O$]	5.5
4) Granular agar [moisture < 15% w/w]	0.75
5) Yeast-extract (water-soluble)	5.0
6) Pancreatic digest of casein	15.0
7) Sodium thioglycollate or	0.5
8) Thioglycollic acid	0.3ml
9) Resazurin [0.1% fresh solution]	1.0ml
10) Distilled water to	1000ml

2) **Alternative Thioglycolate Medium:** This medium is used for turbid and viscid products as well as for devices with tubes of small lumina (**table 9.3**):

Table 9.3: Alternative Thioglycollate Medium

Ingredients	Quantity (g)
1) L-Cystine	0.5
2) Sodium chloride	2.5
3) Dextrose [$C_6H_{12}O_6 \cdot H_2O$]	5.5
4) Yeast-extract (water-soluble)	5.0
5) Pancreatic digest of casein	15.0
6) Sodium thioglycollate or	0.5
7) Distilled water to	1000ml

3) **Fluid Soyabean-Casein Digest Medium:** This media (**table 9.4**) is prepared by dissolving solids in distilled water followed by slight warming. The solution is cooled to room temperature and if necessary sufficient amount of 0.1M NaOH is

added to give a final pH of 7.1 ± 0.2 after sterilisation. Solution is distributed into suitable containers and sterilised in an autoclave at 121°C for 20 minutes.

Table 9.4: Fluid Soyabean-Casein Digest Medium

Ingredients	Quantity (g)
1) Pancreatic digest of casein	17.0
2) Papaic digest of soyabean meal	3.0
3) Sodium chloride	5.0
4) Diabasic potassium phosphate [K_2HPO_4]	2.5
5) Dextrose monohydrate [$C_6H_{12}O_6 \cdot H_2O$]	2.5
6) Distilled water to	1000ml

9.1.3.2. Test Organisms

The test microbes for various media and their incubation conditions are mentioned in **table 9.5**:

Table 9.5: Test Microorganisms Used for Different Medium

Medium	Test Microorganisms (Strains Specified in I.P.)	Incubation	
		Temperature (°C)	Condition
Fluid Thioglycolate	1) *Bacillus subtilis*	30 to 35	Aerobic
	2) *Candida albicans*	30 to 35	Aerobic
	3) *Bacteroides vulgatus*	30 to 35	Aerobic
Alternative Thioglycolate	*Bacteroides vulgatus*	30 to 35	Anaerobic
Soybean-Casein Digest	1) *Bacillus subtilis*	20 to 25	Aerobic
	2) *Candida albicans*	20 to 25	Aerobic

9.1.3.3. Precautions to be Taken

The sterility tests should be performed under highly specific experimental parameters to avoid even least possibility of accidental contamination of the product under analysis. The precautionary measures to be taken are:

1) A sophisticated laminar sterile airflow cabinet provided with effective HEPA filters should be used.
2) Necessary steps should be taken to avoid contamination that they do not affect any microbes need to be revealed in the test.
3) The environmental or working conditions in the laboratory where the sterility tests are being performed should be monitored timely by:
   i) Sampling the air of working area,
   ii) Sampling the surface of working area, and
   iii) Performing the specified control tests.

9.1.3.4. Apparatus

The most suitable apparatus consists of a closed reservoir and a receptacle between which a properly supported membrane of appropriate porosity is present. Some other **features** of the apparatus are:

1) A membrane suitable for sterility testing has a pore size of 0.45µm or less, and diameter of around 47mm. The efficiency of membrane in retaining microbes has been satisfactorily established.
2) The whole unit is assembled and sterilised with the membrane prior to use.
3) If the sample to be used is oil, the membrane is sterilised separately, thoroughly dried, and then the unit is assembled taking the required aseptic precautionary measures.

9.1.3.5. Dilution of Fluids

In sterility test, the following two types of fluids are used:

1) **Fluid A:** 1gm of peptic digest of animal tissue (such as bacteriological peptone) or its equivalent is dissolved in water and volume is adjusted to 1000ml. The solution is filtered or centrifuged to remove any particles present, and pH is adjusted to 7.1 ± 0.2. The resultant solution is dispensed in 100ml flasks and autoclaved at 121°C for 20 minutes.

   For example, in a sterility test of a sample of penicillin or cephalosporin class of antibiotics, the sterile penicillinase is aseptically added to the Fluid A (which is to be used for rinsing the membrane) in amount sufficient to inactivate any residual antibiotic activity on the membrane after the sample solution has been filtered.

2) **Fluid B:** If the test sample contains either oil or lecithin (a phosphoglyceride found in blood and egg yolk, and constitute a part of cell membrane), fluid B is used. To each litre of fluid B, 1ml of polysorbate 80 (non-ionic surface-active agent composed of polyoxyethylene esters of sorbitol) has been added. Fluid B contains associated fatty acids, and is used in preparing pharmaceuticals. After adjusting its pH to 7.1 ± 0.2, it is dispensed into flasks and autoclaved at 121°C for 20 minutes.

   Note: A sterile fluid which can be used for dissolving, diluting, or rinsing a preparation under analysis for sterility should be free from any antimicrobial or antifungal properties.

9.1.3.6. Quantities of Sample to be Used

The exact quantities of sample to be used for determining the tests for sterility are different for injectables, ophthalmics, and other non-injectables, as discussed below:

1) **For Injectable Preparations:** All the contents of the container should be used as a routine practice and wherever possible. However, not less than the quantities mentioned in **table 9.6**, dilutions are made to 100ml with an appropriate sterile diluent, **e.g.,** Fluid A.

Table 9.6: Quantities of Liquids/Solids per Container of Injectables *versus* Minimum Quantity Recommended for Each Culture Medium

Type of Preparation	Quantity in Each Container of Injectables	Minimum Quantity Recommended for Each Culture Medium
1) **For Liquids**	i) Less than 1mL	i) Total contents of a container.
	ii) 1mL or more but < 4mL	ii) Half the contents of a container.
	iii) 4mL or more but < 20mL	iii) 2ml
	iv) 20mL or more but < 100mL	iv) 10% of the contents of a container unless otherwise specified duly in the 'monograph'.
	v) 100mL or more	v) Not less than half the contents of a container unless otherwise specified in the 'monograph'.
2) **For Solids**	i) Less than 50mg	i) Total contents of a container.
	ii) 50mg or more but < 200mg	ii) Half the contents of a container.
	iii) 200mg or more	iii) 100mg.

2) **For Ophthalmic and other Non-Injectable Preparations:** In this case, an amount lying within the range prescribed in Column (A) of **table 9.7** is used, using the contents of more than one container, and mixing thoroughly. For each medium, the amount specified in column (B) of **table 9.7** should be taken from the mixed sample.

Table 9.7: Type of Preparation *versus* Quantity to be Mixed and Quantity to be Used for Each Culture Medium

Type of Preparation	Quantity to be Mixed (A)	Quantity to be Used for Each Culture Medium (B)
1) **Ophthalmic Solutions:** Other non-injectable liquid preparations.	10-100mL	5-10mL
2) **Other Preparations:** Preparations soluble in water or appropriate solvents; insoluble preparations to be suspended or emulsified duly (**e.g.,** creams and ointments).	1-10gm	0.5-1gm
3) Absorbent cotton		Not less than 1gm

9.1.3.7. Test Procedures

The test procedures for membrane filtration may be sub-divided into the following **six categories**:

1) **Aqueous Solutions:** The steps followed for sterility test of such preparations are:
   i) Each membrane is prepared by aseptically transferring a small amount of fluid A to the membrane to moisten it and then filtering it.
   ii) For each medium to be used, not less than the quantity of the preparation under analysis (given either in **table 9.6** or **9.7**) is aseptically transferred in two separate membrane filter funnels or two separate sterile pooling vessels.
   iii) Alternatively, the combined quantities of the preparation under analysis are aseptically transferred in the two media onto one membrane.
   iv) The liquid is quickly sucked in via membrane filter under vacuum (with the help of negative pressure).
   v) If the solution under analysis has significant antibacterial features, the membrane is washed thrice by filtering through it approximately 100ml of sterile fluid A each time.
   vi) The quantities of fluid A used should be sufficient enough to allow growth of small inoculum of microorganisms (nearly 50) sensitive to the antimicrobial substance in the presence of the residual inhibitory material retained on the membrane.
   vii) After filtration, the membrane is aseptically removed from the holder and cut into half; one half is immersed in 100ml of **fluid soyabean-casein digest medium (table 9.4)**, and incubated at 20-25°C for a week.
   viii) Similarly the other half of the membrane is immersed in 100ml of **fluid thioglycolate medium (table 9.2)**, and incubated at 30-35°C for a week.

2) **Liquids Immiscible with Aqueous Vehicles and Suspensions:** The steps followed for sterility test of such preparations are similar to those for aqueous solutions, but a sufficient amount of fluid A is added to the pooled sample for rapid filtration rate.

Special Features

i) Sterile enzyme preparations, **e.g.,** penicillinase and cellulase can be added to fluid A for dissolving the insoluble substances.

ii) If the substance under analysis contains lecithin, fluid B is always used for dilution.

3) **Oils and Oily Solutions:** The steps followed for sterility test of oils and oily solutions are:

i) Oils or oily solutions of low viscosity (i.e., without any dilution) are filtered via dry membrane.

ii) The viscous oils should be diluted using a sterile diluent, **e.g.,** isopropyl myristate not exhibiting any antimicrobial activity under the test conditions.

iii) The oil is allowed to penetrate the membrane, and then filtered by applying gradual suction using a vacuum pump.

iv) The membrane is washed by filtering through it atleast 3/4 successive quantities, each of around 100ml of sterile fluid B or any other sterile diluent.

v) The next steps are as described above for aqueous solutions [**from step (vi)**].

4) **Ointments and Creams:** The steps followed for sterility test of ointments and creams are:

i) Ointments are diluted either in a fatty base or w/o emulsions to yield a fluid of 1% w/v concentration. This can be done by gently heating to 40°C with a sterile diluent, **e.g.,** isopropyl myristate, previously filtered via 0.22µm membrane filter and not exhibiting any antimicrobial activity under the test conditions.

ii) Filtration is performed rapidly as described for the oils and oily solutions [**from step (iii)**].

iii) However, sometimes it becomes a necessity to heat the substance to 45°C, and using such warm solutions for rinsing the membrane effectively.

5) **Soluble Solids:** For each individual culture medium, the substance under analysis in quantity as prescribed in **tables 9.6** and **9.7** is dissolved in a sterile solvent (**e.g.,** fluid A). Then the test is preceded as for aqueous solutions by using a membrane suitable for selected solvents.

6) **Sterile Devices:** A sufficient volume of fluid B is aseptically passed through 20 devices so that atleast 100ml is recovered from each device. The recovered fluids are collected in sterile containers, and together filtered via membrane filter funnel as described for aqueous solutions.

9.1.3.8. Observation and Interpretation of Results

The results of direct inoculation are observed and interpreted by taking the following factors into consideration:

1) No bacterial growth during or after the incubation period indicates the preparation has passed the test.

2) In case of bacterial growth, the test is repeated with the containers showing.

3) The preparation is assumed to pass the test if bacterial growth does not occur after the first re-test.

4) However in case growth is observed in the re-test, the organisms are isolated and identified.
5) If these organisms are not readily distinguishable from those observed in the first test, the preparation is considered to fail the test.
6) On the other hand, if the organisms are distinguishable from the ones observed in the first test, a second re-test is performed using twice the number of samples.
7) If bacterial growth does not occur, the preparation passes the test; while it fails if growth is observed.

9.1.4. Direct Inoculation (or Direct Inoculation of Culture Media)

Following three culture media are commonly used to perform the tests for sterility:

1) **Nutrient Broth:** This medium is used for aerobic microorganisms and have the following features:
   i) The value of redox potential (Eh) of this medium is too high to allow the growth of anaerobes.
   ii) The culture medium, such as soyabean casein digest broth, Hartley's digest broth, only allows the fastidious microorganisms to grow.

2) **Cooked Meat Medium and Thioglycollate Medium:** These two media are discussed as follows:
   i) **Cooked Meat Medium:** This medium is used for culturing *Clostridia*.
   ii) **Thioglycollate Medium:** This medium is used for anaerobic microorganisms, and contains the following ingredients:

Ingredients	Purpose
1) **Glucose**	Energy source.
2) **Sodium thioglycolate**	Inactivate mercury compounds,
	Enhance and promote reducing parameters, and
	Act as a redox indicator.
3) **Agar**	Reduces the resultant 'convection currents'.

3) **Sabouraud Medium:** This medium is mainly used for fungal species, and has the following characteristic features:
   i) It is an acidic medium, and
   ii) It contains glucose or maltose (a rapidly fermentable carbohydrate).

Note:

1) The three media discussed above should be previously evaluated for their nutritive features, i.e., fertility tests to determine the growth of particular microorganisms.
2) The three media should also be previously incubated at specified temperature(s).

The direct inoculation method is now discussed under the following three steps:

1) Quantities of sample to be used,
2) Test method, and
3) Observation and interpretation of results.

9.1.4.1. Quantities of Sample to be Used

The exact quantity of the pharmaceutical preparation to be examined (i.e., to be used for inoculation in the culture media) varies as per the amount present in each container. This quantity along with the exact volume of culture medium to be used is given in **table 9.6**.

9.1.4.2. Test Procedures

Different test method is employed for different substances under analysis:

1) **Aqueous Solutions and Suspensions:** The microbial contamination tests are carried out using the media discussed above for the same sample of the preparation under analysis. In some cases, when the amount present in a container is insufficient to perform the specified tests, the contents of either two or more containers are combined and used for inoculating the media.

   The steps involved are:

   i) Liquid from the test containers are removed using a sterile pipette, or a sterile syringe or needle.

   ii) The required volume of the substance is aseptically transferred from each container to the culture medium vessel.

   iii) The liquid is mixed with the medium in such a way that aeration does not occur.

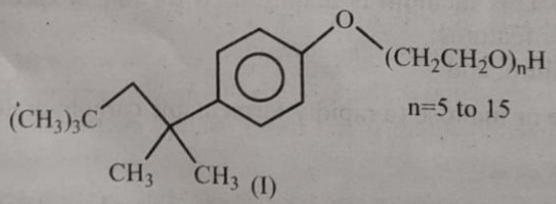
   iv) The inoculated media is incubated at 30-35°C for fluid thioglycollate medium, and 20-25°C for soyabean-casein digest medium for at least 2 weeks [unless it is specifically mentioned in the monograph (Official Compendia, i.e., BP; USP; Int. P., Ind. P.,; Eur. P.,)].

   Important Factors

   i) If the substance under analysis makes the culture medium turbid, the presence or absence of microbial growth cannot be confirmed only by visual examination. In such a case, it is recommended that between the 3rd and 7th days after the test actually began, a suitable part of medium is transformed to other fresh vessels of the same medium.

   ii) Then, these vessels (called as **transfer vessels**) having the transferred medium are again incubated for a week after the transfer, and for a total of 2 weeks.

2) **Oils and Oily Solutions:** The microbial contamination tests for oils and oily solutions are carried out using a culture media containing 0.1% (w/v) solution of **Octoxynol** or **Octylphenoxy polyethoxyethanol (I)**. This emulsifying agent should not show any antimicrobial activity under the prevailing test parameters.



(I)

The required test is performed as mentioned for aqueous solutions and suspensions.

Precautionary Measures

i) The cultures carrying oily preparations should be gently shaken on a regular basis.

ii) When fluid thioglycollate medium is used for detecting anaerobic microorganisms, shaking or mixing should be done at a minimum level to maintain anaerobic experimental parameters.

Ointments: The steps involved are:

i) The test sample is prepared by diluting 10 times with a sterile diluent, **e.g.,** fluid B or any other suitable aqueous vehicle which can homogeneously disperse the sample throughout the fluid mixture.

ii) 10ml of the resultant fluid mixture is mixed with 80ml medium, and the further steps are same as described for aqueous solutions and suspensions.

4) **Solids:** The steps involved are:

i) The required amount of preparation under analysis is transferred to and mixed with the quantity of culture medium specified in **table 9.7**.

ii) The inoculated media is incubated as for aqueous solutions and suspensions.

5) **Sterile Devices:** These devices are completely immersed in not more than 1000ml of the culture medium and incubated as for aqueous solutions and suspensions.

6) **Transfusion or Infusion Assemblies:** These assemblies cannot be immersed, and a sterile 'liquid pathway' should be used. The lumen of each of 20 units is flushed with a sufficient quantity of fluid thioglycollate medium and another lumen of each of 20 units with a sufficient quantity of soyabean-casein digest medium to recover 15ml of each medium. The resultant is incubated with 100ml of each of the two media as for aqueous solutions and suspensions.

Exception: If the lumen of medical devices is so small that fluid thioglycollate medium fails to pass through, the usual fluid thioglycollate medium is replaced with alternative thioglycollate medium and then the inoculated medium is incubated under anaerobic conditions.

Note: In cases, where the sample under analysis, in the culture medium interferes with the test by virtue of the resultant bacteriostatic or fungistatic action, the article is thoroughly rinsed with minimum quantity of fluid A. Finally, the rinsed fluid is recovered and the test is carried out as described for Membrane Filtration for Sterile Devices.

9.1.4.3. Observation and Interpretation of Results

The results of direct inoculation are observed and interpreted by taking the following factors into consideration:

1) The media should be thoroughly examined for bacterial growth during the incubation period and also at its completion.
2) If bacterial growth does not occur, the preparation is said to pass the test.
3) However if growth is observed, the containers showing growth are reserved unless it is proved that growth has occurred due to causes unrelated to the sample under analysis; and, thus, the test is considered invalid.
4) In such cases, the test is repeated using samples and media used in the first test.
5) If after the re-test, bacterial growth is not observed, the preparation is considered to pass the test.
6) And if any evidences of bacterial growth are observed, the organisms are isolated and identified.
7) If these organisms are not distinguishable from those observed in the first test, the preparation is considered to fail the test.
8) On the other hand, if the organisms are distinguishable from the ones observed in the first test, a second re-test is performed using twice the number of samples.
9) If bacterial growth is not observed in the 'second re-test', the preparation passes the test; while it fails if any bacterial growth is observed.

9.2. SUMMARY

The details given in chapter can be summarised as follows:

1) **Sterility test** evaluates whether or not a sterilised pharmaceutical product is free from contaminating microorganisms.
2) **According to I.P. 1996**, the sterility tests are intended for detecting the presence of viable forms of microorganisms in or on the Pharmacopoeial preparations.
3) The wide-spectrum of pharmaceutical products (both pure as well as dosage forms) may be achieved by adopting any one of the two well-recognised, time-tested, and universally accepted methods:
   i) Membrane filtration, and
   ii) Direct inoculation.
4) The membrane filtration method is used to avoid as well as to overcome the activity of antibiotics for which practically little inactivating agents exist.
5) In membrane filtration method, following media are preferred for sterility testing:
   i) Fluid thioglycolate medium,
   ii) Alternative thioglycolate medium, and
   iii) Fluid soyabean-casein digest medium.
6) In direct inoculation method, following three culture media are commonly used to perform the 'tests for sterility':
   i) Nutrient broth,
   ii) Cooked meat medium and thioglycollate medium, and
   iii) Sabouraud medium.

9.3. EXERCISE

9.3.1. True or False

1) Sterility tests are designed for detecting the presence of microorganisms in the 'standard solution'.
2) The membrane filtration method is used to avoid as well as to overcome the activity of antibiotics for which practically little inactivating agents exist.
3) Fluid Soyabean-Casein Digest media is prepared by dissolving solids in distilled water followed by cooling.
4) Thioglycollate medium is used for aerobic microorganisms.
5) Sabouraud medium is mainly used for fungal species.
6) The microbial contamination tests for oils and oily solutions are carried out using a culture media containing 0.1% (w/v) solution of Octoxynol or Octylphenoxy polyethoxyethanol (I).

9.3.2. Fill in the Blanks

7) _____ evaluates whether or not a sterilised pharmaceutical product is free from contaminating microorganisms.
8) The solution of the product under analysis is carefully filtered via_____.
9) Alternative thioglycolate medium is used for _____ and products as well as for devices with tubes of small lumina.
10) Nutrient broth medium is exclusively used for_____.
11) Cooked meat medium is used to culture _____.

Answers:

1) False 2) True 3) False 4) False 5) True
6) True 7) Sterility test 8) Hydrophobic-edged membrane filter
9) Turbid, viscid 10) Aerobic microorganisms 11) *Clostridia*

9.3.3. Very Short Answer Type Questions

1) What is sterility test?
2) Give the composition of fluid thioglycolate medium.
3) Write composition of fluid soyabean-casein digest medium.
4) Give examples of test microbes used in alternative thioglycolate and soybean-casein digest.
5) Write examples of test microbes for fluid thioglycolate medium.
6) What is nutrient broth?
7) Write note on cooked meat medium and thioglycollate medium

9.3.4. Short Answer Type Questions

1) Write a note on guidelines given for the sterility testing of a product.
2) Write a note on culture media used in sterility testing.
3) Give the test procedure of membrane filtration method.
4) Write a note on apparatus used in sterility testing.
5) Give the test procedure of direct inoculation method.
6) Write a note on fluid dilutions.

9.3.5. Long Answer Type Questions

1) Write a detail note on direct inoculation method.
2) What is sterility testing of products? Give detail description.

CHAPTER 10 Aseptic Area

10.1. ASEPTIC AREA

10.1.1. Introduction

Aseptic techniques are employed to provide protection to ophthalmic and parenteral products by preventing the entry of microbial and particulate contamination. Prevention of microbial contamination is also required to remove pyrogens and toxic bacterial products. The terminally sterilised products (product sealed in container and then sterilised) are prepared in clean areas; while products not terminally sterilised are prepared under aseptic conditions using sterile materials or are sterilised by filtration before being packed in sterile containers. Such aseptic products are formulated or prepared in an **aseptic area**, which is a room within a clean area designed, constructed, serviced, and used for controlling and preventing microbial contamination of the product.

Like sterile medicinal products, vaccines containing dead microorganisms, microbial extracts, or inactivated viruses are also filled in aseptic areas; while live or attenuated vaccines are filled in separate areas.

The goal of any facility is to design such an aseptic or sterile environment which provides a controlled environment so that the entrance of viable (microbial) and non-viable (particles) contaminants can be minimised. A controlled environment prevents cross-contamination of **Compounded Sterile Preparations (CSPs)**. To reduce the contamination-related risk, all compounding should be done within primary engineering controls, like a **Laminar Airflow Hood (LAFH)**, also known as **workbenches**, **Biological Safety Cabinet (BSC)**, or a **Compounding Aseptic Isolator (CAI)**.

10.1.2. Designing of Aseptic Area

The sterile production unit and the general manufacturing area within the hospital pharmacy or factory should be located separately. The sterile production unit should be buffered, i.e., unauthorised personnel should not gain access to this unit.

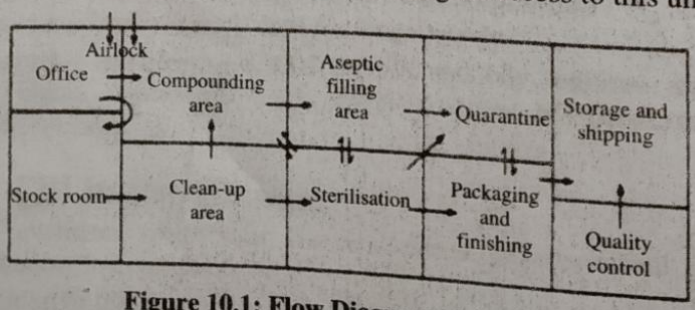


Figure 10.1: Flow Diagram of Aseptic Area

The aseptic unit is designed to carry out **each stage of production separately**. The unit should also ensure a safe and organised workflow so that the need for personnel to move around the clean rooms is minimised. The unit is built and the equipment is positioned in such a manner that the **product remains protected from contamination**.

The layout of aseptic area should be such that **cleaning can be done easily** and **dust accumulation can also be reduced**. Arrangement should be such that the **risk of cross-contamination** (contamination of one product or material with another) is reduced.

The **filling area** should be adjacent to the **compounding area** where the personnel assemble and prepare materials utilised by the staff in the filling area. In **figure 10.1**, the layout of areas and rooms for preparing terminally sterilised products (such as small or large volume injections) is shown.

10.1.2.1. Design and Construction

Only authorised personnel can gain access to the clean and aseptic filling areas. The personnel enter the clean rooms by passing through the **changing rooms** where they put on and remove their clean room uniform.

A **pass-over** (or **cross-over**) **bench** extends across the changing room to form a physical barrier for separating the different areas for changing by the personnel.

Special precautions are taken for preventing clean and aseptic filling areas from getting contaminated while materials are being passed through airlocks or hatchways. Thus, **sterilisers** and **entry ports** are fitted with double-sided doors, which are interlocked to prevent simultaneous opening of both the doors.

10.1.2.2. Surfacing Materials

The floor, wall, and ceiling surface of clean rooms should be **smooth**, **impervious**, and **unbroken** to reduce the release and accumulation of contaminating particles and organisms. The **surface material** should be such that they can withstand the effects of cleaning agents and disinfectants. The **ceilings are scaled** so that the contaminants do not enter from the space above them.

Uncleanable recesses should be avoided within the clean rooms to minimise the accumulation of contaminating particles. Thus, the **wall** and **floor junction** should be **covered**. Minimum shelves, ledges, cupboards, and equipment should be present. **Non-opening** and **sealed windows** should be present to prevent the entry of contaminants.

10.1.2.3. Services

The **piped liquids** and **gases** entering the clean rooms should be **filtered** first, thus ensuring that the liquid or gas at the work position is as clean as the air in the clean room. The position of pipes and ducts should be such that they can be **easily cleaned**. Other fittings, like **fuse boxes** and **switch panels** should be placed **outside** the clean rooms.

Sinks and drains should not be present in areas where aseptic procedures are carried out within the clean room areas. They should nowhere be present in the complete unit. The areas having **sinks** and **drains** should be designed, positioned, and maintained such that **the risk of microbial contamination is reduced**; therefore they are fitted with easily **cleanable traps**, installed with electrically heated disinfection devices.

A **limited** number of **doors** and **ports** should be present for entry of personnel and materials, respectively. The **entry doors** should be **self-closing** to allow easy movement of the personnel.

Airlock doors, wall ports, through-the-wall autoclaves, and **dry heat sterilisers** should have interlocked doors to prevent simultaneous opening of both the doors. All the doors should have an **alarm system** which should ring when more than one door are being opened.

Lights in clean rooms should be **fitted with the ceiling** so that dust accumulation reduces and also the airflow pattern within the room is not disturbed. The equipment in clean rooms should be positioned such that accumulation of particles and microbial contaminants does not occur.

10.1.3. Laminar Flow Equipment (Laminar Airflow Hood)

A Laminar Airflow Hood (LAFH)/ Laminar Aseptic Hoods, or a workbench, is a primary engineering control device which provides the following services during aseptic compounding:

1) Clean air to the critical sites (immediate aseptic compounding area),
2) Constant flow of air out of the work area to prevent the entry of room air, and
3) Outward flow of air from the hood that suspends and removes contaminants which have been introduced in the work area by personnel.

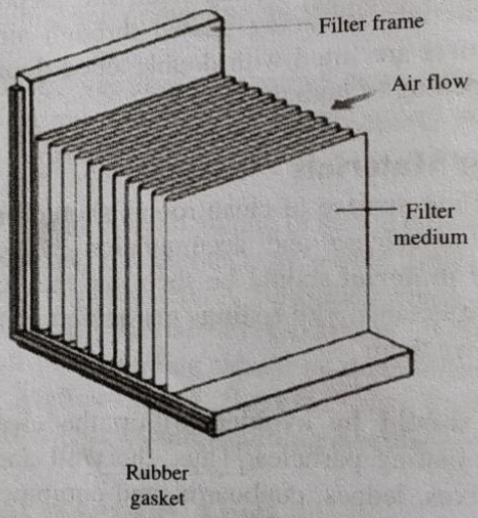


Figure 10.2: Section through a Mini-Pleat High-Efficiency Filter

A **High Efficiency Particulate Air (HEPA) Filter** is the most important part of a LAFH. The air within the room is taken into this filter and passed through a pre-filter which removes the gross contaminants (lint, dust, etc.). The air is then blown at a uniform velocity through the hood and **HEPA filter** in a unidirectional (laminar flow) manner over the critical sites (immediate aseptic compounding area). HEPA filter is a particulate filter which traps the airborne particles and microbes; but allows the gases to pass through.

HEPA filter should be fitted either at or near to the clean room inlet. A pre-filter is fitted upstream of the HEPA filter, thus extending the final filter's life. A fan is also fitted which pumps the air through the filter.

The **filter medium** used in HEPA filters is made up of **pleated fiberglass paper**. Parallelly arranged pleats not only increase the filter surface area but also the air flow through the filter (**figure 10.2**). This parallel arrangement of filter medium also allows the filter to retain a compact volume. In the traditional type of HEPA filters, aluminium

foil was used as spacers, which are no longer used in the modern **mini-pleat type** of filter (now widely used). The mini-pleat filters have a shallower depth in construction than the traditional HEPA filter. The filter material is sealed to an aluminium frame within the filter (**figure 10.2**). One side of the filter is protected with a coated mild steel mesh.

HEPA filters provide:

1) A high air flow rate,
2) A high particulate holding capacity, and
3) A low-pressure drop across the filter.

HEPA filters **remove larger**, **medium**, and **smaller particles** from the air by inertial impaction, direct interception, and by Brownian diffusion, respectively. The HEPA filters are least efficient in removing particles of about 0.3µm size. However, its efficiency of removing particles is affected by air velocity and filter packing. It removes larger and smaller particles with more efficiency.

The LAFH works on the **principle** that twice-filtered laminar layers of aseptic air continuously sweep the work area within the hood to prevent the entry of contaminated room air.

10.1.3.1. Types

A laminar air flow hood is of two types:

1) **Horizontal Airflow Hoods:** These hoods (**figure 10.3**) sweep the filtered air from the back to the front of the hood. An electrical blower draws the contaminated room air using a pre-filter (similar to a furnace filter), to remove gross contaminants. The pre-filter demands timely cleanliness and replacement. The air after passing through the pre-filter is accelerated (but not pressurised) so that a consistent airflow distribution reaches the final filter (HEPA filter), present at the back portion of the hood's working area.

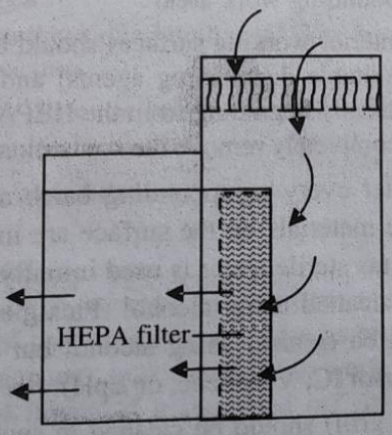


Figure 10.3: Horizontal Laminar Airflow Hood

HEPA filter removes 99.97% of 0.3µ or larger sized particles, thus removing most of the 0.5µ or larger sized airborne microorganisms.

2) **Vertical Airflow Hoods:** These hoods (**figure 10.4**) sweep the filtered air vertically The air passing through HEPA filter merges from the top and passes downward through the working area. The vertical airflow hoods work on the **principle** that the air flow between the HEPA filter and the component used for CSPs preparation should not be interrupted. When a foreign object comes in between the sterile objec

and the HEPA filter, the wind turbulence (zone of turbulence) in the critical area increases, and foreign contaminants are carried to the sterile work surface, thus contaminating the injection port, needle, or syringe.

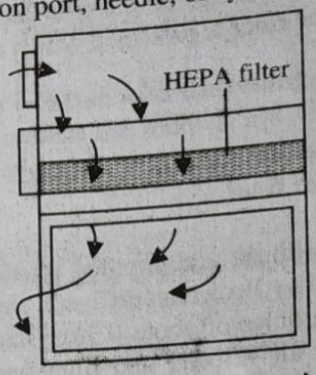


Figure 10.4: Vertical Laminar Airflow Hood

For ensuring complete sterility, nothing should pass behind a sterile object in a horizontal airflow hood; and in case of vertical airflow hood, nothing should pass above the sterile object. The materials within the compounding work area interrupt the pattern of air blowing from the HEPA filter.

10.1.3.2. Operating Principles

A Laminar Air Flow Hood (LAFH) operates on the following general principles:

1) It should be placed away from traffic, doors, air vents, or anything that can produce air currents, to avoid entrance of contaminants.
2) It should remain on for 24 hours otherwise, non-filtered, non-sterile air occupies the compounding work area. Thus, when again it is turned on, it should be left for 30 minutes for disinfection and then used. This also allows the hood to eliminate the non-sterile air from the compounding work area.
3) Before using the LAFH, its interior working surfaces should be disinfected using 70% isopropyl alcohol (or other suitable disinfecting agents) and a clean, lint-free cloth. Cleaning should be done efficiently beginning from the HEPA filter in a top-to-bottom and back-to-front motion, to completely remove the contaminants.
4) Cleaning should be done after every compounding batch and also when the work surface becomes dirty. Some materials on the surface are insoluble in alcohol (**e.g.,** dextrose and amino acids), thus sterile water is used initially. After wiping the water from the surface, it is then cleaned using alcohol. Plexiglas sides present in some types of LAFHs, should not be cleaned using alcohol but with warm water and a germicidal detergent (**e.g.,** Lysol IC, Vesphene, or LpH).
5) HEPA filter grill (protective grill) should be cleaned in such a way that it does not get wet.
6) Objects essential for aseptic compounding should be placed in the hood. Paper, pens, labels, or trays should not be placed in this area.
7) HEPA filter should not be brought into contact with cleaning solution, aspirate from syringes, and glass from ampoules (should not be opened towards the filter).
8) The operators during compounding should not wear any jewellery on hands or wrists since they affect the integrity of personnel clothing (gowns and gloves).
9) The operators should minimise talking and should cough away from the compounding work area.

10) The operators should avoid or reduce the unnecessary movement within or around the LAFH to minimise the airflow turbulence.

11) Smoking, eating, gum chewing, and drinking are strictly prohibited in the aseptic environment.

12) The objects should be arranged to get full benefit of the laminar air flow. Critical items should be placed close to the air source. The objects in horizontal hood should not be placed closer than 3 inches from the back of the hood (ensuring that nothing should be in direct contact with the filter). The pharmacist in some cases, may stack a few objects (such as IVPBs), but from lower to higher starting from the back of the hood. Also, the stack should contain only 3-4 objects.

13) The aseptic procedures should be carried out 6 inches away from the sides and front edge of the hood to prevent contamination.

14) Testing of primary engineering controls (**e.g.,** LAFHs, BSCs, and CAIs) should be carried every 6 months by qualified personnel. Testing is also required whenever the device is moved, or the filter is damaged. Specific tests are used for the certification of integrity of airflow velocity and HEPA filter.

10.1.3.3. Uses

Given below are the uses of a laminar air flow hood:

1) It has various applications, especially where an individual clean air environment is required for smaller items, **e.g.,** particle sensitive electronic devices.
2) It is used for special operations in laboratories.
3) It can be tailor made to the specific requirements of laboratories and are also used for general lab work, especially in medical, pharmaceutical, electronic, and industrial sectors.

10.1.3.4. Advantages

Given below are the advantages of a laminar air flow hood:

1) The sterile area is spacious.
2) Since the sterile area does not become hot, the plant material can stay for a longer duration.
3) Bigger flasks having wide lids can be used.

10.1.3.5. Limitations

The airflow velocity can be overcome easily without any strict work practices and aseptic technique, thus an area of turbulence is created which can introduce room contaminants (particles and microorganisms) into the critical work area. A laminar air flow hood should remain on for 24 hours. If it is turned off, it should be left on for atleast 30 minutes for its disinfection before use.

10.1.4. Different Sources of Contamination in an Aseptic Area

There are various contamination sources in an aseptic area, which are discussed below:

1) **Personnel:** Those supervising, performing, or controlling drug manufacturing are potential source of microbial contamination, due to the following reasons:
   i) Inadequate training,
   ii) Direct contact between the hands and starting materials, primary packagin materials, and intermediate or bulk products,

iii) Improper hygiene,
iv) Unauthorised personnel entering the production, storage, and product control areas,
v) Insufficient gowning and protective equipment, and
vi) Eating, drinking, or smoking within the storage and processing areas.

2) **Buildings and Facilities:** These are also important contributors to microbial contamination due to the following reasons:
i) Inadequate size and organisation of the space, which lead to selection errors (such as mix-ups or cross-contamination between consumables, raw materials, in-process materials, and finished products),
ii) Poor filth and pest controls,
iii) Rough floors, walls, and ceilings,
iv) Absence of air filtration systems,
v) Inadequate lighting and ventilation systems,
vi) Poorly located vents, ledges, and drains, and
vii) Improper washing, cleaning, toilet, locker facilities, sanitary operation, and personal cleanliness.

3) **Equipment and Utensils:** These are used during processing, holding, transferring, and packaging. They are the common sources of microbial contamination due to the following reasons:
i) Unsuitable design, size, corrosion-causing materials, static material accumulation, and/or adulteration with lubricants, coolants, dirt, and sanitising agents,
ii) Inadequate cleaning and sanitisation,
iii) Inefficient cleaning and maintenance due to their designing,
iv) Inappropriate calibration and irregular service, and
v) Using defective equipment.

4) **Raw Materials:** These are used during production and are considered a potential source of contamination due to the following reasons:
i) Improper storage and handling, which leads to mix-ups or selection errors,
ii) Microbial or chemical contamination,
iii) Degradation due to extreme environmental conditions (like heat, cold, sunlight, moisture, etc.),
iv) Wrong labelling,
v) Incorrect sampling and testing, and
vi) Using materials not meeting the acceptance criteria.

5) **Manufacturing Process:** During the manufacturing process, microbial contamination of raw materials, intermediates or packaging materials can widely occur due to the following reasons:
i) Absence of facilities required for manufacturing of a single product,
ii) Improper cleaning between batches for minimising the amount of product changeovers,
iii) Use of an open manufacturing system for exposing the product to the room environment,
iv) Improper zoning,
v) Lacking an area line clearance (as per the approved procedures) after each cleaning process and between each batch, and
vi) Lack of cleaning status labelling on all equipment and materials used within the manufacturing facility.

6) **HVAC (Heating, Ventilation, and Air Conditioning) System:** An inappropriate HVAC system is a possible source of microbial growth and also disperses the contaminants throughout the manufacturing unit. This occurs due to the following reasons:
   i) Organic materials accumulate in or near HVAC air intakes,
   ii) Inadequate air filtration system,
   iii) Inadequate magnitude of pressure differentials, which causes flow of reversal,
   iv) Incorrect ratio of fresh air to re-circulated air,
   v) Incapability of accessing ventilation dampers and filtering from outside the manufacturing areas, and
   vi) Non-directional airflow within production or primary packing areas.

10.1.5. Methods of Prevention of Contamination

The steps by which microbial contamination of products can be prevented in aseptic areas are discussed below:

1) **Personnel:** They can take the following measures:
   i) Access to production areas by the unauthorised personnel should be restricted, and only trained ones should be allowed to enter.
   ii) Adequate personnel hygiene should be maintained.
   iii) The personnel should be given proper and regular training with respect to hygiene as well to ensure that their activities do not hamper the product quality.
   iv) Personnel entering the manufacturing area should wear protective clothing (over-garments, hair cover, beard or moustache cover, and overshoes).
   v) Personnel should avoid touching with naked hands the exposed products or any part of equipment in contact with the product.

2) **Facility Design:** This includes the following measures:
   i) In aseptic rooms, the differential air pressures should be higher than the adjacent controlled areas.
   ii) Air filtration and air change rates should be set to attain the defined clean room class.
   iii) Unidirectional (laminar flow) air flow should be maintained over critical areas at sufficient velocity to sweep particles away from filling/closing area.
   iv) Ambient temperature and humidity should not be very high.
   v) The ventilated cabinets, RABS (Restricted Access Barrier System), isolators systems, etc. should be used depending on the facility/product risk assessment results in order to achieve an absolute or partial barrier to contain microorganisms at their point of use.
   vi) The areas of unequal risk are separated by UV air locks and door barriers.
   vii) All open-container processing should be performed within the isolator/RABS.
   viii) The air pressure of the changing room should be negative with respect to the manufacturing area corridor, but positive with respect to the external adjacent areas.
   ix) The ventilation dampers, specially designed filters, and other services should be positioned such that they can be accessed from outside the manufacturing areas (service voids or service corridors) for maintenance purposes.
   x) An impermeable barrier should be used in appropriate areas to prevent cross-contamination between two zones, such as closed systems, pumped, or vacuum transfer of materials.

xi) HVAC air distribution components should be used for preventing the spreading of contaminants generated within the room.

xii) All rooms and surfaces should be maintained and monitored for viable and non-viable particulates and this facility should be re-certified on a semi-annual basis.

Facility and equipment design cannot eliminate microbial contamination completely, but good risk assessment and consequent hygienic design can reduce these risks several times. **For example,**

i) Flow of man and material should be uniform,
ii) Equipment having smooth surfaces of appropriate material can facilitate effective cleaning, and
iii) Closed Material Transfer Systems.

3) **Access to Areas:** This includes the following measures:
   i) Access to production, packaging, and QC areas by unauthorised personnel should be restricted.
   ii) Personnel should gain access to these areas only via changing rooms.
   iii) Materials should be accessed via specific routes (generally air locks).

4) **Building Requirements:** This includes the following measures:
   i) Smooth, crack-free, and easily cleanable floors, walls, and ceilings should be used as they facilitate easy and effective cleaning.
   ii) Windows or viewing panels should be closed (non-opening), fixed with wall panels, and sealed to prevent accumulation of dust and microbial contaminants.
   iii) The designing of pipe work, ventilation, and light points should be such that creation of recesses which are not easily cleanable is avoided.
   iv) Sinks of stainless steel should be present within the production areas.

5) **Cleaning and Disinfection:** This includes the following measures:
   i) Time-to-time cleanliness and disinfection of the areas should be done.
   ii) Adequate hygiene should be maintained in every aspect of the drug product manfacturing.
   iii) Cleaning agents of proper grades should be used for minimising health risks.
   iv) Contact time, application, temperature, mechanical action, and the chemistry of cleaning agents should be considered during the cleaning process.
   v) The cleaning agents should not be directly applied on the product.
   vi) The cleaning practices should be validated to prove that the process effectively controls microbial contamination.

6) **Utilities:** This includes the following measures:
   i) Water of pharmaceutical grade, microbiologically controlled and monitored should be used in the manufacturing of products.
   ii) Clean and additive-free steam should be used for cleaning and sanitisation of production tools and equipment, and to supply for autoclaves and humidification.

10.1.6. Clean Area Classification

The **clean** or **buffer room** is the secondary engineering control. It houses the primary engineering controls (the LAFH, BSC, or CAI) where the aseptic compounding is actually performed. Clean room is a specially constructed enclosed area, which contains one or more clean zones, where the concentration of airborne particles is controlled using

HEPA filters, continuous air circulation, and a physical barrier to non-filtered (or outside) air. Clean rooms establish appropriate environmental levels for airborne particulates, temperature, humidity, air pressure, and airflow patterns. Clean rooms are categorised by their constant air quality or class. Clean rooms are rated as given in **table 10.1**:

Table 10.1: Classes of Clean Rooms and their Properties

Clean Room	Properties
Class 10,000 (ISO class 7)	10,000 or less particles of 0.5μ and larger size exist in a given cubic foot of air.
Class 1,000 (ISO class 6)	1,000 or less particles of 0.5μ and larger size exist in a given cubic foot of air.
Class 100 (ISO class 5)	100 or less particles of 0.5μ and larger size exist in a given cubic foot of air.

Lower the classification number, cleaner is the air.

Apart from the above mentioned **basic components**, a clean room also includes:

1) UV irradiation,
2) Air-lock entry portals,
3) Special filtration or treatment systems for incoming air,
4) Sticky mats for removing particulate matter from shoes, and
5) Positive room air pressure for controlling entry of contaminants from adjacent rooms.

Clean rooms are divided into different classes in standards. **Table 10.2** lists the equivalence of classes from different international standards. For manufacturing sterile products, a certain classification (**table 10.3**) with grades A to D are characterised to activity category (**tables 10.3** and **10.4**) is used.

Table 10.2: Class Limits for Federal 209D and ISO Standards

Class	Measured Particle Size (Micrometers)						
Federal 209D	ISO	0.1	0.2	0.3	0.5 (a)	1.0	5.0
1	3	1000	237	102	(1)	8	
10	4	10000	2370	1020	(10)	83	
100	5	100000	23370	10200	(100)	832	29
1000	6	1000000	237000	102000	(1000)	8320	293
10000	7				(10000)	832000	2930
100000	8				(100000)	8320000	293000

(a) - Particle count for this particular size is per ft^3 while others are per m^3.

Table 10.3: Air Particle Classification System for the Manufacturing of Sterile Products

Grade	Maximum Permitted Number of Particles per m^3 Equal to or Above			
	At Rest (b)		In Operation (b)	
	0,5μm(d)	5μm	0,5μm(d)	5μm
A	3500	0	3500	0
B(a)	3500	0	350000	2000
C(a)	350000	2000	3500000	20000
D(a)	3500000	20000	Not defined (c)	Not defined (c)

Remarks

1) In order to reach the air grades B, C, and D, the number of air changes should be related to the room size, and the equipment and personnel in the room. The air system should be provided with HEPA filters for grades A, B, and C.
2) After 15-20 minutes of "clean up" period, an unmanned state (no manual acitivity) should be received.
3) Appropriate alert and action limits should be fixed for particulate and microbiological monitoring results. A corrective action in the operating procedures should be taken in case these limits are exceeded.

The need for other parameters (like temperature, relative humidity, etc.) depends on the nature of product and manufacturing procedure. These parameters do not have any relation with the purifying classes.

Table 10.4: Terminally Sterilised Products

Grade	Examples of Operation
A	Filling of products, when unusually at risk.
C	Preparation of solutions, when unusually at risk. Filling of product.
D	Preparation of solutions and components for subsequent filling.

Most products should be prepared in grade D environment; while grade C environment should be used in case of an unusual risk.

Table 10.5: Aseptic Parameters

Grade	Examples of Operation
A	Aseptic preparation and filling.
C	Preparation of solutions to be filtered.
D	Handling of components after washing.

The components after washing should be handled in grade D environment. The sterile starting material should be handled in grade A environment with grade B background. Solutions to be sterile filtered should be prepared in grade C environment; while if not filtered, the preparation should be done in grade A environment with grade B background. The aseptically prepared products should be handled and filled in grade A environment with grade B background. The sterile ointments, creams, suspensions, and emulsions should be prepared and filled in grade A environment with grade B background, when the product is exposed and is not subsequently filtered. Control of purity of zones by the particles in operation state and microbial control at an aseptic production area is required; and the recommended levels are listed in **table 10.6**:

Table 10.6: Recommended Limits for Microbial Contamination in the Operation State (Average Values)

Grade	Air Sample, cfu/m^3	Settle Plates (Diameter 90mm), cfu/4 hours (a)	Contact Plates (Diameter 55mm), cfu/plate	Glove Print, 5 Fingers cfu/glove
A	< 1	< 1	< 1	< 1
B	10	5	5	5
C	100	50	25	-
D	200	100	50	-

(a) - Individual settle plates are exposed for 4 hours or less; **cfu** - colony-forming unit.

Warning limitation and action for contamination by particles and microbes depend on the controlling results. Also corrective action should be provided in case of exceeding the above limits.

10.2. SUMMARY

The details given in chapter can be summarised as follows:

1) Aseptic techniques are employed to protect the ophthalmic and parenteral products from the entry of microbial and particulate contamination.
2) Aseptic techniques are used to design such an aseptic or sterile environment which provides a controlled environment so that viable (microbial) and non-viable (particles) contamination can be minimised. A controlled environment prevents cross-contamination of **Compounded Sterile Preparations (CSPs)**.
3) The layout of aseptic area should be such that **cleaning can be done easily** and **dust accumulation can also be reduced**.
4) The **filling area** should be adjacent to the **compounding area** where the personnel assemble and prepare materials utilised by the staff in the filling area.
5) The floor, wall, and ceiling surface of clean rooms should be **smooth**, **impervious**, and **unbroken** to reduce the release and accumulation of contaminating particles and organisms. The **surface material** should be such that they can withstand the effects of cleaning agents and disinfectants.
6) During aseptic compounding, a Laminar Airflow Hood (LAFH)/ Laminar Aseptic Hoods, or a workbench, is a primary engineering control device which provides the following services:
   i) Clean air to the critical sites (immediate aseptic compounding area),
   ii) Constant flow of air out of the work area to prevent the entry of room air, and
   iii) Outward flow of air from the hood that suspends and removes contaminants which have been introduced in the work area by personnel.
7) A **High Efficiency Particulate Air (HEPA) Filter** is the most important part of a LAFH.
8) The **filter medium** used in HEPA filters is made up of **pleated fiberglass paper**.
9) HEPA filters provide:
   i) A high air flow rate,
   ii) A high particulate holding capacity, and
   iii) A low-pressure drop across the filter.
10) HEPA filters **remove larger**, **medium**, and **smaller particles** from the air by inertial impaction, direct interception, and by Brownian diffusion, respectively.
11) A laminar air flow hood is of two types:
   i) Horizontal airflow hoods, and
   ii) Vertical airflow hoods.
12) Different sources of contamination in an aseptic area are:
   i) Personnel,
   ii) Buildings and facilities,
   iii) Equipment and utensils,
   iv) Raw materials,
   v) Manufacturing process, and
   vi) HVAC (heating, ventilation, and air conditioning) system.
13) The **clean** or **buffer room** is the secondary engineering control, houses the primary engineering controls (the LAFH, BSC, or CAI) where the aseptic compounding is actually performed.

10.3. EXERCISE

10.3.1. True or False

1) The clean or buffer room is the secondary engineering control, houses the primary engineering controls (where the aseptic compounding is actually performed.
2) The filling area should be adjacent to the compounding area.
3) The layout of aseptic area should be such that cleaning can be done easily and dust accumulation can also be increased.
4) The aseptic techniques are used to design such an aseptic or sterile environment which provides an uncontrolled environment.
5) A laminar air flow hood should not remain on for 24 hours.

10.3.2. Fill in the Blanks

6) HEPA filters remove larger, medium, and smaller particles from the air by inertial impaction, direct interception, and by_____, respectively.
7) A High Efficiency Particulate Air (HEPA) Filter is the most important part of a_____.
8) Aseptic techniques are employed to protect the _____ and _____ products from the entry of microbial and particulate contamination.
9) The filter medium used in _____ filters is made up of pleated fiberglass paper.
10) A controlled environment prevents cross-contamination of_____.

Answers:

1) True 2) True 3) False 4) False 5) False
6) Brownian diffusion 7) LAFH 8) Ophthalmic, parenteral
9) HEPA 10) Compounded Sterile Preparations (CSPs)

10.3.3. Very Short Answer Type Questions

1) Draw a layout of aseptic area.
2) Enlist the methods of prevention of contamination.
3) Give uses of laminar flow equipment.
4) Give types of laminar flow equipment.
5) What are vertical airflow hoods?
6) What is HVAC system?
7) Give uses of laminar air flow hood.

10.3.4. Short Answer Type Questions

1) Give the classification of clean area.
2) Write a note on methods of prevention of contamination.
3) What are the sources of contamination in an aseptic area?

10.3.5. Long Answer Type Questions

1) Write a detail note on laminar flow equipment.
2) Give details on designing of aseptic area.

CHAPTER 11

Microbiological Assay

11.1. MICROBIOLOGICAL ASSAY

11.1.1. Introduction

The **microbiological** or **microbial assay** is a type of biological assay in which the relative potency of activity of a compound is determined by measuring the amount required for producing the predicted effect on a suitable test organism under standard conditions.

The principles involved in microbial assays are similar to those applied to assays of higher plants or animals. An existing **difference** is the **relative size of experimental population**. In bioassays, the response each test animal produces is observed and results are obtained when a few animals have gone through statistical analysis to calculate mean activity, standard error, etc. In microbial assays, each evaluation is performed using a microbial culture, and the results obtained indicate the average response produced by large number of test organisms. Often in bioassays, the log dose and the response have a linear relationship; while in microbial assays; the dose and the response (within certain limits) have a linear relationship.

The microbiological assay is a biological assay performed using microorganisms, **e.g.,** bacteria, yeast, and moulds. These assays are much easier than those performed using mammals for isolating essential vitamin; **for example**, a growth factor for *Lactobacillus* proved to be similar to a haematopoietic factor in pernicious anaemic patients.

Many **therapeutic agents**, such as antibiotics inhibiting microbial growth or the essential growth factors (vitamins and amino acids), are **standardised by microbiological assays**. The activity of antibiotics (extent of inhibiting the microbial growth), vitamins, or amino acids (extent of supporting the microbial growth) is determined by microbiological assays; while the potency (concentration or amount) of such substances is determined by chemical assays. Thus, **microbiological assays of antibiotics**, **vitamins**, and **amino acids are significantly important**.

11.1.2. Principles

A microbiological assay relies on the principle that **when certain compounds are present in limited amounts, the amount of microbial growth corresponds to the amount of these compounds**. The basic procedure of most of the microbial assays is same; however the test conditions vary. The test substance is added to a liquid or gel medium, test microorganism is inoculated on the medium, and the resultant response (which depends on the substance's biochemical effect on the test organism) is observed. It may be a growth response (positive in the assay of nutrients and negative in the assay of antibiotics), which is determined by counting, optical density, weight, or area; the growth response may be a definite end-point, or an all-or-none response.

In case of either a positive or negative metabolic response, the metabolic products or changes in some function may be measured. **Examples** of some measurable metabolic responses are acid production, CO_2 production, oxygen uptake, nitrate reduction

haemolysis of RBCs, anti-luminescent activity, or inhibition of spore germination. In **1962**, **Cancellien** and **Morpurgo** proposed a determination method of amino acids, vitamins, and purine and pyrimidine bases. Substances analogous and antagonistic to regular metabolites inhibit the growth of wild, prototrophic strains of bacteria. The nutrilite content can be assayed by adding graded amounts of nutrilite for re-activating the growth.

11.1.3. Merits

A microbiological assay has the following merits:

1) It is suitably used for compounds which cannot be assayed by either physical or chemical methods.
2) It is used for the assay of naturally occurring therapeutic agents.
3) It minimises the mortality rate of animals.
4) It is a simple and rapid method as compared to bioassays.
5) It is used for accurate standardisation of medicinal compounds.
6) It determines the concentration as well as activity of compounds.
7) It does not require large amount of sample and instruments.
8) Its complete procedure can be automated to minimise the duration.

11.1.4. Demerits

A microbiological assay has the following demerits:

1) It requires a specific test organism for the assay of a particular compound.
2) It demands maintenance of sterile conditions within the laboratory.
3) It may give invalid results due to a slight variation in incubation temperature.
4) It is a time-consuming method.
5) It requires well trained and expert individuals.

11.1.5. Standardisation of Antibiotics

Antibiotics are the agents that **kill** or **reduce the bacterial growth**. They belong to a class of antimicrobials (a larger group also comprising of anti-viral, anti-fungal, and anti-parasitic drugs). Antibiotics are chemicals either derived from or produced by the microorganisms (i.e., bugs or germs such as bacteria and fungi).

The methods of microbiological assay of antibiotics are based on the following principles:

1) **Method A (Serial Dilution Method):** The dilution of test antibiotic (which will inhibit the growth of a susceptible organism) is compared with the dilution of the standard antibiotic preparation having an identical effect. Through the potency of standard, the strength of the unknown can be calculated.

2) **Method B (Diffusion Method):** Due to the ease with which quantitative results can be obtained, this method is preferred over serial dilution method.

   Dilutions of the test and standard antibiotic preparations are made in geometric proportions.

   Test organism is inoculated on the agar plates, which are then placed on a horizontal surface so that agar occupies a constant depth of the plate. The test organism is either mixed with agar and then poured on the plates or is later applied on the agar surface already set on plates.

Antibiotic solutions are applied in:

i) **Cups** in medium using a sterile cork borer of 10mm diameter; a vacuum device or a splayed-out pen nib is used for removing the cut agar disc.

ii) **Cylinders** of stainless steel, glazed porcelain, Pyrex glass, or sterilisable plastic of 8mm external diameter and 10mm height are slightly warmed so that they sink to a constant depth when placed on the agar.

iii) **Filter paper** or **cellulose discs** absorb a fixed volume of solution.

iv) **Standard ceramic insulation beads** (or **fish-spine beads**) are when touched on the solution surface attract a definite volume of it. When using this method, the agar medium surface should be dry.

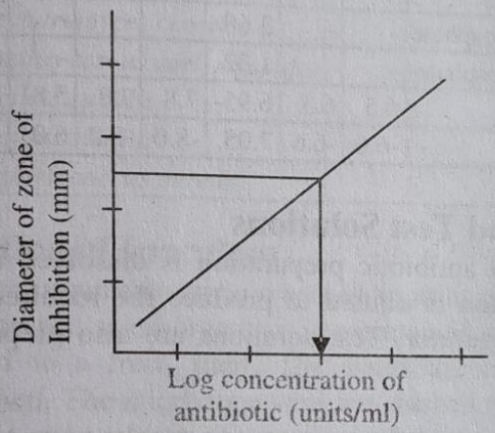


Figure 11.1: Curve of Diameter of Zone of Inhibition *versus* Log Concentration of Antibiotic

Thereafter the agar plates are left for 2 hours at room temperature so that the antibiotic solution diffuses throughout the medium for further growth of organism.

Growth inhibition is observed after incubation as a **clear zone of inhibition** around each container. Diameter of this zone of inhibition is proportional to the log of antibiotic concentration.

The diameters of zone of inhibition are best measured using an **'antibiotic zone reader'** in which an optical system forms an image of plate on a large grid. Two diameters at right angles are used to check the ellipticity of zone.

On plotting log concentration of the standard antibiotic against the diameter of zone of inhibition, a **standard curve** (**figure 11.1**) is obtained.

11.1.5.1. Principle

The microbiological assay of antibiotics as per I.P. relies on the principle that the inhibition of bacterial growth by the measured concentration of test antibiotic is compared with the inhibition of bacterial growth by the known concentration of standard antibiotic preparation having a known activity.

11.1.5.2. Media Used

The ingredients required for preparing the inoculum of test microorganism are given in **table 11.1**. These ingredients are dissolved in sufficient water to make 1000ml and then 1M sodium hydroxide or hydrochloric acid is added in sufficient amount, so that after sterilisation the pH is adjusted to values given in **table 11.1**:

Table 11.1: Composition of Media (Quantities in gm per 1000ml Medium)

Ingredients	A	B	C	D	E	F	G	H	I	J
Peptone	6.0	6.0	5.0	6.0	6.0	6.0	9.4	-	10.0	-
Yeast extract	3.0	3.0	1.5	3.0	3.0	3.0	4.7	-	-	-
Beef-extract	1.5	1.5	1.5	1 .5	1.5	1.5	2.4	-	10.0	-
Pancreatic digest of casein	4.0	-	-	4.0	-	-	-	17.0	-	15.0
Dextrose	1.0	-	1.0	1 .0	-	-	10.0	2.5	-	-
Papaic digest of soybean	-	-	-	-	-	-	-	3.0	-	5.0
Agar	15.0	15.0	-	15.0	15.0	15.0	23.5	12.0	17.0	15.0
Glycerin	-	-	-	-	-	-	-	-	10.0	-
Polysorbate 80	-	-	-	-	-	-	-	10.0	-	-
Sodium chloride	-	-	3.5	-	-	-	10.0	5.0	3.0	5.0
Dipotassium hydrogen phosphate	-	-	3.68	-	-	-	-	2.5	-	-
Potassium dihydrogen phosphate	-	-	1.32	-		-	-	-	-	-
Final pH	6.5	6.5	6.95-	7.8	7.8	5.8	6.0	7.1	6.9	7.2
	-6.6	-6.6	7.05	-8.0	-8.0	6.0	-6.2	-7.3	-7.1	-7.4

11.1.5.3. Standard and Test Solutions

A quantity of the standard antibiotic preparation is dissolved in the specific solvent (**table 11.2**). The preparation is diluted to produce the required concentration (**table 11.2**) and stored in a refrigerator. Test solutions are also prepared in concentrations shown in **table 11.2**:

Table 11.2: Preparation of Standard and Test Solutions of Antibiotics

Antibiotics	Assay Method	Solvent Used for Standard Solution	Final Stock Concentration of Std. Solution/ml	Median Dose µg or Units/ml of Test Solution
Amikacin	B	Water	1 mg	10 µg
Amphotericin B	A	Dimethyl formamide	1 mg	1 µg
Bleomycin	A	Buffer solution	2 units	0.04 unit
Carbenicillin	A	Buffer solution	1 mg	20 µg
Doxycyline	B	0.1 M HCl	1 mg	0.1 µg
Erythromycin	A	Methanol	1 mg	1.0 µg
Gentamicin	A	Buffer solution	1 mg	0.1 µg
Kanamycin sulphate	A	Buffer solution	800 units	10 units
	B	Water	1000 units	1.0 unit
Neomycin	A	Buffer solution	1 mg	1.0 µg
Novobiocin	A	Ethanol	1 mg	0.5 µg
Nystatin	A	Dimethyl formamide	1000 units	20 units
Oxytetracycline	A	0.1 M HCl	1 mg	2.5 µg
Rifampicin	A	Methanol	1 mg	5.0 µg
Streptomycin	A	Water	1 mg	1.0 µg
	B	Water	1 mg	30 µg
Tetracycline	A	0.1 M HCl	1 mg	2.5 µg
	B	0.1 M HCl	1 mg	0.24 µg

11.1.5.4. Test Microorganisms

The test microorganisms used in the assay of each antibiotic are listed in **table 11.3**. A culture is maintained on slants of the medium under the incubation conditions and transferred weekly to fresh slants.

Table 11.3: Test Microorganisms for Microbiological Assay of Antibiotics

Antibiotic	Test Microorganisms	Medium Used	Incubation Conditions (°C)
Amikacin	*Staphylococcus aureus*	C	32–35
Amphotericin B	*Saccharomyces cerevisiae*	G	29–31
Bleormycin	*Mycobacterium smegmatis*	I	30–37.5
Carbenicillin	*Pseudomonas aeruginosa*	H	36–37.5
Doxycycline	*Staphylococcus aureus*	C	32–35
Erythromycin	*Micrococcus luteus*	D	32–35
Gentamicin	*Staphylococcus epidermidis*	D	32–35
Kanamycin sulphate	*Bacillus pumilus*	D	32–35
Neomycin	*Staphylococcus epidermidis*	D	32–35
Novobiocin	*Staphylococcus epidermidis*	A	32–35
Nystatin	*Saccharomyces cerevisiae*	G	29–31
Oxytetracycline	*Bacillus cereus var. mycoides*	F	32–35
Rifampicin	*Bacillus subtilis*	B	32–35
Streptomycin	*Bacillus subtilis*	E	32–35
Tetracycline	*Staphylococcus aureus*	E	32–35

11.1.5.5. Preparation of Inoculum

Suspensions of test microorganism can be prepared by utilising the methods given below:

1) **First Method:** The test microorganism is maintained on slants of medium A and weekly transferred to a fresh slant. The slants are incubated for 1 day at the temperature specified. The microorganisms are washed (with 3ml of saline solution) from the agar slant onto a large agar surface of medium A (such as a Roux bottle containing 250ml of agar). It is then incubated for 1 day at appropriate temperature. The bacterial growth is washed from the nutrient surface using 50ml of saline solution. The test microorganisms are stored under refrigeration. The dilution factor is determined which will give 25% light transmission at 530nm. The amount of suspensions to be added to each 100ml of agar of nutrient broth using test plates or test broth is also determined. The suspensions are then stored in refrigerator.

2) **Second Method:** This method is started as described in the first method but the Roux bottle (used for growing mass cultures and single or monolayer cultures) is incubated for 5 days (and not for 1 day). The supernatant liquid is centrifuged and decanted. The sediment is re-suspended with 50-70ml of saline solution and the suspension is heated at 70°C for 30 minutes. The spore suspension is washed thrice using 50-70ml of saline solution and again heated for 30 minutes. Test plates are used to determine the amount of suspension required for 100ml agar. The suspensions are then stored in refrigerator.

3) **Third Method:** The test microorganism is maintained on 10ml agar slant of medium G. It is then incubated at 32-35°C for 1 day. 100ml of nutrient broth is inoculated and incubated at 37°C for 18-24 hours. Then the method is continued as described in the first method.

4) **Fourth Method:** This method is started as described in the first method but the growth is washed from the nutrient surface using 50ml of medium A (prepared without agar) instead of saline solution.

11.1.5.6. Official Methods

Pharmacopoeias suggest the following two antibiotic assay methods:

1) Turbidimetric or tube assay method, and
2) Cup-plate or cylinder-plate method.

11.1.5.7. Turbidimetric or Tube Assay Method

This method depends on the microbial culture growth in a uniform solution of antibiotic in a fluid medium favourable to its rapid growth in the absence of antibiotic. This method involves a **shorter incubation period** (4-5 hours) for the growth of test microorganism. However, this assay method is affected by the presence of solvent residues or other inhibitory substances. This method is **not used for cloudy** or **turbid preparations**.

The **steps** involved in turbidimetric method are given below:

1) The standard solution is prepared in five different concentrations by diluting the stock solution for making the standard curve.
2) A **median concentration** is selected and the sample of test antibiotic solution is diluted up to this concentration.
3) 1ml of each concentration of the standard and sample solutions is placed in each tube in duplicate.
4) 9ml of nutrient medium inoculated with the test microorganism is added to each tube.
5) Simultaneously, **three control tubes**, one with the inoculated culture medium (culture control), second similar but treated with 0.5ml of dilute formaldehyde solution (blank), and third with un-inoculated culture medium are prepared.
6) All the tubes are **incubated** for 4-5 hours at the specified temperature.
7) 0.5ml of dilute formaldehyde solution is added to each incubated tube.
8) By measuring the absorbance of each solution in the tubes against the blank at about 530nm, the growth of test microorganism is determined (**figure 11.2**).

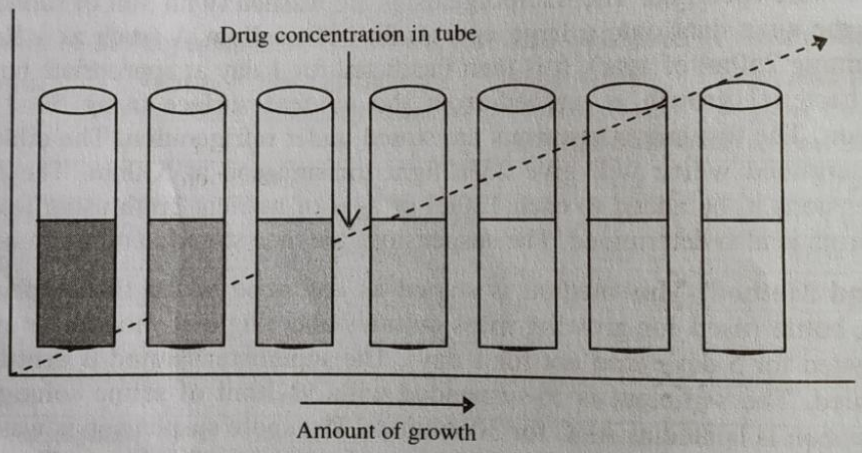


Figure 11.2: Microbiological Assay by Tube Assay Method

11.1.5.8. Cup-Plate or Cylinder-Plate Method

This method relies on the diffusion of an antibiotic from a vertical cavity or a cylinder through the solidified agar layer in a petri plate. The growth of test microorganisms is observed to be inhibited in a circular area or zone around the cavity containing antibiotic solution.

The **steps** involved in cup-plate method are given below:

1) A liquefied assay medium (43-45°C) is inoculated by the suspension of test microorganisms.
2) This inoculated test culture medium is poured and spread on sterile petri plates or pre-prepared agar plates.
3) Standard and test antibiotic solutions of known concentrations are prepared in appropriate solutions, which are then added to sterile cavities prepared on solid medium (**figure 11.3**).

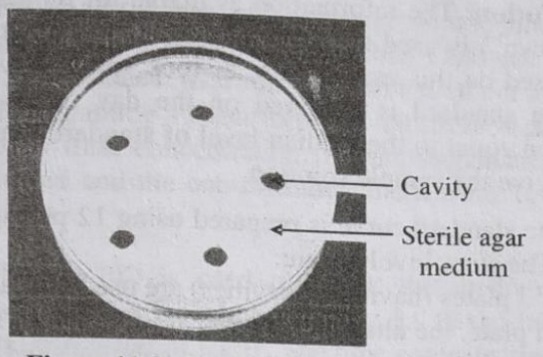


Figure 11.3: Cavities Prepared in Sterile Agar Medium for Assay of Antibiotics

4) Uniform volume of solution should be added to each cavity to fill them sufficiently. If paper discs are used, they should be sterilised first, then dipped in the standard or test solutions, and finally placed on the medium surface.

5) The plates are allowed to stand at room temperature or at 4°C for 1-2 hours. This is the period of pre-incubation diffusion which minimises the effects of variation in time between the applications of different solutions.

6) All plates are then incubated at the temperature listed in **table 11.3** for 18-24 hours.

7) The diameters or areas of the circular inhibition zones (**figure 11.4**) produced by standard and test antibiotic solutions are accurately measured.

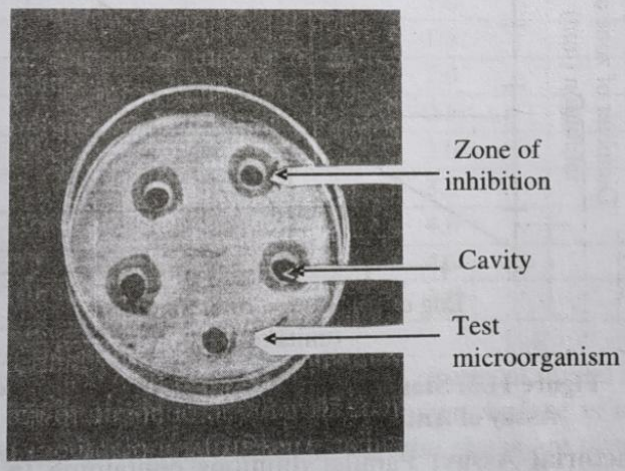


Figure 11.4: Zone of Inhibition Observed in Plate (Assay of Streptomycin)

In cup-plate method, the assay designs should be selected based on the requirements stated in individual monographs. Some of the common assay designs are discussed below:

1) **One-Level Assay with Standard Curve:** This is carried out as follows:
   i) **Standard Solution:** It is prepared as follows:
      a) An accurately weighed and previously dried standard antibiotic preparation is dissolved in the specified solvent, and diluted up to the desired concentration to obtain the stock solution.
      b) It is then stored under refrigeration and should be used within the specified period.
      c) The day on which assay has to be performed, 5 dilutions (solutions S_1 to S_5) representing five test levels of the standard and increasing stepwise in the ratio of 4:5 are prepared from the stock solution.

ii) **Sample Solution:** The information available for the test antibiotic preparation (the "unknown") is used for assigning an assumed potency per unit weight or volume. Based on this assumption, a stock solution with the same solvent as used for the standard is prepared on the day of assay. A dilution up to a concentration equal to the median level of standard is prepared from this stock solution to give the sample solution.

iii) **Method:** The standard curve is prepared using 12 petri plates for 72 cavities (or cylinders). The steps involved are:
   a) A set of 3 plates (having 18 cavities) are used for each dilution.
   b) On each plate, the alternate cavities are filled with solution S_3 (representing the median concentration of standard solution) and the remaining 9 cavities are filled with one of the other 4 dilutions of the standard solution.
   c) The process is repeated for the other 3 dilutions of the standard solution.
   d) For each unknown preparation, a set of 3 plates (having 18 cavities) are used, their alternate cavities are filled with sample solution, and the remaining 9 cylinders are filled with solution S_3.
   e) The plates are incubated at the specified temperature for 18 hours.
   f) The diameters of the zones of inhibition are measured, plotted against the logarithm of antibiotic concentration (**figure 11.5**), and the concentration of test antibiotic is calculated from the graph obtained.

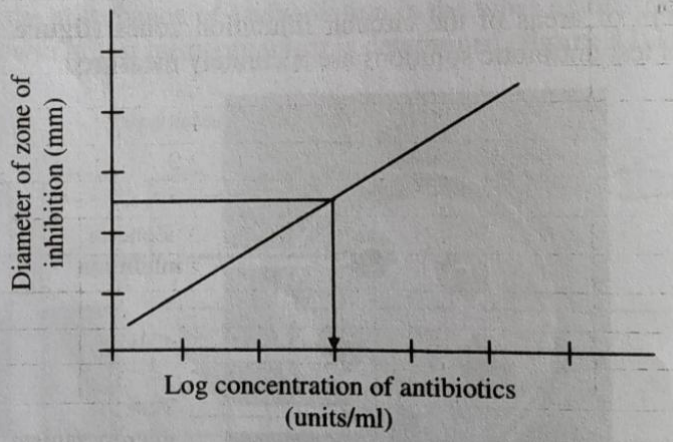


Figure 11.5: Standard Curve of Microbiological Assay of Antibiotics (Cup-Plate Method)

2) **Two-Level Factorial Assay:** Parallel dilutions containing two levels of both the standard (S_1 and S_2) and the unknown (U_1 and U_2) solutions are prepared. Four or more plates, each having 4 cavities are alternately filled with standard and unknown solution. The plates are then kept at room temperature and the diameters of the zones of inhibition are measured.

3) **Other Designs:** These include:
   i) Factorial assay having parallel dilution of three test levels of standard and the unknown, and
   ii) Factorial assay involving two test levels of standard and two test levels of two different unknowns.

11.1.5.9. Minimum Inhibitory Concentration

The lowest concentration of antimicrobial compound inhibiting the growth of a particular test microorganism is termed the **Minimum Inhibitory Concentration (MIC)**. The MIC is calculated in terms of **µg/ml** or **units/ml**.

The **methods** given below are used for **determining the MIC** of different antimicrobials:

1) **Liquid Dilution or Test Tube Method:** A series of test tubes containing double strength medium and labelled as shown in **(table 11.4)** are used. The first un-inoculated tube (0) is not added with the inoculum used for checking the medium sterility; while in the remaining 11 test tubes (0′-10), 3 or 4 drops of the inoculum are added to reach the final concentration of microorganisms (105-106 cells/ml). Except the un-inoculated and the control tubes, the remaining ones are added with 0.5-5ml of test chemical.

   The second control tube (0′) is used to check the medium suitability for test microorganism growth and the inoculum viability. In all the tubes, the final volume is adjusted up to 10ml using sterile water. The tube contents are mixed and incubated at 37°C for 2-3 days. Then all the tubes are examined for microbial growth in the form of turbidity, results are recorded, and MIC is calculated. A preliminary experiment should also be carried out for determining the approximate range (test solution) suitable for the test.

Table 11.4: Series of Test Tubes Containing Double Strength Medium and Labelled

Tube Number	Volume of Double Strength Medium (ml)	Volume of Test Chemical (ml)	Volume of Sterile Water (ml)
0 (uninoculated)	5	0.0	5
0′ (control)	5	0.0	5
1	5	0.5	4.5
2	5	1.0	4.0
3	5	1.5	3.5
4	5	2.0	3.0
5	5	2.5	2.5
6	5	3.0	2.0
7	5	3.5	1.5
8	5	4.0	1.0
9	5	4.5	0.5
10	5	5.0	0.0

2) **Solid Dilution Method:** In this method, the test chemical mixed with molten agar is poured on petri plates. After solidification, the agar surface is inoculated and then incubated at 37°C for 2-3 days. Thereafter, all the plates are observed for inoculum growth and the MIC of test chemical is calculated.

   This method has some **advantages**:

   i) By using a multipoint inoculator, several microorganisms can be tested at the same time.
   ii) Contaminations can be easily detected as the colony on solid media is more distinctive than turbidity differences in fluid media.

11.1.6. Standardisation of Vitamins

Vitamins are essentially required for the growth and multiplication of microorganisms (which are highly sensitive even to small amounts of growth factors). These test microorganisms can synthesise the factor being assayed which forms the basis of microbiological assay of vitamins and amino acids. The test microorganisms used for the standardisation of water-soluble vitamins are given in **table 11.5**:

Table 11.5: Test Microorganisms and Conditions for the Microbial Assay of Vitamins

Vitamins	Test Microorganisms	Incubation Temperature (°C)	Assay pH
Vitamin B_{12}	*Lactobacillus leichamannii* ATCC 7830	37	6.1
	Poteriochromonas stipitata ATCC 11532	30	5.5
Vitamin B_6	*Saccharomyces uvarum* ATCC 9080	30	4.5
	Tetrahymena thermophila ATCC 30008	30	6.1
Riboflavin	*Lactobacillus casei* ATCC 7469	37	6.8
	Tetrahymena thermophila ATCC 30008	30	6.1
Thiamine	*Lactobacillus viridescens* ATCC 12706	30	6.0
	Ochromonas danica ATCC 30004	30	5.5
Biotin	*Lactobacillus plantarum* ATCC 8014	37	6.8
	Ochromonas danica ATCC 30004	30	5.5
Niacin	*Lactobacillus plantarum* ATCC 8014	37	6.8
	Tetrahymena thermophila ATCC 30008	30	6.1
Pantothenate	*Lactobacillus plantarum* ATCC 8014	37	6.7
	Tetrahymena thermophila ATCC30008	30	6.1

11.1.6.1. Principle

Vitamins are essential growth factor for microorganisms. In the standardisation of vitamins, the ability of test organisms to use the substance being standardised under a proper nutritional condition is measured. These growth factors (vitamins) are required by the organisms in micro or nano grams. The growth response produced by the test organism is proportional to the amount of growth factor added to the medium.

11.1.6.2. Standardisation of Calcium Pantothenate

The reagents and the methods utilised for the standardisation of calcium pantothenate are discussed below:

Reagents

Standard Stock Solution of Calcium Pantothenate

The stock solution is prepared by dissolving 50mg calcium pantothenate in 500ml water. To the resultant solution, 10ml of 0.2N acetic acid and 100ml of 1.6%w/v sodium acetate solution are added. Volume is adjusted up to 1000ml by adding water. This solution is stored in a refrigerator under toluene. Each ml of this resultant stock solution contains 50µg of calcium pantothenate.

Standard Solution

Each ml of standard solution contains 0.04µg of calcium pantothenate. This solution is prepared by diluting the standard stock solution with water.

Test Solution

The concentration of calcium pantothenate in test solution is approximately equivalent to that in the standard solution prepared in water.

Medium

The medium utilised contains the following ingredients:

Acid-hydrolysed casein solution	25ml
Cystine-tryptophane solution	25ml
Polysorbate-80 solution	0.25ml

Dextrose (anhydrous)	10gm
Sodium acetate (anhydrous)	5gm
Adenine-guanine-uracil solution	5ml
Riboflavin-thiamine HCl-biotin solution	5ml
P-amino benzoic acid-niacin-pyridoxine HCl solution	5ml
Salt solution A	5ml
Salt solution B	5ml

The medium is prepared by dissolving the anhydrous dextrose and sodium acetate in previously mixed solutions. The pH of solution is adjusted to 6.8 using 1N NaOH. Ultimately, the solution is diluted to 250ml with water and mixed.

Stock Culture of Organism

The stock culture is prepared by dissolving 2gm water-soluble yeast extract in 100ml water; 500mg each of anhydrous dextrose and sodium acetate, and 1.5gm agar is also added. The resultant mixture is heated to dissolve the agar. 10ml of this hot solution is added to test tubes and sterilised at 121°C by mounting in vertical position. Stab culture of *Lactobacillus plantarum* is prepared in 3 tubes, incubated for 16-24 hours at 30-37°C, and stored in a refrigerator.

Preparation of Inoculum

The inoculum is prepared by transferring cells from the stock culture of organism to 10ml of culture medium in a sterile tube, and then incubating for 16-24 hours at 30-37°C.

Principle

Pantothenate assay medium (a dehydrated medium) is free from pantothenic acid or pantothenate, but contains other essential nutrients and vitamins used for preparing the culture of *L. plantarum*. Addition of calcium pantothenate in increasing concentrations as specified yields a growth response which is either turbidimetrically or titrimetrically measured.

Procedure

1) Standard solution in 1, 2, 3, 4, and 5ml is added to test tubes in duplicate.
2) Each of these tubes and another four similar tubes containing no standard solution are added with 5ml medium solution and volume is adjusted to 10ml with water.
3) Volumes of test solution corresponding to 3 or more of the levels as taken above are added to similar test tubes, in duplicate.
4) Each tube is added with 5ml medium solution and volume is adjusted up to 10ml with water.
5) A complete set of standard and assay tubes is placed in a tube rack and the duplicate set is kept in the other rack.
6) Tubes of both the series are autoclaved for 5 minutes at 121°C. After cooling, a drop of inoculum is added to each tube [except 2 of the 4 tubes which do not have standard solution (un-inoculated blanks)], mixed, and incubated for 16-24 hours at 30-37°C.
7) Spectrophotometer is used to measure the transmittance of the tubes at a specific wavelength lying between 540-660nm.

Calculation

A **standard concentration-response curve** is obtained by plotting the transmittance against the logarithm of the ml of standard solution per tube. The response is calculated by summing the two transmittances for each level of the test solution. This curve helps in determining the concentration of calcium pantothenate in the test sample.

11.1.6.3. Standardisation of Niacin

The selected organism should have the ability to utilise free niacin, niacinamide, nicotinuric acid, cozymase, and niacinamide nucleoside. This requirement is satisfied by *Lactobacillus plantarum* (an acid forming organism), cannot synthesise niacin for its metabolic processes. This bacterium is also non-pathogenic, can be easily cultured, and is negligibly affected by stimulatory or inhibitory substances present in niacin-containing pharmaceutical preparations.

This organism is grown on a simple stab culture containing gelatin, yeast extract, and glucose. For assay it is cultured in assay tubes by transferring to the liquid medium containing the basic medium having an optimum amount of added niacin. A measurable response can range from 0.05-0.5µg of niacin per tube.

For preparing the niacin test solution, sample is extracted, either in acid or alkali medium by adding such amount of alkali or acid that each ml of final volume contains 0.1gm niacin.

Reagents

1) **Standard Niacin Stock Solution I:** It contains 100µg/ml of U.S.P. Niacin RS.
2) **Standard Niacin Stock Solution II:** It contains 10µg/ml of U.S.P. Niacin RS, and is prepared by diluting solution I with water.
3) **Standard Niacin Solution:** It contains 10-40ng of niacin per ml and is prepared by diluting solution with water.
4) **Basal Medium Stock Solution:** It contains the following ingredients:

Acid-hydrolysed casein solution	25ml
Cystine-tryptophane solution	25ml
Dextrose (anhydrous)	10gm
Sodium acetate (anhydrous)	5gm
Adenine-guanine-uracil solution	5ml
Riboflavin-thiamine hydrochloride-biotin solution	5ml
Aminobenzoic acid-calcium pantothenate-pyridoxine Hydrochloride solution	5ml
Salt Solution A	5ml
Salt Solution B	5ml

The medium is prepared by dissolving anhydrous dextrose and sodium acetate in the previously mixed solutions. The pH is adjusted to 6.8 using 1N NaOH solution, and volume is adjusted to 250ml by adding water.

Culture Medium

The culture medium is prepared in a series of test tubes each containing 5ml of basal medium stock solution. Further, each tube is added with 5ml water containing 1.0µg of niacin, plugged with cotton, autoclaved at 121°C for 15 minutes, and cooled.

Preparation of Inoculum

The inoculum is prepared by transferring cells from the stock culture of *Lactobacillus plantarum* to 10ml culture medium in a sterile tube, and then incubating for 16-24 hours at 30-37°C.

Principle

Niacin assay medium (a dehydrated medium) is free from nicotinic acid and its analogues, but contains other essential nutrients and vitamins used for preparing the culture of *L. plantarum*. Addition of nicotinic acid or its analogues in increasing concentrations as specified yields a growth response which is either turbidimetrically or titrimetrically measured.

Procedure

1) The spectrophotometer is calibrated as per the Pharmacopoeia.
2) Standard niacin solution in 0.0, 0.5, 1.0, 1.5, 2.0,.... 5.0ml is added to test tubes in duplicate.
3) Each tube is added with 5ml of basal medium stock solution and volume is adjusted to 10ml with water.
4) Each tube is added with 5ml medium solution and volume is adjusted up to 10ml with water.
5) Tubes containing the material to be assayed are prepared by taking in duplicate, 1.0, 2.0, 3.0, and 4.0ml, respectively of the test solution of the material to be assayed.
6) Again each tube is added with 5ml basal medium stock solution and volume is adjusted up to 10ml with water.
7) The contents in tubes are mixed, plugged with cotton, and autoclaved.
8) After cooling, each tube is aseptically inoculated with a drop of inoculum and incubated for 16-24 hours at 30-37°C.
9) The transmittance for the un-inoculated blank is adjusted at 1.0, using which the transmittance of inoculated blank is read.
10) Finally, using the transmittance of the inoculated blank set at 1.0, the transmittance for the remaining tubes is measured.

Calculation

A standard curve of niacin standard transmittances for each level of standard niacin solution is plotted against µg of niacin present in the respective tubes. By interpolating this standard curve, the niacin content of test solution in each tube is determined. The niacin content of the test material is calculated from the average values obtained from atleast 6 tubes not varying by more than ±10% from the average. For expressing the results as niacinamide, the obtained values are multiplied by 0.992.

11.1.6.4. Standardisation of Vitamin B_{12}

Assay Preparation

1) An accurately weighed quantity of the material to be assayed is taken in the form of fine powder in a vessel.
2) An aqueous extracting solution is prepared containing 1.29gm disodium phosphate, 1.1gm anhydrous citric acid, and 1.0gm sodium meta bisulphite in each 100ml.
3) For each gm or ml of material taken, 25ml of this freshly prepared aqueous extracting solution is added and mixed.
4) The resultant mixture is autoclaved at 121°C for 10 minutes.
5) The undissolved particles of the extract are allowed to settle, and later filtered or centrifuged.
6) An aliquot of this so obtained clear solution is diluted with water so that the final test solution contains vitamin B_{12} having activity equivalent to the standard cyanocobalamin solution added to the assay tubes.

Standard Cyanocobalamin Stock Solution
The standard stock solution is prepared by adding sufficient amount of 25% alcohol to an accurately weighed quantity of U.S.P. cyanocobalamin RS. The solution obtained having a known concentration of 1.0µgm of cyanocobalamin per ml is stored in a refrigerator.

Standard Cyanocobalamin Solution
A fresh standard cyanocobalamin solution is prepared for each assay. It is prepared by diluting a suitable volume of standard cyanocobalamin stock solution with water up to a measured volume such that after incubation the difference in transmittance between the inoculated blank and 5.0ml of the standard cyanocobalamin solution is not less than that which corresponds to a difference of 1.25mg in a dried cell weight. This concentration falls between 0.01-0.04µg/ml of standard cyanocobalamin solution.

Basal Medium Stock Solutions
The basal medium utilised contains the following ingredients:

Table 11.6: Basal Medium Ingredients

Ingredients	Quantity
L–Cystine	0.1gm
L-Tryptophane	0.05gm
1 N Hydrochloric acid	10ml
Adenine-guanine-uracil solution	5ml
Xanthine solution	5ml
Vitamin solution I	10ml
Vitamin solution II	10ml
Salt solution A	5ml
Salt solution B	5ml
Asparagine solution	5ml
Acid-hydrolysed casein solution	25ml
Dextrose (anhydrous)	10gm
Sodium Acetate (anhydrous)	5gm
Ascorbic Acid	1gm
Polysorbate 80 Solution	5ml

Cysteine and tryptophan are dissolved in hydrochloric acid and then the next 8 solutions are added. To the resultant solution, 100ml water is added and mixed. To this mixture, dextrose, sodium acetate, and ascorbic acid are added. The solution is filtered and polysorbate 80 solution is added. The pH is adjusted between 5.5 and 6.0 using 1N sodium hydroxide, and volume is adjusted to 250ml with purified water.

Culture Medium
The culture medium is prepared by dissolving 0.75gm water-soluble yeast extract, 0.75gm dried peptone, 1.0gm of anhydrous dextrose, and 0.20gm of potassium biphosphate in 60-70ml of water. To the resultant solution, 10ml tomato juice and 1ml polysorbate 80 solution are added. The pH of the solution is adjusted to 6.8 using 1N NaOH solution, and volume is adjusted to 100ml with water. 10ml portions of the solution are taken in test tubes, plugged with cotton, and autoclaved at 121°C for 15 minutes. The solution is immediately cooled to avoid colour formation which may result due to overheating of the medium.

Suspension Medium
The suspension medium is prepared by diluting a measured volume of basal medium stock solution using an equal volume of water. 10ml portions of this diluted medium are taken in test tubes, sterilised, and cooled same as for the culture medium.

Stock Culture of *Lactobacillus Leichmannii*

1) The stock culture of the organism is prepared by heating with continuous stirring of 100ml of culture media with 1.0-1.5gm of agar medium on a steam bath till the agar dissolves.
2) Approximately, 10ml portions of the hot solution are taken in test tubes, plugged with cotton, and autoclaved at 121°C for 15 minutes.
3) After cooling the tubes in a vertical position, 3 or more tubes are inoculated by transferring stab of a pure culture of *Lactobacillus leichmannii*.
4) The inoculum is incubated for 16-24 hours at 30-40±0.5°C temperature, and stored in a refrigerator.
5) Fresh stab cultures are prepared at least thrice a week, and are not used for inoculum preparation if more than four days old.
6) The microorganism activity can be increased by transferring daily or twice-daily the stab culture to the point where definite turbidity in the liquid inoculum can be observed after 2-4 hours of inoculation.
7) A slow-growing culture rarely yields a suitable response curve, resulting in irregular results.

Preparation of Inoculum

1) The inoculum is prepared by transferring cells from the stock culture of organism to two sterile tubes, each containing 10ml culture medium.
2) These culture tubes are then incubated for 16-24 hours at 30-40±0.5°C temperature.
3) The incubated tubes are aseptically centrifuged, and the supernatant is decanted.
4) The cells from the culture are suspended and mixed with 5ml sterile suspension medium.
5) The volume is adjusted using sterile suspension medium so that 1 in 20 dilutions in saline TS produces 70% transmittance on a spectrophotometer at 530nm wavelength, and read against saline TS set at 100% transmittance.
6) 1 in 400 dilution of the adjusted suspension is prepared using basal medium stock solution to use for the test inoculum.

Principle

Vitamin B_{12} assay medium is free from vitamin B_{12}, but contains other essential nutrients and vitamins used for the culture of *Lactobacillus leichmannii*. Addition of U.S.P. cyanacobalamin reference in increasing concentrations as specified yields a standard curve providing growth response which is either titrimetrically or turbidimetrically measured.

Procedure

1) Standard cyanacobalamin solution in 1.0ml, 1.5ml, 2.0ml, 3.0ml, 4.0ml, and 5.0ml is added to thoroughly cleaned hard-glass test tubes in duplicate.
2) These tubes and other 4 identical empty tubes are added with 5.0ml of basal medium stock solution and volume is adjusted to 10ml with water.
3) Assay preparation in 1.0ml, 1.5ml, 2.0ml, 3.0ml, and 4.0ml is added to similar tubes in duplicate.
4) Each of these tubes is added with 5ml basal medium solution and volume is adjusted to 10ml with water.
5) A complete set of standard and assay tubes is placed in a tube rack and the duplicate set is placed in the other rack in random order.
6) The tubes are covered to prevent bacterial contamination and autoclaved for 5 minutes at 121°C.

7) The tubes are immediately cooled to prevent colour formation which may result due to overheating of the medium.
8) Each tube [except 2 of the 4 without standard cyanocobalamin solution (the un-inocoulated blanks)] are added with 0.5ml inoculum.
9) These tubes are incubated for 16-24 hours at 30-40±0.5°C temperature. Bacterial growth is terminated by heating for 5 minutes at temperature not lower than 80°C.
10) After cooling to room temperature, the contents are agitated, and when a steady state is reached, transmittance is recorded in a spectrophotometer at the wavelength of 530nm. This steady state is observed after a few seconds of agitation when the reading remains unchanged for 30 seconds or more.
11) Reading for each tube is recorded after the same time interval.
12) With the transmittance set at 100% for the un-inoculated blank, the transmittance of the inoculated blank is recorded. If the difference is greater than 5% or if contamination with a foreign microorganism is observed, the assay results are ignored.
13) With the transmittance set at 100% for the un-inoculated blank, the transmittance of the remaining tubes is recorded. If the slope of the standard curve indicates a sensitivity problem, the assay results are ignored.

Calculation

1) For preparing a **standard concentration-response curve**, any abnormal individual transmittance is prevented.
2) For each level of the standard, the response is calculated by adding the duplicate values of the transmittances (Σ) as the difference, $y = 2.00 - \Sigma$.
3) On plotting this response against the logarithm of the ml of standard cyanocobalamin solution per tube, a straight line or a smooth curve is produced.
4) The response (y) is calculated by adding the two transmittances for each level of the assay preparation.
5) From the standard curve the logarithm of the volume of standard preparation corresponding to each value of y falling within the range of the lowest and highest points plotted for the standard is determined.
6) From this logarithm value the logarithm of the volume (ml) of assay preparation is subtracted to obtain the difference (x) for each dosage level.
7) Average the values of x for each of three or more dosage levels to obtain x = M (the log-relative potency of assay preparation).
8) The quantity of U.S.P. Cyanocobalamin RS (µg) corresponding to the cyanocobalamin in the material being standardised is determined by the equation:

$$\text{Antilog } M = \text{antilog } (M + \log R)$$

Where,

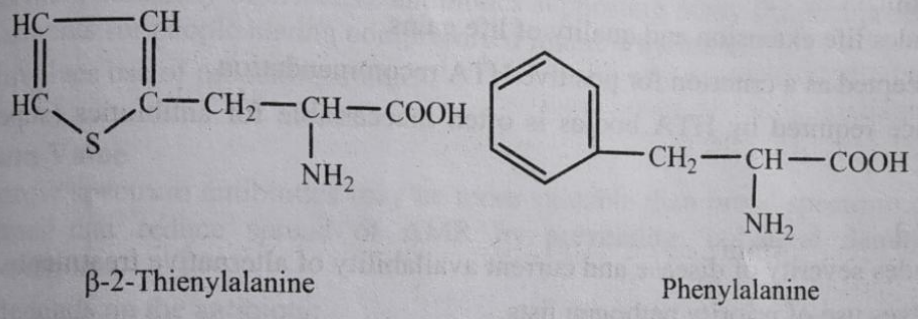
R = Number of µg of cyanocobalamin present in each mg of the material standardised.

11.1.7. Standardisation of Amino Acids

Microbiological assays were used for quantitation of amino acids since many years as they were the most reliable, sensitive, and specific available tests. They can be used for any type of biological material; however sometimes they are affected by the activators or inhibitors present in the material being standardised. These inexpensive methods are used for analysing large batches of samples, but are time-consuming and also not suitable for all amino acids.

Amino acids are essential for the growth and replication of some microorganisms. Many strains of such microbes depend on a particular amino acid. Thus, if such microorganisms are cultured using a small amount of that particular amino acid, a limited degree of growth will be observed, which is measured by turbidimetry or by measuring the increased lactic acid production by either microtitration or pH change.

Guthrie and **Susi** introduced a modified version of microbiological assay utilising diffusion in gels in clinical biochemistry laboratories for screening blood samples having increased phenylalanine levels. This test was named as the **Guthrie test** (after its inventors) and is the most widely used microbiological assay method of an amino acid. This bacterial inhibition assay is based on the ability of phenylalanine to counteract the effects of β-2-theienylalanine (a competitive metabolic antagonist) on the growth of a special strain of *Bacillus subtilis* which depends on phenylalanine for its growth (**figure 11.6**).



β-2-Thienylalanine Phenylalanine

Figure 11.6: Guthrie Test: The similarity in structure between phenylalanine and its metabolic antagonist β-2-thienylalanine provides the basis for a microbiological assay for phenylalanine

The test is carried out on an agar layer in which a mixture of the suspension of *B. subtilis* spores, minimum amount of growth nutrients, and a fixed amount of β-2 thienylalanine is added.

Filter paper discs of 4mm diameter are soaked in blood and placed on the agar surface along with the blood soaked discs of phenylalanine standards. These agar plates are incubated at 37°C overnight. Bacterial growth is observed only when phenylalanine concentration in the blood discs is sufficient to overcome the effects of β-2 thienylalanine. Growth is observed in zones of growth around each disc. The next day diameter of each zone of growth is measured and related to phenylalanine concentration.

11.1.8. Assessment of a New Antibiotic

Antimicrobial Resistance (AMR) occurs when microbes (bacteria, viruses, fungi, and parasites) change in ways making the medications used for curing the infections ineffective. If new antibiotics are not introduced, the patients will die due to the previously treatable infections.

However, a major issue is adequate evaluation of the antibiotics, particularly by payers and/or **Health Technology Assessment (HTA) Bodies**, to take account of AMR and reflect the full benefit provided to patients and society.

Two Key Challenges

1) Clinical trials are designed for establishing non-inferiority, while HTA bodies require demonstration of clinical superiority.

2) HTA bodies do not have a mechanism to evaluate the health benefits of antibiotics, and control the rise in AMR.

Table 11.7: Additional Elements of Value Relevant to Antibiotics

Relevant Benefits included in Traditional HTA	Other Types of Benefit of Possible relevance to Antibiotics
Health gain	Insurance value
Unmet need	Diversity value
Cost offsets	Diagnostic value
Productivity benefits	Uniqueness or innovation value
	Enablement value
	Spectrum value

Health Gain

1) It includes life extension and quality of life gains.
2) It is accepted as a criterion for positive HTA recommendation.
3) Evidence required by HTA bodies is often inaccessible for antibiotics (superiority trials).

Unmet Need

1) It includes severity of disease and current availability of alternative treatments.
2) It involves use of priority pathogen lists.

Cost Offsets

1) It includes the reduction in costs in other areas as a result of use of new medicine.
2) It involves use of modelling studies and/or evidence from clinical trials.

Productivity Benefits

1) It involves profits or losses related to value of patient's time, receiving medical care or out of work.
2) It involves use of modelling studies and/or evidence from clinical trials/observational studies.

Insurance Value

1) It is the value of treatment available in case of catastrophic health events, **e.g.,** outbreak of MDR infections which cannot be controlled by existing last-line antibiotics.
2) It is analogous to availability of a fire engine.
3) It also needs to add in the "precautionary principle".
4) It involves use of modelling studies.

Diversity Value

1) It is the selection pressure, i.e., the antibiotic is able to eliminate susceptible species of bacteria but no other resistant pathogens; thus these pathogens survive and multiply, making the antibiotic ineffective.
2) It involves evidence that reducing selection pressure by withdrawing antibiotic for a period of time may result in restoration of susceptibilities.
3) It involves use of modelling studies.

Diagnostic Value

1) If infection is diagnosed at an early stage, appropriate antibiotic therapy can be started earlier.
2) It needs evidence of test accuracy.

Uniqueness or Innovation Value

1) It is the potential value associated with new or unique mechanism of action.
2) Antibiotics with novel mechanism of action may avoid problems of cross-resistance occurring in many existing classes.
3) Discovery of new mechanism of action of an antibiotic makes it easier for "follow on" products to enter market.
4) It needs evidence of new mechanism of action.

Enablement Value

1) It is the availability of effective antibiotics supporting many surgical procedures and treatments for people having compromised immune systems.
2) It involves use of modelling studies.

Spectrum Value

1) Narrow spectrum antibiotics may be more valuable than broad spectrum ones as the former can reduce spread of AMR by preventing 'collateral damage' to the microbiome.
2) It depends on the antibiotic.

Given below are the reasons for which additional elements of value for antibiotics are required:

1) **AMR is a Public Health Priority:** The rise of AMR is a serious threat, control of which is a priority for the national and international health organisations. The current HTA methods do not account for the value of reducing this public health threat, **e.g.,** of the insurance value of having a treatment available in case of a future major or rapidly intensifying resistance problem.

2) **Diverse Set of Non-Inferior Antibiotics is Valuable to Society:** Since AMR is increasing widely, it has become valuable developing a new antibiotic for MDR pathogens, even if it is ineffective than the existing antibiotics in treating susceptible (non-resistant) pathogens. This concept is unique to antibiotics and is not considered by HTA bodies.

3) **Non-Clinical and Microbiology Data are Important for Demonstrating the Value of Antibiotics:** Non-clinical and microbiology data for antibiotics can predict their results. Due to the difficulties in conducting clinical trials for antibiotics for MDR pathogens, regulators accept these alternative types of evidence as part of the approval process in areas of high unmet need.

4) **Antibiotics have Benefits that go Beyond the Patient Treated:** When one patient is treated with an antibiotic, the spread of the infectious disease is reduced thus benefitting the population.

5) **Antibiotics Enable Other Types of Treatment and Procedures:** Apart from treating infections, antibiotics also reduce the risk associated with other types of treatment such as surgery and chemotherapy.

11.2. SUMMARY

The details given in chapter can be summarised as follows:

1) The **microbiological** or **microbial assay** is a type of biological assay that determines the relative potency of activity of a compound by measuring the amount needed to produce an anticipated effect on a suitable test organisms under standard conditions.
2) A microbiological assay relies on the principle that **when certain compounds are present in limited amounts, the amount of microbial growth corresponds to the amount of these compounds**
3) The methods used for microbiological assay of antibiotics are based on the following principles:
   i) Method A (serial dilution method), and
   ii) Method B (diffusion method).
4) Pharmacopoeias suggest the following two antibiotic assay methods:
   i) Turbidimetric or tube assay method, and
   ii) Cup-plate or cylinder-plate method.
5) **Turbidimetric** or **tube assay method** depends on the growth of a microbial culture in a uniform solution of antibiotic in a fluid medium favourable to its rapid growth in the absence of antibiotic. This method involves a **shorter incubation period** (4-5 hours) for the growth of test microorganism.
6) **Cup-plate** or **cylinder-plate method** relies on the diffusion of an antibiotic from a vertical cavity or a cylinder through the solidified agar layer in a petri plate.
7) **Standardisation of vitamins** relies on the measurement of the ability of test organisms to use the substance being standardised under a proper nutritional condition.
8) A **standard concentration-response curve** is obtained by plotting the transmittance against the logarithm of the ml of standard solution per tube.
9) **Minimum Inhibitory Concentration (MIC)** is the lowest concentration of antimicrobial compound inhibiting the growth of a particular test microorganism. The MIC is calculated in terms of **µg/ml** or **units/ml**.
10) MIC is determined by the following methods:
    i) Liquid dilution or test tube method, and
    ii) Solid dilution method.
11) **Antimicrobial Resistance (AMR)** occurs when microbes (bacteria, viruses, fungi, and parasites) change in ways making the medications used for curing the infections ineffective.
12) **Diversity value** is the selection pressure, i.e., the antibiotic is able to eliminate susceptible species of bacteria but no other resistant pathogens; thus these pathogens survive and multiply, making the antibiotic ineffective.
13) **Innovation value** is the potential value associated with new or unique mechanism of action.
14) **Enablement value** is the availability of effective antibiotics supporting many surgical procedures and treatments for people having compromised immune systems.
15) Narrow spectrum antibiotics may be more valuable than broad spectrum ones as the former can reduce spread of AMR by preventing 'collateral damage' to the microbiome.

11.3. EXERCISE

11.3.1. True or False

1) The microbiological or microbial assay is a kind of biological assay that determines the relative potency of activity of a compound.
2) In serial dilution method, the dilution of the antibiotic to be tested is compared with the dilution of the standard antibiotic preparation having an identical effect.
3) The highest concentration of antimicrobial compound inhibiting the growth of a particular test microorganism is termed the Minimum Inhibitory Concentration (MIC).
4) Cup-plate method relies on the diffusion of an antibiotic from a vertical cavity or a cylinder through the solidified agar layer in a petri plate.
5) Standardisation of vitamins relies on the measurement of the ability of test organisms to use the substance being standardised under a proper nutritional condition.

11.3.2. Fill in the Blanks

6) The principle of _____ is that when certain compounds are present in limited amounts, the amount of microbial growth corresponds to the amount of these compounds.
7) _____ are the agents that kill or reduce the bacterial growth.
8) The diameters of zone of inhibition are best measured using an _____ in which an optical system forms an image of plate on a large grid.
9) A standard curve is obtained by plotting _____ of the standard antibiotic against the zone of inhibition.
10) _____ occurs when microbes change in ways that render the medications used to cure the infections they cause ineffective.

Answers:

1) True 2) True 3) False 4) True 5) True
6) Microbiological assay 7) Antibiotics 8) Antibiotic zone reader
9) Log concentration 10) Antimicrobial Resistance (AMR)

11.3.3. Very Short Answer Type Questions

1) What is minimum inhibitory concentration?
2) Give the principle involved in standardisation of vitamins.
3) Give the principle of standardisation of cyanocobalamin.
4) What is Guthrie test?
5) Define Antimicrobial Resistance (AMR).
6) What is enablement value?
7) Give principle of microbiological assay.
8) Enlist official methods involves in the standardisation of antibiotics.

11.3.4. Short Answer Type Questions

1) Give the principle of standardisation of vitamin.
2) Write a note on cup-plate method.
3) Write a note on standardisation of amino acids.
4) Give standardisation procedure of niacin.
5) What is tube assay method?
6) Give a note on standardisation of vitamin B_{12}.

11.3.5. Long Answer Type Questions

1) What is microbial assay? Give details.
2) Write a note on standardisation of antibiotics.
3) Write a detail note on standardisation of vitamins.
4) Give a descriptive note on assessment of new antibiotic.

CHAPTER 12

Microbial Contamination and Preservation of Pharmaceutical Products

12.1. SPOILAGE

12.1.1. Introduction

The process of deterioration of pharmaceutical products by the contaminant microbes is known as **microbial spoilage**. A stable and effective medicine may get contaminated and damaged due to microbial growth. These microbes can enter the product either during manufacturing or use by the patient or medical staff. Such spoilage either results in immediate product loss or increased litigation cost that whether the spoilage will harm the user, thus leading to financial problems to the manufacturer.

Based on its proposed use, a pharmaceutical product is considered to be microbiologically spoiled due to:

1) Presence of low levels of acutely pathogenic microbes or higher levels of opportunist pathogens,
2) Presence of toxic microbial metabolites even after death or removal of initially present microorganisms, and
3) Occurrence of detectable physical or chemical changes in the product.

Formulating an economic product of desirable efficiency, stability, and patient acceptability requires incorporation of various ingredients in a complex physical balance. This potentially increases the risk of microbial attack and even microbial growth in the product. Since the demand of sterile or self-sterilising products is increasing, the formulators should establish a system having low levels of microbial contamination, or even if it occurs the microbes either fail to multiply or multiply minimally during the product life.

12.1.2. Types of Spoilage

Spoilage may be of the following types:

1) **Infection Induced by Contaminated Pharmaceutical Products:** Processing faults and contamination of injections during use produce localised infections or septicaemia. Presence of a few microbial survivors in an injection may also induce infection. Microbial growth occurring during storage of the product prior to its administration, results in a highly infective or toxic product. **For example**, severe eye infections have occurred in patients using eye drops contaminated with *Pseudomonas aeruginosa*. Ointments and creams (required to be clean but not sterile) if gets contaminated, causes dermatoses, burns, respiratory infections, and other diseases transmitted via shared containers. Ineffectively sterilised and contaminated wash-out solutions cause urinary tract infections.

   Oral medications have a variety of microorganisms, few of which are infective. However, tablets or capsules of yeast, thyroid, pancreatin, and carmine are

contaminated with *Salmonella* species, thus causing infections. The antacid suspensions (mainly those flavoured with peppermint water) often get contaminated with *Pseudomonads* and causes bowel infections.

High levels of infection-causing *Pseudomonads* are often present in dilute antiseptic and disinfectant solutions of quaternary ammonium type. These pathogenic agents either cause infection or serve as reservoirs of contamination. Such preparations become readily inactive during use and have poor activity towards these microorganisms, which thus survive and start multiplying in them.

The available regulatory restrictions of recent years have failed to reduce the incidence of drug-induced infections. At the current time, the main concerns are the high occurrence of in-use contamination of infusion and Total Parenteral Nutrition (TPN) fluids; and the antiseptics and aqueous oral mixtures (contaminated with gram-negative bacteria) which cause harmful infections to large number of patients receiving immunosuppresive therapy.

Microbial toxins present in pharmaceutical products are the pyrogens (microbial lipopolysaccharides) liberated by gram-negative bacteria and blue-green algae (cyanobacteria), and pyrogenic substances liberated by fungi. Pyrogens present in infusion fluids may cause acute febrile shock. Pyrogenic reactions resulting from contaminated haemodialysis fluids have been recorded.

Also, there are some evidences that pharmaceutical products contain bacterial toxins causing food poisoning; although pharmaceutical products are detected with toxigenic fungi including aflatoxin-producing *Aspergilli*. The irritant products of degradation (such as salicylic acid) produced by microbial hydrolysis of aspirin or of the allergenicity of high levels of microorganisms cause a potential problem.

2) **Chemical and Physicochemical Deterioration of Pharmaceutical Products:** The degradation frequency of microorganisms is such that all organic matter are subjected to chemical modifications under conditions milder than those for reactions performed by non-biological processes. Biological half-lives of materials released into the environment ranges from hours (phenol) to months (surfactants) to years (halogenated pesticides).

Biodegradable material accumulating in the environment is the result of localised adverse conditions or the absence of particular microorganisms. **For example**, lignins accumulate as peat and persistence of oil slicks in sea water. However, this discussion is restricted only to the fate of ingredients within the specialised microenvironments of various types of pharmaceutical formulation, rather than their subsequent fate when released into the biosphere.

The degradation rate of ingredients depends on their chemical structure, the physicochemical properties of the product, and the microbial contamination level present. The liability to attack ingredients and the physical character of some pharmaceuticals lead to microbial growth from metabolites, thus the degradation rates of recalcitrant components are enhanced along with rapid onset of formulation destruction. Initial attack by a group of spoilage organisms makes the product vulnerable to a second group of organisms, thus causing more damage.

3) **Ingredients Subject to Microbial Attack:** The ingredients easily attacked by microbes are:

i) **Cationic Surfactants:** The surfactants used as antiseptics and disinfectants in pharmacy are resistant, although there are reports of microbial attack at both

'use' concentrations and diluted, as occurring in sewage. Gram-negative bacteria grow extensively in these solutions at the expense of other ingredients, **e.g.,** using ammonium acetate buffer as a vehicle.

ii) **Anionic Surfactants:** Alkali-metal and amine soaps of fatty acids are readily degraded in sewage but are normally stable in their slightly alkaline formulations. Alkyl, alkylbenzene sulphates and sulphonates undergo metabolism when the terminal methyl group is attacked with sequential β-oxidation of the alkyl chain and cleavage of sulphate from the molecule after aromatic ring fission. The ease of degradation decreases with increase in length and complexity of alkyl chain branching. Alkyl sulphates and alkyl ether sulphates undergo oxidation readily compared to the recalcitrant polypropylene alkylbenzene sulphonates. The released sulphate reduces to hydrogen sulphide by the sulphate-reducing anaerobic bacteria in shampoos; thus indicating the generation of very low oxygen tensions in viscous formulations.

iii) **Non-Ionic Surfactants:** Alkylpolyoxyethylene alcohol emulsifiers readily undergo metabolism by various microorganisms. The ease of degradation again decreases with increase in length and complexity of alkyl chain branching. Alkylphenol polyoxyethylene alcohols are attacked in the same way, but are more resistant. After lipolytic release of fatty acids from sorbitan esters, polysorbates, and sucrose esters, the cyclic nuclei degrade to produce nutrient for microbial growth.

iv) **Ampholytic Surfactants:** Phosphatides and some betaine derivatives readily undergo degradation. Accumulation of the early, recalcitrant, anionic detergents in the biosphere result in the designing of such anionic and non-ionic surfactants which readily metabolise, and thus cause nutritional hazards to pharmaceuticals formulated with them.

v) **Organic Polymers:** Various thickening and suspending pharmaceutical agents result in nutritive fragments and monomers when subjected to microbial depolymerisation by extracellular enzymes, **e.g.,** amylases (starches), pectinases (pectins), celluloses (carboxymethylcellulose), uronidases (tragacanth and acacia), dextranases (dextrans), and proteases (proteins); the enzyme names are mentioned along with their substrates in parenthesis.

Agar is used as an inert support for culture media because even if agar-degrading microorganisms are present, agar rarely undergoes depolymerisation. Polyethylene glycols readily undergo degradation by sequential oxidation of hydrocarbon chains, except for the very high molecular weight congeners. Recalcitrant polymers are used in plastic packaging; however cellophane is an exception subjected to cellulolytic attack in some cases.

vi) **Humectants:** High concentrations of glycerol and sorbitol used in pharmaceutical formulations support microbial growth.

vii) **Fats and Oils:** These hydrophobic materials are attacked by microbes when dispersed in aqueous formulations; although fungal growth is observed in condensed moisture films on the surface of bulk oils or when the bulk phase is contaminated during storage by water droplets.

The lipolytic rupture of triglycerides releases glycerol and fatty acids; in the latter, β-oxidation of the alkyl chain occurs producing odorous ketones.

Microbial metabolism of hydrocarbon oils poses a problem in engineering and fuel technology when water also acts as a contaminant; although only limited evidences of this case have been reported in pharmaceuticals. *Cladosporium resinae* causes metabolism of aviation kerosene, thus inducing corrosion in aircraft, resulting in the build-up of acid products.

viii) **Sweetening, Flavouring, and Colouring Agents:** Sugars and other sweetening agents used in pharmaceutical preparations act as substrates for microbial attack. Concentrated stock solutions of sugars (syrups) having low water activities are resistant to microbial attack, although evidences of their spoilage by growth of osmophilic yeasts have been reported and therefore preservatives are added. Aqueous stock solutions of flavouring agents (**e.g.,** peppermint water and chloroform water) and colouring agents (**e.g.,** amaranth or tartrazine) support growth of bacteria and yeasts; while simple stock solutions of flavouring agents are no longer used, and of colouring agents are still commonly used for dispensing.

ix) **Potent Therapeutic Agents:** It has been demonstrated by laboratory experimental reports that many drugs undergo gross degradation by various microbes, which either destroys or changes the therapeutic efficiency. Materials like alkaloids (**e.g.,** morphine, strychnine, atropine, etc.), analgesics (**e.g.,** aspirin, paracetamol, etc.), thalidomide, barbiturates, steroid esters, or mandelic acid can undergo metabolism and serve as substrates for microbial growth.

Formation of irritant salicylic acid from aspirin or of inactive products from penicillin (by β-lactamase) or chloramphenicol (by chloramphenicol acetylase) or certain other antibiotics are **examples** of such spoilage. Microbial transformations of steroid molecules form the basis of a valuable biotechnology tool used in the production of potent drugs from inert steroids.

Other drugs such as dicophane are highly recalcitrant; however, such attacks to pharmaceutical formulations have been rarely reported. Loss of potency of some alkaloids (**e.g.,** atropine in eye drops and in an oral medication) and degradation of aspirin and paracetamol in aqueous formulations have been recorded. Penicillin injections have been found to be inactivated by β-lactamase-forming bacteria. Localised transformation of steroids occurs around fungal colonies developing on steroid tablet surface and in steroidal creams.

x) **Preservatives and Disinfectants:** Most of the organic preservatives and disinfectants undergo metaboilsm by various bacteria and fungi; and even sometimes act as growth substrates at concentrations below the 'use' levels, as in sewage and effluent with the exception of quaternary ammonium antimicrobial agents.

Halogenation or nitration increases recalcitrance up to considerable level. Rapid microbial conversion of organomercurial preservatives is of the main concern. These preservatives are used and released in industrial effluent in large quantities that infect humans via an ascending food chain. Microbial degradation at 'use' levels of these antimicrobial agents is rarely reported but includes a few metabolisms including that of chlorhexidine, cetrimide, phenolics, phenylethyl alcohol, and benzoic acid.

It has been reported that benzalkonium chloride is utilised at concentrations below the 'use' levels; but rapid utilisation of *p*-hydroxybenzoate esters (at

concentrations previously recommended for preservation of eye drops and other aqueous pharmaceutical mixtures) occurs by various *Pseudomonads* and related bacteria, including *Pseudomonas aeruginosa*, often using them as substrates for growth.

4) **Observable Effects of Microbial Attack in Pharmaceutical Products:** In a pharmaceutical product, microbial spoilage becomes visible if there is a very high contaminant level or extensive multiplication. However, bacterial growth is observed on a surface moisture film (and not in the bulk of the product) or is indicated by the production of odoriferous products.

Aqueous products and water in the early stages of microbial attack develop earthy tastes. Microbial spoilage results in unpleasant tastes and odours; **for example,** sour-tasting fatty acids and ketones; fishy odour of amines, H_2S, and ammonia; or metabolites with bitter, sickly or alcoholic tastes and odours. Apart from the coloured microbial colonies or sediments, the formulations may also become green, pink, brown, black, or yellow coloured due to the diffusible microbial pigments.

Depolymerisation of thickening and suspending agents reduces the viscosity and sedimentation of suspended materials. The sugary products and concentrated shampoos are reported to develop polymeric, slimy strands. Spoiled creams become lumpy or gritty to feel. The product pH varies due to build-up of metabolites. Yeast attack of some acidic products raise the pH to an extent allowing microbial attack, which was formerly inhibited. Bubbles of gaseous metabolites accumulate in viscous formulations and also cause ballooning of flexible plastic packages.

The o/w (oil-in-water) emulsions and creams serve as rich substrates for microbial attack and undergo gross deterioration in their physicochemical structure. Surfactant degradation and pH reduction by lipase attack of triglycerides induces progressive coalescence of lipid droplets, ultimately leading to cracking (i.e., complete separation of the two phases) and forming an intermediary, unstable, w/o emulsion state. Most of the surfactants lose their surface activity at a very early stage due to metabolism. Plastics (**e.g.,** polyethylene) may undergo a pink discolouration after limited fungal colonies are formed.

12.1.3. Factors Affecting the Microbial Spoilage of Pharmaceutical Products

The physical and chemical properties of a pharmaceutical formulation affect the type and extent of spoilage from the microbe it is at risk. Some specific conditions within a product may favour its degradation by a specific group of microorganisms; these conditions are not necessarily being those required for its optimal growth in standard laboratory media. The initial attack modifies the conditions to an extent allowing further degradation by a group of microorganisms. The factors potentially influencing spoilage should be considered as vital part of formulation development, and the conditions minimising the risk of microbial deterioration should be specified. Such factors are discussed below:

1) **Size of Inoculum:** Microbial attack can be avoided by the removal or destruction of viable microorganisms present in a pharmaceutical preparation. Maintaining the preparation in a sterile condition is also necessary to avoid microbial attack. Conventional sterilisation methods, with the exception of filtration sterilisation, also inactivate the already present degradative enzymes; but some pyrogens remain

active. However, economic considerations limit this solution to products which are specifically valuable or are used in areas at infection risk. Low levels of contaminants not growing in the product cause deterioration at low rates, while high levels of contaminants introduced through ingredients during processing or by the user results in an attack even without proliferation. The lag period before rapid growth period can be considered in products with originally very low levels of microorganisms and decreases as the contaminant load increases. Some control measure for microbial levels will benefit the product stability.

2) **Nutritional Factors:** Usually, gross microbial deterioration of a pharmaceutical product occurs in the presence of appreciable growth of the contaminating microbes within it. The attack of organic ingredients mostly yields metabolites (carbon, or carbon and nitrogen) which serve as potential substrates for microbial growth. Normally, sufficient amount of inorganic salts are present to support this growth. The complexity of many formulations offers nutritional variety for various microorganisms; other factors usually determine whether or not growth will occur. Presence of crude vegetable or animal products in a formulation introduces a nutritious adjunct and the active component(s). However, good-quality glass-distilled water is also sufficiently nutritive to allow gram-negative bacteria (*Pseudomonas aeruginosa*) to multiply to levels in excess of 1×10^5 organisms per millilitre within 48 hours at room temperature. Demineralised water produced by ion exchange methods support microbial growth in greater amounts.

3) **Shelf-Life of Product:** A product's expected life should be considered an important factor when the degree of microbial spoilage which may occur is being determined. If a parenteral solution containing a very low level of viable but damaged bacteria (resulting from improper heat sterilisation) is stored for a prolonged period, the bacteria may undergo multiplication and release pyrogen, thus making the innocuous preparation toxic. Presence of non-pathogenic microorganisms in moderate levels in a product can be tolerated for several days; however initial levels is intolerable by a factory-produced formulation, which being many weeks old have suffered microbial attack before patient administration.

4) **Moisture Content:** The components dissolved in an aqueous preparation form a complex with water molecules via hydrogen and other bonds. This lowers the proportion of water available to contaminant microorganisms in the product. **Water activity (Aw = Vapour pressure of solution/Vapour pressure of water at same temperature)** is a measure of available water, and is inversely proportional to solute concentration; therefore greater the solute concentration, lower is the water activity. Microorganisms mostly grow best at high values of Aw, while halophilic bacteria depend for stability on high solute levels. By lowering the Aw values or by adding sugars, polyethylene glycols, or sodium chloride (for foodstuffs) in suitable amounts, or by drying, preparations can be protected from microbial attack.

The growth-inhibiting property of solutes is not limited to their water-binding properties; thus efficiency of lithium chloride is more than that of equimolar proportions of sodium chloride, but has undesirable pharmacological activity. The **lower Aw limits for the growth of spoilage microorganisms** are in the following order: Gram-negative rods, 0.95; *Staphylococci*, *Micrococci*, and *Lactobacilli*, 0.91; and yeasts, 0.88. Syrup-fermenting osmotolerant yeasts can grow in solutions having Aw values of 0.73. Filamentous fungi such as *Aspergillus glaucus* can grow at Aw values as low as 0.61.

Condensed moisture films are formed on the surface of dry products (such as tablets) due to the fluctuations in storage temperatures. The moisture content of air may be sufficient to initiate growth of *A. glaucus* fungi. In a closed container, the localised Aw values of metabolically produced water further increase to allow spore germination. Moisture films formed on the surface of viscous syrups or exuded by syneresis from hydrogels exhibit very high Aw values to allow growth of yeast and fungi.

5) **Redox Potential:** The oxidation-reduction balance of a preparation is partially determined by its oxygen content and partially by its ingredients. Many aerobic and facultative microorganisms multiply slowly at low redox potentials; and spoilage prevention by complete removal of oxygen is not normally practiced for pharmaceutical products. However, pressurised carbon dioxide provides protection to some foodstuffs by a more specific microbicidal action. Facultative anaerobic microorganisms lower the redox potentials to allow the obligate anaerobes to grow, while anaerobic spoilage of pharmaceutical products (a recognised food spoilage problem), would remain infrequent.

6) **Storage Temperature:** Spoilage of pharmaceutical products could occur from –20 to 60°C temperature, although poorly at the extremes. Storage within specific, narrower, temperature range supports the growth of particular groups of spoilage microorganisms. Microbial growth and spoilage are negligible below –20°C. It is recommended that to prevent pyrogen production, the distilled water used for preparing water for injections should be stored at 80°C before sterilisation. Storing a prepared solution near a radiator and not in a cool place increases its risk of microbial degradation. In such a case, the transportation effect and product storage at ambient temperatures in the tropics or sub-tropics should be considered.

7) **pH Value:** High pH prevent microbial attack, although feeble growth of mould is observed in dilute solutions of hydrochloric acid. Under neutral pH, microbial spoilage prevails. Under mildly alkaline conditions (**e.g.,** soap emulsions having pH 8.0-9.5 and mixtures of magnesium hydroxide or aluminium hydroxide gel), microbial spoilage is discouraged. Under acidic pH, fungal and yeast proliferation is favoured, **e.g.,** at pH 3.5 the fruit-flavoured pharmaceutical products preserved under improper conditions encourage microbial growth. Yeasts can metabolise organic acids and increase the pH value up to levels sufficient for secondary microbial attack. Protein degradation increases the pH in acidic preparations by favoured decarboxylase activity or decreases the pH in slightly alkaline formulations by favoured deaminase activity.

8) **Package Design:** The risk of microbial contamination of residual product while using multi-dose containers by medical staff or patients is reduced by using unit-dosage containers. However, their high cost has limited their use to a greater extent than required. Cap liners of cork or cardboard (used earlier) readily absorb moisture and support microbial growth, thus are infused with preservative. Certain packaging plastics encourage microbial spoilage by allowing the moisture vapour and oxygen to penetrate the product. To prevent microbial entry into multi-dose injection containers after they are punctured with a hypodermic needle, self-sealing rubber wads are used. Wide-mouthed ointment and cream jars promote the entry of contaminants and nutrients via coughs, fingers, etc.

9) **Protection of Microorganisms within Pharmaceutical Products:** The product components apart from directly reducing the efficiency of antimicrobial preservatives may also increase the resistance and durability of contaminant

microorganisms. There are some evidences in which certain proteins, suspending agents, and low concentrations of surfactants (polysorbates and cetomacrogols) are involved in increased resistance of certain organisms to phenolic and other preservatives. Adsorption of microorganisms on suspended particles (**e.g.,** kaolin, magnesium trisilicate, or aluminium hydroxide gel) increases their life. Formulation pH will influence the sensitivity of many organisms towards preservatives.

12.1.4. Sources and Types of Microbial Contaminants

Pharmaceutical formulations are contaminated by the materials used in their formulation (**e.g.,** raw materials, water, and equipment) and by the environment in which they are manufactured (**e.g.,** from atmosphere and human sources). Various sources and types of microbial contamination are described here:

1) **Atmosphere:** In the atmosphere, number of microorganisms depends on the activity in the environment and the amount of dust. Air does not provide natural environment for the growth and multiplication of microbes as it does not contain the favourable factors (**e.g.,** moisture and nutrients). Microbes are carried into the atmospheric air by suspending on dust particles, skin, clothing, droplets of moisture, or sputum coming from sneezing or coughing. Presence of microbes in the atmospheric air is increased during handling of contaminated materials during dispensing, blending, and formulating. Fungi count may be increased due to the use of starch and some sugars in the dry state. The microbes present in atmosphere are isolated and identified by settle plate and different air sampling techniques. **Examples** of bacteria and fungi commonly isolated from atmosphere include *Staphylococcus* spp., *Streptococcus* spp., *Clostridium* spp., *Bacillus* spp., *Penicillium* spp., *Aspergillus* spp., *Mucor* spp., *Cladosporium* spp., etc.

   The microbial count of atmosphere or air can be decreased by filtration, chemical disinfection, radiation (UV light), and gaseous agents.

2) **Water:** It is the most common solvent and is also used for other processes like washing and cooling. Various types of microorganisms are found in fresh water and some microbes may contaminate the water with sewage (**e.g.,** *Pseudomonas* spp., *Proteus* spp., *Escherichia coli*, *Streptococcus faecalis*, *Flavobacterium* spp., *Clostridium* spp., *Chromobacter* spp., etc.). Containers used during the preparation of various pharmaceutical formulations are washed with soft water; however soft water prepared by Base Exachange Method may get contaminated by *Bacillus* and *Staphylococcus* spp.

   For the preparation of pharmaceutical products, **deionised water**, **distilled water**, and **water for injections** are used. To reduce microbial growth, water may be treated with ultraviolet light, filtration, or stored at high temperature.

3) **Raw Materials:** Raw materials, mainly of natural origin are a potential source of microorganisms. Various products obtained from animal sources (like gelatin, desiccated thyroid, pancreas, cochineal, etc.) may be contaminated with various pathogens. Various products obtained from plant sources (like agar, starch, acacia, tragacanth, etc.) also contaminate the pharmaceutical products. Synthetic raw materials are generally free of microbes, but may get contaminated accidentally. **Examples** of bacteria and fungi commonly present in raw materials are *Bacillus* spp., *Pseudomonas* spp. *Lactobacillus* spp., *Salmonella* spp., *Cladosporium* spp., *Fusarium* spp., etc.

   Microbial load in raw materials can be reduced by heat treatment, filtration, recrystallisation, irradiation, or any other sterilisation process.

4) **Process Operators:** The pharmaceutical formulations may be contaminated during process operators by the natural skin flora microorganisms. The contamination-causing bacteria and fungi are *Staphylococcus aureus*, *Sarcina* spp., *Alcaligenes* spp., and various dermatophytic fungi (**e.g.,** *Epidermophyton* spp., *Microsporon* spp. and *Trichophyton* spp.). Wounds with signs of infection may include *Staphylococcus aureus*, *Staphylococcus pyogenes*, *Proteus* spp., and *Pseudomonas aeruginosa*. The respiratory tract and nasal passage may have excessive concentration of *Staphylococcus aureus*, *Staphylococcus salivarius*, *Neisseria pharyngis*, *Klebsiella pneumoniae*, etc.

5) **Equipment:** The equipment involved in the animal cell culture should be simple and have minimum junctions, valves, and pumps for facilitating cleaning by circulation of detergents or other chemical antimicrobial agents. This type of microbial contamination and degree of microbial growth depends on the source of contamination, environmental conditions, and nutrients. The cleansing efficiency is checked by plating out a sample of the final rinsed water with a nutrient agar or by swab tests.

   For cleaning pipelines, plates and tubular types of heat exchangers, pumps, homogenisers, and all larger equipment, **'in-place' cleaning system** are used. Various agents like heat, gases, and chemicals are used for the sterilisation and disinfection of culture equipment. Autoclaving (steam under pressure) is used to sterilise tanks, containers, small equipment, and reaction vessels.

6) **Packing:** Microbial contamination depends on the composition of packing materials and storage conditions of packaging. The packaging materials act as containers for storage of the product, and also prevent the entry of microorganism or moisture. Various packaging materials (**e.g.,** cardboard boxes, closure liners) should be sterilised as they may carry different types of microbes (**e.g.,** *Bacillus* spp., *Penicillium* spp., *Aspergillus* spp., *Cladosporium* spp., and *Micrococcus* spp.). **For examples,** vials and ampoules (used for injectable and ophthalmic preparations) are sterilised by dry heat at 170°C. Moist heat, chemicals, gaseous agents, or radiations are used to sterilise caps and closures of containers.

7) **Buildings:** Some moulds and bacterial species are commonly present on the walls and ceilings, **e.g.,** *Aspergillus niger*, *Aspergillus flavus*, *Cladosporium* spp., *Penicillium* spp., *Bacillus* spp., etc. They are mostly growing on poorly ventilated buildings. These microorganisms obtain their nutrients from plaster paint. To control microbial growth, all the walls, ceilings, and floors should be smooth and washable. A resistant sealing and jointing materials are used in areas where acid or alkaline chemicals or cleansing fluids are applied.

   Doors and windows should be fit flush with walls, thus the entry of dust particles and microorganisms can be prevented. The windows of the manufacturing areas should only allow the entry of light, and should not be used for ventilation.

12.1.5. Assessment of Microbial Contamination and Spoilage

Microbial contamination and spoilage are assessed by the following methods:

1) **Physical and Chemical Changes:** Microbial contamination and spoilage in various pharmaceutical formulations can be identified by the physical and chemical changes (like change in viscosity, pH, emulsion stability, and loss of surface activity) occurring in it. Amount of oxygen consumed by the available product indicates the degree of oxidative attack and microbial growth.

2) **Sterility Test:** It is essential that materials subjected for sterility testing should not be contaminated by the operator or the environment during the testing process. Thus, it is essential to conduct a sterility test by competent and experienced personnel in an adequately clean room with laminar flow cabinet facilities. Sterile preparations (like injectables and ophthalmic preparations) should undergo sterility testing.

3) **Assessment of Viable Microorganisms in Non-Sterile Products:** Viable microorganisms in non-sterile products are tested for detection of pathogens and total viable counts.

4) **Estimation of Pyrogens:** An agent causing rise in temperature when injected into a patient is known as pyrogen. The lipopolysaccharides and lipoproteins which are chief constituents of the cell wall of gram-negative bacteria are called endotoxins. These endotoxins are commonly known as pyrogens. Pyrogens are detected by two main procedures:
   i) In **BP pyrogen test**, pyrogens are injected in laboratory rabbits and their body temperature is monitored for a specific period of time.
   ii) In **Limulus Amoebocyte Lysate test (LAL)**, the pyrogen-containing sample causes gel formation in the lysis product of amoebocyte cells of the giant horseshoe carb (*Limulus polyphemus*).

12.1.1. Preservation of Pharmaceutical Products Using Antimicrobial Agents

Antimicrobial preservatives are added to non-sterile dosage forms to protect them from microbial growth or from microorganisms that are introduced inadvertently during or after the manufacturing process. Antimicrobial preservatives should be added if sterile articles are packaged in multiple-dose containers, thus they inhibit the growth of microorganisms introduced from repeated withdrawal of individual doses.

Preservatives are natural or synthetic chemicals added to prevent decomposition by microbial growth or by undesirable chemical changes in various products like foods, pharmaceuticals, paints, biological samples, wood, etc. Thus, preservatives are added to various foods and pharmaceuticals in order to enhance their shelf-life. Chemical preservatives do not totally keep products free from getting spoiled by microbes, rather they slow down the spoiling process. Preservatives are not commonly added in frozen and canned foods. In processed foods, the harmful bacterium which may contaminate the food is killed by different processes, which are assumed to be beneficial for preserving the products, but they can also add harmful substances.

During the processing of natural food, it is crushed, heated, and added with chemicals that may destroy all the nutritional value of the food. Often, additives are added to the products to put back some vitamins and nutrients that were lost when the food products were processed.

Preservatives are added to keep the food fresh and free from spoiling. Some commonly used **natural preservatives** are vinegar, salt, and sugar. Most of the synthetic food and cosmetic preservatives are considered safe but some of them were found to be carcinogenic and toxic, and therefore have limited uses. Generally, all synthetic chemical additives and preservatives may be avoided because most of them are not tested properly.

The food colouring chemicals have harmful effects, therefore use of food colourings made from synthetic chemicals should be avoided. The efficiency of antimicrobial preservatives depends on their ability to kill live cells; thus their toxicity is unavoidable. Many plants and compounds of natural origin (like essential oils) have the power to destroy bacteria, yeast, and fungi; however in many cases these substances produce toxic effects to humans. **For example,** citrus or grapefruit seed extracts have natural antimicrobial properties and some of their constituents are responsible for life-threatening hormonal imbalances.

An **antimicrobial agent** should possess the following **ideal characteristics**:

1) It should be efficient against many species of microorganisms.
2) It should possess high water solubility or easy dissolution in commonly used cosmetic ingredients.
3) It should be safe and should cause no irritation.
4) It should be neutral with no effect on product pH.
5) It should not have any effect on product efficiency.
6) It should not have any adverse effect on product appearance.
7) It should be stable over wide range of temperature and pH.
8) It should be available readily and its supply should be stable.
9) It should be economical.

Table 12.1 enlists some antimicrobial agents used in pharmaceutical products:

Table 12.1: Antimicrobial Agents Currently Used in Pharmaceutical Products

1)	Single-dose multiple-dose injections Chlorocresol (0.1%) Cresol (0.3%) Phenol (0.5%) Chlorbutol (0.5%)	Benzylalcohol (1.0%) Phenylmercuric nitrate, acetate, or borate (0.002%)
2)	Eye drops and contact lens solutions Chlorhexidine acetate or gluconate (0.1%) Benzalkonium chloride (0.3%)	Thiomersal (0.1%) Chlorbutol (0.5)
3)	Oral liquids Methyl, ethyl and propyl *p*-hydroxybenzoate (parabenz) (0.3%) Benzoic and sorbic acid (0.3-0.5%) Chloroform (0.25%) Bronopol (0.5%)	
4)	Creams Parabenz (0.3%) Chlorocresol (0.1%) Bronopol (0.5%)	Cetyltrimethylammonium bromide (1.0%) Phenylmercuric nitrate (0.01%)

12.1.2. Evaluation of Microbial Stability of Formulations

Microbial stability testing involves the following five steps:

1) **Sampling**
   i) The number of containers to be used and volume to be tested are specified in the Pharmacopoeia.
   ii) Samples of aseptically manufactured product are collected in the beginning of, during, and end of batch fill, and also after any kind of interventions and stoppages.
   iii) Samples from terminally sterilised product are collected from previously identified cool spots within load methods as specified in the Pharmacopoeia.

iv) If the product can be filtered, membrane filtration method is preferred, while direct inoculation is used as an alternative method.

2) **Growth Media**

i) Soybean Casein Digest (SCD) medium and Fluid Thioglycollate Medium (FTM) are used for the detection of aerobic and anaerobic organisms.

ii) The efficiency of media to support growth of a range of low numbers of organisms in the presence of product is demonstrated by validation studies.

iii) Bacterial growth should occur after 3 days, and growth of moulds should occur after 5 days.

iv) Media is either procured or made in-house by validated sterilisation techniques.

v) Before using the media, its growth supporting qualities (for low number of organisms) should be tested.

vi) Batch number and shelf-life of the media should be allotted.

Incubation Period

The SCD media is incubated at 20-25°C and the FTM at 30-35°C, both for 14 days in test containers, which should be periodically examined.

Negative Test Controls

i) Before using the media, either a portion of it or the complete batch should be incubated for 14 days. Incubation may be done simultaneously with the test.

ii) The negative product control items identical in type and packaging to actual product under test should be included in each test session. This makes interpretation of test results easy.

Positive Test Controls

i) Bactiostasis/fungistasis test should prove that media can support the growth of range of low numbers of organisms in the presence of product.

ii) Bacterial growth should occur after 3 days, and that of moulds should occu after 5 days.

Results

Any type of growth identified (Genotypic) by automated/semi-automated system should be verified from time-to-time using reference strains.

Interpretation and Repeat Tests

i) No contaminated units should be found.

ii) A test is repeated when it proves out to be invalid due to reasons dissimilar the product under test.

3) **Methods of Evaluation**

i) **Method A (Membrane Filtration)**

a) The sample is collected and diluted as per the requirement.

b) A suitable nutrient or culture medium is selected. The broth is dispensed a sterile petri dish. As a result, the absorbent pad gets uniformly saturated

c) The forceps are passed through flame and then used for removing membrane from the sterile package.

d) The membrane filter is placed in the funnel assembly.

e) The pouring lip of the sample container is flamed, after which the sampl poured in the funnel.

f) The vacuum is turned on and the sample is completely drawn through the filter.
g) The funnel is washed with sterile buffered water.
h) The vacuum is turned on and the liquid is completely drawn through the filter.
i) The forceps are again passed through flame and the membrane filter is removed from the funnel.
j) The membrane filter is then placed in the prepared petri dish.
k) The dish is incubated at suitable temperature for suitable time period.
l) After incubation the number of colonies formed on the plate is counted under a microscope with 10-15X magnification.
m) The presence of colonies is confirmed and the results are reported.

A care should be taken to maintain aseptic conditions during the test.

ii) **Method B (Direct Inoculation)**

a) The contents of the container(s) to be tested are transferred to the culture medium.
b) A small number of viable microorganisms (not more than 100CFU) are inoculated to the medium.
c) A growth promotion test is carried out as a positive control.
d) All the medium containing containers are incubated for not more than 5 days.
e) If after incubation the microbial growth is clearly visible, this growth is visually compared to that in the control vessel (without the product). Either the product exhibits no antimicrobial activity under the conditions of the test or the activity has been eliminated.
f) The sterility test is then performed without any modification.
g) If microbial growth is not clearly visible in the presence of the product to be tested, this growth is visually compared to that in the control vessel (without the product). The product possesses antimicrobial activity that has not been eliminated under the conditions of the test.
h) The conditions are further modified to eliminate the antimicrobial activity and the method suitability test is repeated.

The method suitability test is performed due to any one of the following reasons:

a) When the sterility test has to be carried out on a new product, or
b) When the experimental conditions of the test are changed.

The method suitability test is simultaneously performed with the sterility test of the product to be examined.

Sterility Test of the Product to be Examined

a) Sterility test is performed using either the membrane filtration method or by direct inoculation of the culture media with the product to be examined.
b) Appropriate negative controls are used.
c) Membrane filtration method is used when the product nature permits for filterable aqueous preparations, for alcoholic or oily preparations, and for preparations miscible with or soluble in aqueous or oily solvents. It is important to notice that these solvents should not show their antimicrobial effect on the test conditions.

4) **Endotoxin Test:** Endotoxin is a lipopolysaccharide found in the cell wall of gram-negative bacteria. The raw materials, water for injection used in manufacture, and the finished product should undergo endotoxin test, which is of two types:
   i) Gel clot (limit test), and
   ii) Gel clot (semi-quantitative test).

   The equipment used for endotoxin test should be free from any endotoxins.

   Validation of accuracy and dependability of the test method for each product is:
   i) Gel clot method,
   ii) Original method,
   iii) The official "referee test",
   iv) The sample is incubated with LAL of known sensitivity. A gel clot formation shows positive endotoxin test, and
   v) Gel clot is a quantitative method as it quantifies bacterial endotoxins in the test solution by titration to an end point.

5) **Microbial Limit Test (Test for Specified Microorganism)**
   i) This test is designed for determining whether or not a preparation fulfils the established specifications of microbiological quality.
   ii) Total Viable Aerobic Count (TVAC).
   iii) Total Aerobic Microbial or Bacterial Count (TAMC or TABC).
   iv) Trypticase Soy Agar (TSA).
   v) Total Yeast and Mound Count (TYMC or Total Fungal Count).
   vi) Sabouraud Dextrose Agar (SDA).
   vii) TVAC = TAMC + TYMC.
   viii) Report – ml or gm.

12.2. SUMMARY

The details given in chapter can be summarised as follows:

1) **Microbial spoilage** is the process of deterioration of pharmaceutical products by the contaminant microbes.
2) A pharmaceutical product is considered to be microbiologically spoiled due to:
   i) Presence of low levels of acutely pathogenic microbes or higher levels of opportunist pathogens,
   ii) Presence of toxic microbial metabolites even after death or removal of initially present microorganisms, and
   iii) Any detectable physical or chemical changes in the product.
3) Spoilage may be of the following types:
   i) Infection induced by contaminated pharmaceutical products,
   ii) Chemical and physicochemical deterioration of pharmaceutical products,
   iii) Ingredients subject to microbial attack, and
   iv) Observable effects of microbial attack in pharmaceutical products.
4) Following factors affect the microbial spoilage of pharmaceutical products:
   i) Size of inoculum,
   ii) Nutritional factors,

iii) Shelf-life of product,
iv) Moisture content,
v) Redox potential,
vi) Storage temperature,
vii) pH value,
viii) Package design, and
ix) Protection of microorganisms within pharmaceutical products.

5) **Water activity** (Aw = Vapour pressure of solution/Vapour pressure of water at same temperature) is a measure of available water. Water activity is inversely proportional to solute concentration; therefore greater the solute concentration, lower is the water activity.

6) Various sources and types of microbial contamination are:
i) Atmosphere,
ii) Water,
iii) Raw materials,
iv) Process operators,
v) Equipment,
vi) Packing, and
vii) Buildings.

7) Microbial contamination and spoilage are assessed by the following methods:
i) Physical and chemical changes,
ii) Sterility test,
iii) Assessment of viable microorganisms in non-sterile products, and
iv) Estimation of pyrogens.

8) In **BP pyrogen test**, pyrogens are injected in laboratory rabbits and their body temperature is monitored for a period of time.

9) In **Limulus Amoebocyte Lysate test (LAL)**, the pyrogen-containing sample causes gel formation in the lysis product of amoebocyte cells of the giant horseshoe carb (*Limulus polyphemus*).

10) **Preservatives** are natural or synthetic chemicals that are added to prevent decomposition by microbial growth or by undesirable chemical changes in various products like foods, pharmaceuticals, paints, biological samples, wood, etc.

11) Some commonly used **natural preservatives** are vinegar, salt, and sugar.

12.3. EXERCISE

12.3.1. True or False

1) Vinegar, salt, and sugar are examples of natural preservatives.
2) In Limulus Amoebocyte Lysate test (LAL), the pyrogen-containing sample causes gel formation in the lysis product of amoebocyte cells of the giant horseshoe carb.
3) Water activity is directly proportional to solute concentration; therefore greater the solute concentration, lower is the water activity.
4) Microbial spoilage is the process of deterioration of pharmaceutical products by the contaminant microbes.
5) For the preparation of pharmaceutical products, deionised water, distilled water, and Water for Injections are used.

12.3.2. Fill in the Blanks

6) The preservatives are _____ or _____ chemicals that are added to prevent decomposition by microbial growth or by undesirable chemical changes in various products.

7) In_____, pyrogens are injected in laboratory rabbits and their body temperature is monitored for a period of time.

8) For cleaning pipelines, plates and tubular types of heat exchange units, pumps, homogenisers, and all larger equipment, _____ is used.

9) Water activity is a measure of ___ water and is ____ proportional to solute concentration.

Answers:

1) True 2) True 3) False 4) True 5) True
6) Natural, synthetic 7) BP pyrogen test
8) 'in-place' cleaning system 9) Available, inversely

12.3.3. Very Short Answer Type Questions

1) What is water activity?
2) Enlist the factors affecting the microbial spoilage of pharmaceutical products.
3) Give the sources and types of microbial contaminants.
4) What is BP pyrogen test?
5) What is Limulus Amoebocyte Lysate test (LAL)?
6) Give ideal characteristics of antimicrobial agents.
7) What are surfactants? Give examples.

12.3.4. Short Answer Type Questions

1) Write a note on factors affecting microbial spoilage of pharmaceutical products.
2) Give the assessment of microbial contamination and spoilage.
3) Write a note on pharmaceutical products using antimicrobial agents.
4) Write a note on sources and types of microbial contaminants.

12.3.5. Long Answer Type Questions

1) What is spoilage? Write down the factors affecting the spoilage of pharmaceutical products.
2) Write a note on evaluation of microbial stability of formulations.
3) Write a detail note on types of spoilage.

CHAPTER 13 Animal Cell Culture

13.1. CELL CULTURE

13.1.1. Introduction

The process of removal of cells from an animal or plant, and their subsequent growth in a favourable artificial environment is referred to as **cell culture**. The cell to be cultured may be obtained from the tissue directly and disaggregated by enzymatic or mechanical means before cultivation, or they may be obtained from a cell line or cell strain that has already been established.

The culture media used to culture animal cells should deliver nutritional, hormonal, and stromal factors. For the growth and maintenance of *in vitro* animal cell, the culture conditions should be similar to *in vivo* conditions with respect to temperature, oxygen and carbon dioxide concentration, pH, osmolality, and nutrition.

Vertebrate cells can be cultured using a media rich in essential amino acids, vitamins, peptides, or protein growth factors, and frequently provided by serum. Therefore, tissue culture media used in the previous times were made up of biological fluids such as plasma, lymph, serum, and tissue extracts of embryonic origin. Most of the cultured vertebrate cells grow when attached to negatively charged substratum, identical to the extracellular matrix of animal tissues.

Following **types of cells** are involved in the animal cell culture:

1) **Primary Cells:** These cells are derived from animal tissues, grow limitedly in culture, and give rise to a cell strain.

2) **Transformed Cells:** These cells are derived from animal tumours or arise from primary rodent cells, and grow indefinitely in culture. They have an unstable, aneuploid complement of chromosomes, including abnormal chromosomes. Transformed cells derived from a single parental cell are known as **cell lines**.

3) **Cultured Cells:** These cells are treated with certain viruses or polyethylene glycol to be fused into heterokaryons (hybrids between cells of different species, which lose the chromosomes of one species on dividing).

Culture medium is the most essential factor for culturing cells and tissues. A **cell culture medium** is comprised of the following **two parts**:

1) **Basal Medium:** It supports the cellular requirements for nutrients, salts, and pH control.

2) **Set of Supplement:** It supports other cellular requirements and allows the cells to grow in the basal medium.

Culture medium provides:

1) Optimum conditions of factors like pH, osmotic pressure, etc., and
2) Chemical constituents which cannot be synthesised by cells.

13.1.2. Growth of Animal Cells in Culture

Animal cell culturing is more difficult than microbial culturing because animal cells demand more nutrients and should be attached to specially coated surfaces for their growth. Still there are various undifferentiated and differentiated animal cell types which have been cultured.

The nine amino acids, known as the **essential amino acids** (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine), are not synthesised by adult vertebrate animals and therefore must be obtained from external sources. Apart from these, most of the cultured cells require cysteine, glutamine, and tyrosine also, synthesised by the intact animal cell, **e.g.,** liver cells synthesise tyrosine from phenylalanine; liver and kidney cells synthesise glutamine. All animal cells both within the organism and in culture can synthesise the remaining eight amino acids; therefore these amino acids need not be present in the diet or culture medium. Other essential components of media of the animal cell culture are **vitamins** (cannot make at all or in adequate amounts), various salts, glucose, serum, and the non-cellular part of the blood.

Most of the cultured animal cells require special solid surfaces for growth. Within the intact animal tissues, the cells are tightly packed and interact with other cells through the cellular junctions. The cells are also in contact with the extracellular matrix (a complex network made up of secreted proteins and carbohydrates to fill the intercellular spaces). The constituents of matrix secreted by the cells participate in binding the cells in tissues together; as well as it also provides a lattice through which cells can move, especially at the early phase of animal differentiation.

In various animal tissues, the **extracellular matrices** consist of several common **components**:

1) Fibrous collagen proteins,
2) Hyaluronic acid,
3) A large mucopolysaccharide,
4) Covalently linked polysaccharides,
5) Proteins in the form of proteoglycans (mostly carbohydrate), and
6) Glycoproteins.

The exact matrix composition is different in different tissues, thus giving a specialised function to each tissue. **For example**, the extracellular matrix in connective tissue mainly contains a type of collagen protein which forms insoluble fibres exhibiting a very high tensile strength. Fibroblasts, the major cells of connective tissue, secrete this type of collagen and other components of the matrix. Receptor proteins present on the plasma membrane of a cell bind various matrix elements, and provide strength and rigidity.

Given below are the **conditions** need to be maintained for the growth of animal cells:

1) **Balanced Salt Solution (BSS):** It is made up of inorganic salts which maintain the osmotic pressure and buffer medium at physiological pH. Some inorganic ions, **e.g.,** Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, SO_4^{2+}, PO_4^{3+}, etc., are also required to stimulate the process growth.
2) **Buffering Systems:** These are required to compensate for CO_2 evolution and lactic acid production from carbohydrate metabolism. Bicarbonates are used as a buffer for the media.

3) **pH:** Neutral pH value (i.e., 7) is considered to be optimum for the growth of cells. It facilitates inspection and control of cultures.

4) **Energy Sources:** The major source of energy is carbohydrates which initiate growth in cultured cells. The most commonly used sugar is glucose; while others like maltose, sucrose, fructose, galactose, and mannose are also used.

5) **Amino Acids:** Arginine, cysteine, histidine, isoleucine, lysine, etc., are the essential amino acids required for the growth of most animal cells.

6) **Vitamins:** Biotin, choline, folic acid, nicotinic acid, panthoic acid, pyridoxal, riboflavin, thiamine, and inositol are some of the vitamins required for the growth and multiplication of animal cells.

7) **Hormones and Growth Factors:** Hormones and growth factors are essential for a variety of different effects on the survival and proliferation of cells. Insulin and hydrocortisone are the most commonly used ones. Also, interleukins, colony stimulating factors, epidermal growth factor, and fibroblast growth factor are required.

8) **Proteins and Peptides:** Fetuin, fibronectin, albumin, and transferrin are the protein supplements commonly used for stimulating animal cell proliferation and growth.

9) **Fatty Acids and Lipids:** These are important components of some serum-free media, but their effect has not been yet established.

10) **Accessory Factors:** Some additives like iron, zinc, copper, and selenium trace elements also aid in effective cell growth.

13.1.3. General Procedure for Cell Culture

The cell culture methods are either applied to free-living organisms (bacteria or eukaryotic microorganisms), or to cells removed from multicellular tissues. Cells can be cultured for a prolonged time if they split regularly. After that the growth medium is replaced and the cells are diluted (after first detaching them by trypsin of NaOH from the support).

For culturing cells efficiently, the environment in which they found themselves should be maintained before they are transplanted to the artificial environment (an extracellular fluid derived from blood). This extracellular fluid is recreated using Ham's tissue culture medium (commonly for mammalian cells).

Some cells (like those in bloodstream) survive without attaching to a surface; while others (like those derived from solid tissues) require a special surface. There are also cells like yeast and some bacteria types which survive under either condition and exhibit different phenotypes depending on whether or not they are attached to a surface. In most of the cells derived from tissues, nutrients are provided by a liquid broth that washes the cells attached to a surface. Some cells require an "air-liquid interface" for their growth; the cells, in this case, are grown on a raft of organic material floating on the surface of a nutrient broth and acting like a wet sponge to feed the cells from underneath while the tops are exposed to the air.

For bacteria and yeast, small quantities of cells are grown on a solid support enriched with nutrients embedded in its stiff nutritious Jello, while large-scale cultures are grown with the cells suspended in a nutrient broth.

A population of cells derived from a single parental cell is called a **clone**, which may be derived from continuous cell lines or from primary culture. Cloning within a cell population reduces the degree of genetic and phenotypic variation.

Initiation of cell culture can be discussed under the following heads:

1) Preparation and sterilisation of the substrate and culture vessel,
2) Preparation and sterilisation of the medium,
3) Isolation of cells and tissues,
4) Disaggregation of tissues,
5) Primary cell culture,
6) Sub-culture,
7) Cloning of cell lines, and
8) Contamination in cell culture.

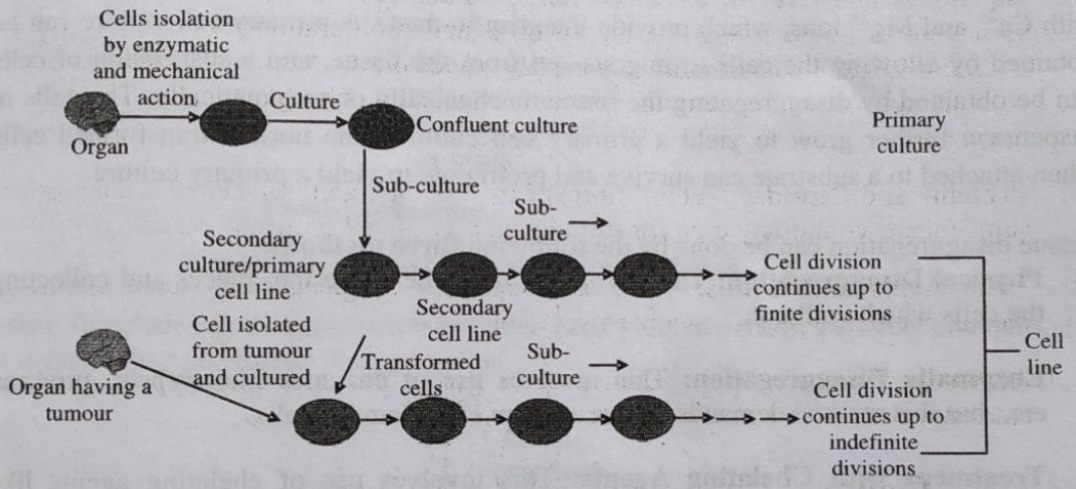


Figure 13.1: Animal Cell Culture

13.1.3.1. Preparation and Sterilisation of the Substrate and Culture Vessel

The **glassware** used for culturing should be soaked overnight in non-toxic detergent, followed by thorough washing with tap water and then with distilled water. Thereafter, they are kept in containers or wrapped in aluminium foil followed by dry heat sterilisation; while the screw caps are sterilised by autoclaving separately. The quality of glassware washing and sterilisation should be timely monitored.

Plastic wares are an alternative to glass which are available in pre-sterilised and ready to use forms; thus do not require any treatment. However plastic wares have a **disadvantage** of their high cost as they are not reusable and should be disposed off.

13.1.3.2. Preparation and Sterilisation of the Medium

The constituents of culture media and the reagents used for culturing should be sterilised by either **autoclaving** or **filtration**. Autoclaving is preferred more as it is economic, uniformly effective, and requires less labour.

Nowadays culture media available in the market come in pre-sterilised form. Water, salts, supplements like peptone or trypsin, etc., being thermostable constituents are autoclaved at 12°C for 20 minutes; while serum trypsin, and proteins are thermolabile constituents and should be sterilised by filtering through a membrane filter having porosity of 0.2mm.

13.1.3.3. Isolation of Cells and Tissues

In general, embryos and other contents of the uterus are free from contamination; while in adult tissues, bacterial contamination may be common. Before isolation of the tissue, the site is sterilised with **70% alcohol** and tissues are removed aseptically. The isolated tissues are transferred to **Balanced Salt Solution** (BSS), **e.g.,** EBSS or HBSS supplemented with antibiotics or to a suitable culture medium. Care should be taken not to disrupt ethical or legal boundaries in obtaining the desired explant.

The isolated tissues should be stored under refrigeration before transferring them to a culture medium, as viable cells may be recovered from chilled tissue several days after explanation.

13.1.3.4. Disaggregation of Tissues

Some tissues are made up of tightly aggregated cells. Epithelial tissues are impregnated with Ca^{2+} and Mg^{2+} ions, which provide integrity to them. A primary cell culture can be obtained by allowing the cells to migrate out from the tissue, and a suspension of cells can be obtained by disaggregating the tissue mechanically or enzymatically. The cells in suspension further grow to yield a primary cell culture. The normal transformed cells when attached to a substrate can survive and proliferate to yield a primary culture.

Tissue disaggregation can be done by the following **three methods**:

1) **Physical Disaggregation:** This involves slicing the tissue into pieces and collecting the cells which spill out.

2) **Enzymatic Disaggregation:** This involves use of enzymes like trypsin, pronase, etc.; but this method demands labour and can even damage cells.

3) **Treatment with Chelating Agents:** This involves use of chelating agents like citrate, and EDTA for producing suspension of cells from established cultures.

Physical (Mechanical) Disaggregation

The physical disaggregation method is a **force based technique**. In this method, the tissue is aseptically removed with much care from a given spot and kept in a sterile petri dish containing buffered medium with balanced salt solution. The spilled cells are passed through sieves of decreasing pore size (50µm to 20mm mesh). The process can be repeated if more disaggregation of cells is required.

The debris left on sieves is discarded and the medium containing cells is collected, followed by counting the cells with a haemocytometer. The medium can also be diluted with serum if one wants to increase the level of cells up to 10^4cells/ml (**figure 13.2**). The cells can also be collected using **syringe needles**. Sometimes cells are repeatedly pipetted, thus causing mechanical damage to the cells.

The viable cells so obtained are called **primary cells**, which are seeded on the culture medium in high density to allow their growth. Thus primary viable cells of primary cell culture are called **adherent cells** and the culture is called **adherent culture**. In case some non-viable cells also grow along with the adherent cells, they are separated using a second medium. In the same manner, primary culture can be grown in suspension; and the non-viable cells are removed from primary disaggregates in suspension by centrifugation using Ficoll and sodium.

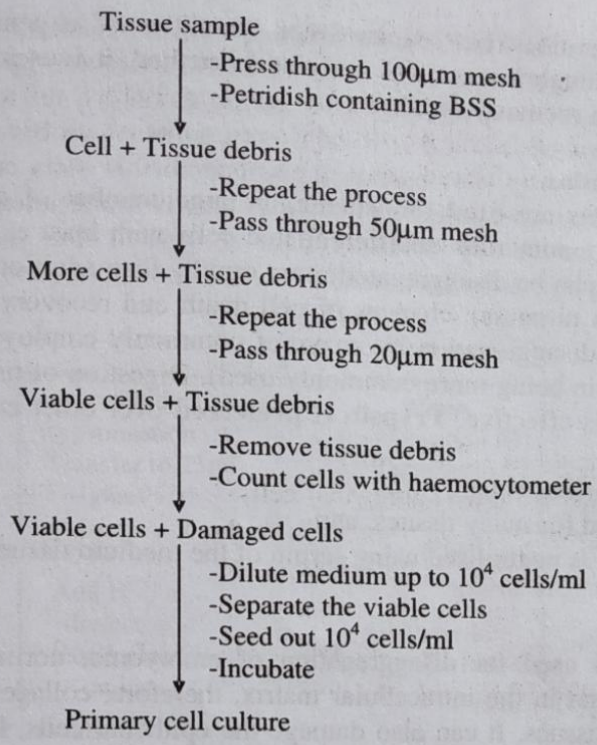


Figure 13.2: Physical Disaggregation of Tissues

Physical (mechanical) disaggregation provides good yield of cells in a shorter time period as it is faster than enzymatic disaggregation.

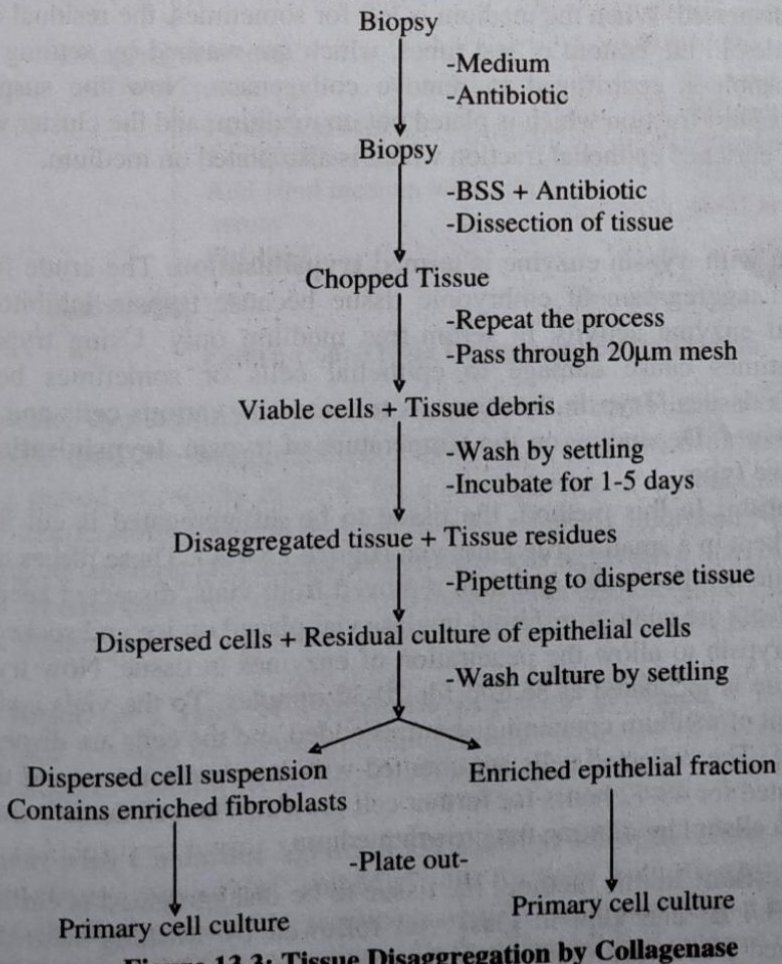


Figure 13.3: Tissue Disaggregation by Collagenase

Physical disaggregation does not require large quantities of expensive enzymes for isolation of cells. Although it is a quick and **cheap** method, it causes **more damage to cells**, thus **lowering the recovery rates**.

Enzymatic Disaggregation

In this method, enzymes are used for isolating a large number of cells from tissues. Embryonic tissues carry numerous undifferentiated cells with least extracellular matrix; thus embryonic tissues can be disaggregated more rapidly than adult or new born tissues. In fragile tissues (as in tumours), chances of cell death and recovery are more than in normal tissues. Tissue disaggregation by enzymes commonly employs **collagenase** and **trypsin** enzymes (trypsin being more commonly used). Digestion of tissue by incubation in proteolytic enzyme is effective. **Trypsin is preferred** over other enzymes due to the following **reasons**:

1) It is tolerable by various cells,
2) It is effectively used for many tissues, and
3) Its residual activity is neutralised using serum of the medium tissue.

Use of Collagenase

Collagenase enzyme is used for disaggregation of embryonic, normal, and malignant tissues. Collagen is found in the intracellular matrix, therefore, collagenase disaggregates normal and malignant tissues. It can also damage the epithelial cells, but not the fibrous tissues. Some non-specific proteases are also present in crude collagenase. The method involves cutting the tissue to be disaggregated into pieces in basal salt solution containing antibiotics (**figure 13.3**). The tissue pieces are washed with sterile distilled water and transferred in a medium containing collagenase. After 5 days the mixture is pipetted out so that the medium get dispersed. When the medium is left for sometimes, the residual culture of epithelial cells settles in the bottom of test tubes, which are washed by settling or the dispersed cell suspension is centrifuged to remove collagenase. Now the suspension contains enriched fibroblast fraction which is plated out on medium; and the cluster washed by settling consists of enriched epithelial fraction which is also plated on medium.

Use of Trypsin

Tissue disaggregation with trypsin enzyme is termed **trypsinisation**. The crude form of trypsin is used for disaggregation of embryonic tissue because trypsin inhibitors can neutralise its residual enzyme activity in serum-free medium only. Using trypsin for disaggregation sometimes cause damage to epithelial cells or sometimes becomes ineffective for fibrous tissues. Trypsin, however, is tolerated by various cells and is also effective for many tissues. Depending on the temperature of trypsin, **trypsinisation** may be of the following **two types**:

1) **Cold Trypsinisation:** In this method, the tissue to be disaggregated is cut into 2-3 small pieces and kept in a small sterile glass vial (**figure 13.4 A**). These pieces are then washed with sterile distilled water and then removed from vials, dissected keeping on BSS. All the contents are again transferred in glass vial placed on ice and soaked for 4-6 hours in cold trypsin to allow the penetration of enzymes in tissue. Now trypsin is removed and tissue is incubated at 36.5°C for 20-30 minutes. To the vials containing tissue pieces, 10ml of medium containing serum is added and the cells are dispersed by repeated pipetting. The collected cells are counted with haemocytometer and then are plated and incubated for 48-72 hours for further cell growth. The cell density should be maintained to 10^4cells/ml by diluting the growth medium.

2) **Warm Trypsinisation:** In this method, the tissue to be disaggregated is cut into 2-3 pieces (**figure 13.4 B**) and kept in glass vial followed by washing with distilled water. The washed pieces are then transferred to a 250ml flask containing 100ml

warm trypsin (maintained at 36.5°C temperature). The flask contents are stirred for 4 hours, and the pieces are allowed to settle. The dissociated cells are treated with warm trypsin. After 3-4 hours during which complete tissue may be disaggregated, trypsin is removed by centrifugation. The dissociated cells are collected after every 30 minutes. The glass vials containing dispersed cells in medium are placed on ice. After the trypsinisation time, samples are pipetted, cells are counted with haemocytometer, cell density is maintained to 10^4cells/ml, cells are plated on medium, and finally incubated for 48-72 hours for further cell growth.

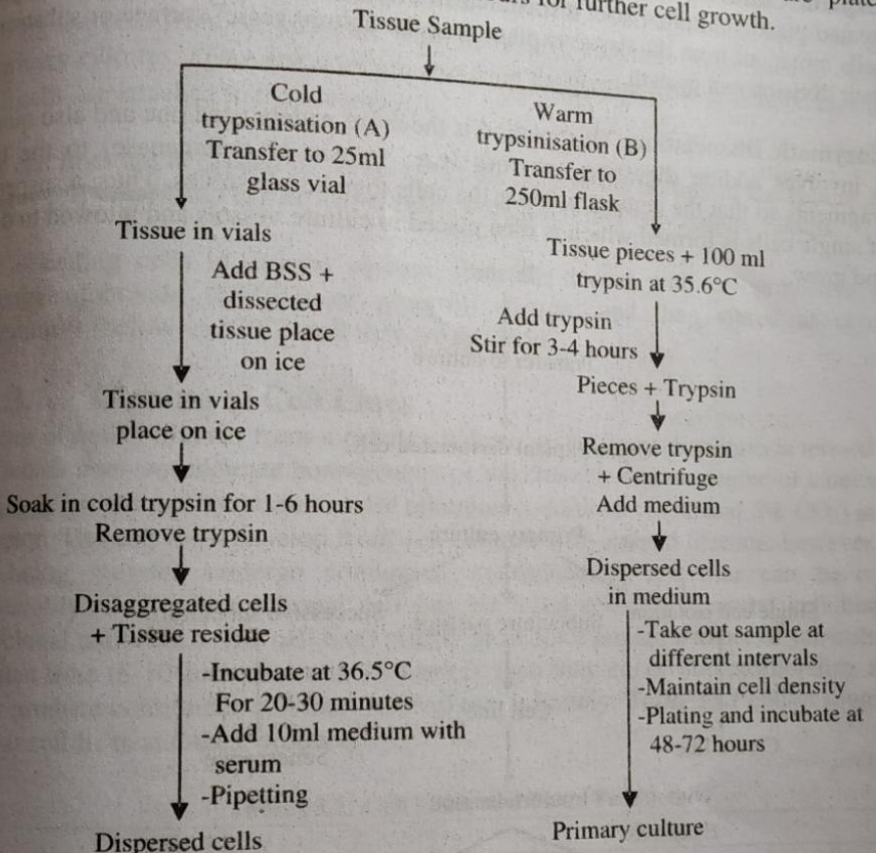


Figure 13.4: Tissue Disaggregation by Trypsin

Elastase, hyaluronidase, pronase, dispase, or a combination of these enzymes can also be used for disaggregation. Digestion is performed either slowly at 4°C for a long period or rapidly at 37°C for a short period, or a combination of the two. At a low temperature, less cell damage occurs, resulting in higher yields. Tissue digestion using collagenase and dispase enzymes is less aggressive than digestion using trypsin and pronase enzymes; thus less cell damage occurs. Enzymatic disaggregation results in a higher yield of representative cells within a short time period.

Perfusion is a **type of enzymatic digestion** in which a proteolytic solution is pumped through the tissue and the cells are collected in the enzyme solution. This technique is used for isolating cells from liver.

Treatment with Chelating Agents (EDTA, Ethyl Diamine Tetra Acetic Acid)

Epithelial tissues require Ca^{2+} and Mg^{2+} ions for their integrity maintenance. Such tissues can be easily disaggregated by treatment with EDTA prepared in a balanced salt solution. This technique is used for separating cells from established cultures of epithelial cells.

13.1.3.5. Primary Cell Culture

The surgically removed cells of an organism attach, divide, and grow when placed into a suitable culture environment; the resultant culture is the **primary culture**. These cultures are obtained from intact or dissociated tissues or organ fragments (**figure 13.5**). After a primary culture is sub-cultured, it is termed as **cell line**. This can be done by the following **two ways**:

1) **Explant Cultures:** In this method, small pieces of tissue attached to a glass or to a treated plastic culture vessel are bathed in a culture medium. After a few days, the cells move out from the tissue explant onto the culture vessel surface or substrate for their division and growth.

2) **Enzymatic Dissociation:** This method is the more widely used one and also quick as it involves adding digesting enzymes (**e.g.,** trypsin or collagenase) to the tissue fragments so that the cement holding the cells together dissolves. Thus, a suspension of single cells is formed which is then placed in culture vessels and allowed to divide and grow.

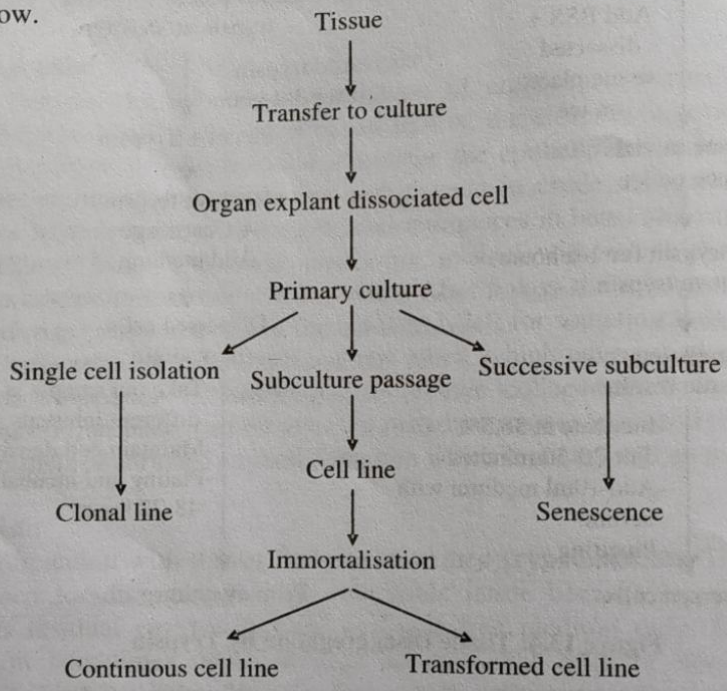


Figure 13.5: Culturing of Animal Cells

Primary cultures do not contain variety of cells present in the tissue because they cannot attach to the substrate and survive. The dead cells get easily eliminated when the medium is poured out during sub-culture. After some time, the available substrate is occupied by the monolayer. Then the cells are harvested from primary culture.

Advantages

1) **Capacity for Biotransformation:** In many cases, primary cell culture metabolism has greater similarity to *in vivo* than that to sub-cellular fractions used as an exogenous source for biotransformation.

2) **Tissue-Specific Functions:** The primary culture retains tissue-specific functions. **For example**, on exposing primary cultures of rat myocardial cells (having synchronously beating cells) to tricyclic cardiotoxic antidepressants, beating is observed.

The main **disadvantages** of the primary cultures are the necessity to isolate cells for each experiment, and the procedure followed for isolation disrupts the tissues, along with proteolytic enzymes. Thus, specific membrane receptors are damaged or lost, integrity of the membrane is damaged, and cellular products are lost.

13.1.3.6. Sub-Culture (Secondary or Continuous Cell Culture)

After the cells in primary culture vessel have grown and occupied the entire culture substrate, they are sub-cultured for further growth. Sub-culturing involves gently removing the cells from the substrate with the help of enzymes (those used in obtaining the primary culture). These enzymes are also used for breaking the protein bonds through which cells are attached to the substrate.

Some cell lines are harvested by scraping off the cells in the bottom of the culture vessel. Then the cell suspension can be sub-divided and placed into new culture vessels.

After obtaining cells in desired amount, they are treated with cryoprotective agents [Dimethylsulphoxide (DMSO) or glycerol], frozen, and then stored at cryogenic temperatures (below −130°C) until they are required.

13.1.3.7. Cloning of Cell Lines

A group of cells obtained from a primary culture at the first sub-culture is termed a **cell line** (which does not indicate homogeneity or the characterisation degree of a culture). A cell line is grown and incubated under optimum conditions (37°C and 5% CO_2) in a cell incubator. The cell lines develop from cell cultures with limited lifetime; however, some cells being mutated undergo continuous multiplication, and thus can be cultured considerably. Sometimes, normal cell can be fused with an immortal cell line, **e.g.,** monoclonal antibodies. The cell lines mostly grow for a limited number of sub-cultures in a limited time (8-10 sub-culture in 12 weeks); then they either stop proliferating and die out or produce continuous cell lines to be grown infinitely. **Table 13.1** enlists some of the popular cell lines and their products:

Table 13.1: Cell Lines and Their Products

Cell Lines	Products
Human tumour	Angiogenic factor
Human leucocyte	Interferon
Human kidney	Urokinase
Human kidney cell	Human protein
Dog kidney	Canine distemper vaccine
Cow kidney	Foot and mouth disease
Chick embryo fluid	Vaccines for influenza, measles, and mumps
Duck embryo fluid	Vaccines for rabies and rubella
Mouse fibroblast	Interferon
Cell line of mouse, rat or human origin	Monoclonal antibodies
Chinese hamster cells	Factor VIII, Tissue plasminogen activator

A large number of cell lines derived from human and animal tissues are either finite or continuous cell lines. A cell line is multiplied and divided into:

1) **Seed Stock:** This is characterised and frozen in liquid nitrogen at −196°C. Seed stock ampoules are used to produce working stock so that the cell line changes with time due to genetic instability, selection senescence, or transformation.

2) **Working or Distribution Stock:** This consists of material multiplied from the seed stock and used for further studies or distribution. A **cryopreservation agent** (glycerol) is used for minimising cell injuries during freezing and thawing.

Various types of animal cells, **e.g.,** tumour cells, pigmented melanoma cells, neuroblastoma cells, steroid-producing adrenal cells, growth hormone prolactin secreting cells, teratoma cells (capable of differentiating in artificial conditions), pigmented or cartilage cells, etc. can grow *in vitro*.

A continuous (immortal) cell line can be developed from cultured tissues on the basis of the experimental purpose. Healthy animal tissues which can undergo division are cultured on artificial nutrient media which proliferate and differentiate into heterogeneous mixture of different cell types (**figure 13.6**).

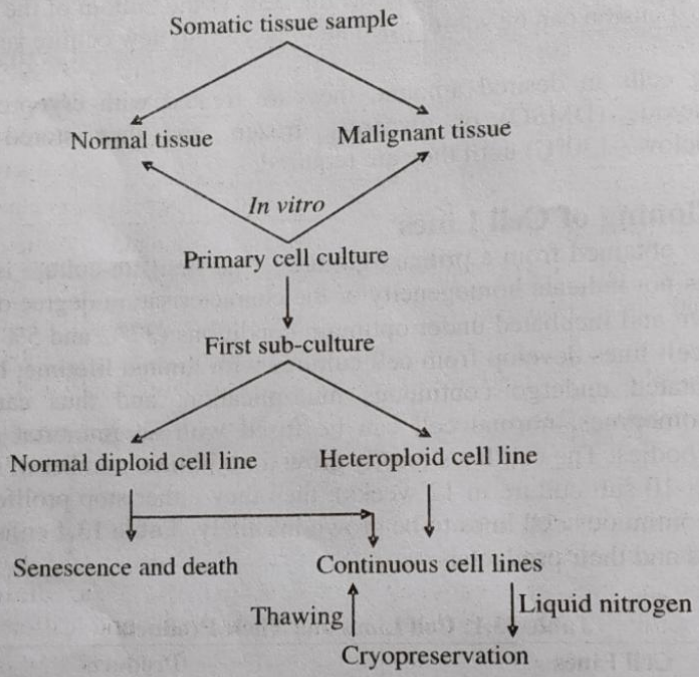


Figure 13.6: Cell Culture from Somatic Tissue

Continuous Cell Lines

In vitro **transformation** is the phenomenon generating continuous cell lines; this process either occurs spontaneously, or is induced by means of chemicals or viruses. Since the continuous cell lines are similar to malignant cells and are sometimes tumerogenic, thus the term **transformation** is used. Continuous cell lines exhibit the following **features**:

1) These cells are smaller, round, less adherent, and have a higher nucleus/cytoplasm ratio,
2) These cells have reduced dependence on serum,
3) These cells have increased cloning deficiency,
4) These cells are capable of growing up to higher cell densities,
5) Many cell lines become malignant,
6) These cells have increased growth ratio, and
7) These cells have aneuploid chromosome.

List of finite and infinite cell lines and cell strains in regular usage are given in **table 13.2**:

Table 13.2: Cell Lines and Cell Strains in Regular Usage

Name	Morphology	Origin	Tissue	Ploidy	Characteristics
Finite Cell Lines					
MRC5	Fibroblast	Human lung	Normal	Diploid	Susceptible to viral infection
MRC9	Fibroblast	Human lung	Normal	Diploid	Susceptible to viral infection
WI38	Fibroblast	Human lung	Normal	Diploid	Susceptible to viral infection
IMR90	Fibroblast	Human lung	Normal	Diploid	Susceptible to viral infection
Continuous Cell Lines					
A9	Fibroblast	Mouse subcutaneous	Neoplastic	Aneuploid	HGPRT –ve; derive L929
BHK21C13	Fibroblast	Syrian hamster kidney	Normal	Aneuploid	Transformable by polyoma virus
BR13A	Epithelial	Rat liver	Normal	Diploid	Produce MSA
CHOK1	Fibroblast	Rat liver	Normal	Diploid	Simple karyotype
EB 3	Lymphotic	Human lung	Neoplastic	Diploid	EB virus +ve
GH1, GH3	Epithelial	Rat liver	Neoplastic	Aneuploid	Produce growth hormone
HeLa	Epithelial	Human lung	Neoplastic	Aneuploid	G6PD Type A
L1210	Lymphotic	Mouse	Neoplastic	Aneuploid	Rapidly growing; suspension

Continuous cell lines develop from normal or tumour tissues by their own. Continuous cell lines have the **advantages** of faster growth rate, low serum requirement, ability to grow in suspension, and independency from cell density. Their **disadvantages** are chromosome instability, loss of tissue specific markers, and difference from the phenotypes of donor tissue. The characteristic features of finite and infinite cell lines are listed in **table 13.3**:

Table 13.3: Finite and Continuous Cell Lines

Properties	Finite	Continuous
Ploidy	Euploid	Aneuploid, heteroploid
Transformation	Normal	Immortal and tumerogenic
Anchorage dependence	Yes	No
Contact inhibition	Yes	No
Mode of growth	Monolayer	Monolayer or suspension
Maintenance	Cyclic	Steady state possible
Cloning efficiency	High	Low
Markers	Low	High
Growth rate	Tissue specific	Chromosomal, enzymic, antigenic
Yield	Low	High
Serum requirement	High	Low
Density limitation of cell proliferation	Yes	Reduced or lost

Evolution of Cell Lines

In single cell suspension, the cell mixtures are used as a **primary** or **starter culture**, which is sub-cultured by transferring into special growth nutrient medium contained in culture vessels at optimal growth conditions.

Thereafter, some cells despite being damaged in suspension attach to the surface and proliferate to yield a single cell line. Thus during sub-culturing, the suspension should be diluted using a certain ratio of fresh medium and then transferred to a flask.

It is only after sub-culturing that a primary culture becomes a cell line. Sub-culturing is a necessity when the nutrient present in medium for cell growth becomes deficient. These may be sub-cultured repeatedly on fresh medium and propagated to maintain continuous growth of cell, thus resulting in multiple copies of a single type of cell.

Now the cell line gets properly evolved and established, and comprises of cells with the ability to proliferate rapidly. So, the unaltered form of cell line propagates in logarithmic ways. Changes in continuous cell line due to the effect of chemicals, spontaneous mutation, or viruses will hinder the increase in cell number. This phenomenon of alteration in a continuous cell line is termed as ***in vitro* transformation**.

A cell line consists of several similar or dissimilar cell lineages. A defined cell lineage with specific properties is termed a **cell strain**, which is identified when bulk cells are present in a culture. The cell line or cell strain is either continuous or finite, based on the culture's lifespan. The remaining cell line may be immortal. A finite cell line has a lifespan of up to 20-80 generations, after which they die.

Factors Affecting Sub-Culture *in Vitro*

Following are the factors influencing the differentiation and proliferation of cells undergoing *in vitro* sub-culture:

1) The mammalian cells need to be attached to a suitable surface.
2) The serum in media is beneficial.
3) Many additives (**e.g.,** cellulose and starch derivatives, protein mixtures, pluronic polyals, polyvinyl pyrolidones, dextrans polyvinyl glycol, polyvinyl alcohols, etc.) are added to provide protection from agitation or aeration damage to the freely suspended animal cells in culture.
4) The pH should be optimum, i.e., 7.0-7.6; and in animal cell culture, the medium is buffered with bicarbonate buffering system.
5) Animal cells are temperature-sensitive, thus a thermostat is used to maintain the temperature of bioreactor medium at 37°C.

13.1.3.8. Contamination in Cell Culture

The cell culture may get contaminated as follows:

1) **Chemical Contamination:** This type of contamination is caused by invisible agents (**e.g.,** endotoxins, plasticisers, metal ions, or traces of chemical disinfectants); thus, is detected with much difficulty.

2) **Biological Contamination:** This type of contamination is caused by fast growing yeast, bacteria, and fungi; and generally has visible effects on the culture (changes in medium turbidity or pH). However, biological contamination by mycoplasmas and viruses demand special detection methods as they cannot be visually detected.

Following are the chief **requirements** for **avoiding contamination**:

1) The cell culturist should be properly trained in aseptic technique.
2) Properly designed, maintained, and sterilised equipment, plastic ware, glassware, and media should be used. Some antibiotics which are designed to be used in tissue culture also prevent biological contamination.

13.1.4. Cell Culture Systems

For growing cells, two basic culture systems are used. These culture systems are mainly based on the ability of the cells to either grow on attached glass or treated plastic substrate (monolayer culture systems) or floating free in the culture medium (suspension culture systems). These two cell culture systems are discussed as follows:

1) **Monolayer Cultures:** These are normally grown on dishes, T-flasks, roller bottles, or multiple well plates treated with tissue culture. The equipment is selected based on the number of cells required, nature of the culture environment, cost, and personal reference. Cell lines derived from normal tissue require a surface where they can attach themselves and grow, thus are **anchorage-dependent**.

2) **Suspension Cultures:** These are grown either:
   i) In magnetically rotated spinner flasks or shaken Erlenmeyer flasks containing cells suspended in the medium, or
   ii) In stationary culture vessel (such as T-flask and bottle) containing cells unable to attach to the substrate.

Some cell lines despite being **anchorage-independent** require a substrate for attachment or float freely in suspension; while some normal blood cells grow in suspension only, without requiring any substrate.

13.1.5. Types of Cultured Cells

The cultured cells are described on the basis of their morphology (shape and appearance) or their functional characteristics are as follows:

1) **Epithelial-Like Cells:** These flat and polygonal-shaped cells are attached to a substrate.

2) **Lymphoblast-Like Cells:** These spherical-shaped cells do not attach to a substrate, but remain free in a suspension.

3) **Fibroblast-Like Cells:** These elongated and bipolar cells, forming swirls in heavy cultures are attached to a substrate.

Note: The culture conditions determine the shape and many cell cultures exhibit multiple morphologies.

The cultured cells are also divided into:

1) **Precursor or Stem Cells:** These cells are also called **totipotent** or **pluripotent** or **multipotent cells**. They can proliferate but not differentiate until they are provided with proper environmental conditions. They can differentiate into one of several kinds of cells only under accurate inducing conditions.

2) **Undifferentiated but Committed Cells:** These cells are derived from stem cells, can proliferate, and also have the ability to differentiate, but still remain undifferentiated.

3) **Mature Differentiated Cells:** These differentiated cells cannot divide.

13.1.6. Transformed Cell Culture

Transformation means a phenotypic change which depends on the uptake of new genetic material. Transformation of cultured cells means a spontaneous or induced permanent phenotypic change due to a genetic change in DNA and gene expression. Transformed cells can be grown easily at a faster rate, can be often grown in suspension, and have extra or abnormal chromosomes.

Transformation of cells results in the following changes:

1) **Genetic Instability:** A cell line does not always remain stable. Normal finite cell lines are genetically stable, but continuous cell lines are unstable because of higher mutation rate *in vitro* and mutant cells cannot be eliminated till their growth capacity is diminished. Thus, genotypic variation also results in phenotypic variation.

2) **Immortalisation:** The normal cells usually have a lifespan of 20-100 generations, but some cells can produce continuous cell lines with an infinite lifespan. They have a growth advantage and subsequent cultures are rapidly overgrown by the randomly growing cell. This is called **tumerogenic cell**.

3) **Aberrant Growth Control:** Cells cultured from tumours, and *in vitro* transformed cultures show aberrations (abnormalities) in growth control, such as growth to higher saturation densities and growth of monolayers of homologous cells. These cell lines slightly depend on the lower serum or growth factor and acquire autonomous growth control by over-expression of oncogenes.

   Growth control is generally autocrine. The transformed cells are anchorage-independent, and have lost the property of cell-cell recognition, contact-inhibition of cell lines, and density limitation of cell proliferation. Transformation is a multi-step process with production of neoplastic cells as its last step.

4) **Malignancy:** The cells having the capacity to generate invasive tumours indicate the condition of malignancy. A large number of properties can be recognised in *in vitro* transformed cells, or cells cultured from *in vivo* tumours, and associated with malignant invasion. Example of such type of transformation is **tumerogensis**.

13.1.7. Advantages of Animal Cell Culture

Animal cell culture has the following advantages:

1) It provides controlled physiochemical environment like pH, temperature, osmotic pressure, O_2, etc.
2) It provides controlled and defined physiological conditions.
3) It achieves homogeneity of cell types through serial passages.
4) It requires smaller quantities of reagents (as compared to that required for *in vivo*), thus is economical.
5) It avoids legal, moral, and ethical questions of animal experimentation.

13.1.8. Disadvantages of Animal Cell Culture

Animal cell culture has the following disadvantages:

1) It demands experts for interpreting and controlling the behaviour of cells in culture.
2) It is ten times more expensive for same quantity of animal tissue.
3) It has unstable aneuploid chromosome.

13.1.9. Application of Cell Cultures in Pharmaceutical Industry and Research

Animal cell culture has the following applications:

1) **Model Systems:** A good model system is provided by the cell cultures for the following studies:
   i) Basic cell biology and biochemistry,
   ii) Interactions between disease-causing agents and cells,
   iii) Drug effects on cells,
   iv) Ageing process and the trigger factors, and
   v) Nutritional studies.
2) **Toxicity Testing:** Cell cultures are used for studying the effects of new drugs, cosmetics, and chemicals on survival and growth in various cell types. For this purpose they are either used alone or together with animal tests. Liver- and kidney-derived cell cultures are particularly used for toxicity testing.
3) **Cancer Research:** The difference between normal and cancer cells can be studied by growing them in culture. Even the normal cultured cells can be converted into cancer-causing cells with the help of chemicals, viruses, and radiation; therefore, the mechanisms involved can be studied. Cultured cancer cells can be used as a test system for determination of drugs and methods suitable for destroying cancer.
4) **Virology:** Replication of viruses in cell cultures to be used for vaccine production is one of the earliest uses of cell culture. Clinical detection, isolation of viruses, their growth, and their process of causing infection are also studied by cell cultures.
5) **Cell-Based Manufacturing:** Cell cultures are used for large-scale production of viruses to be used for the production of polio, rabies, chicken pox, hepatitis B, and measles vaccines. Cell cultures are also used for large-scale production of genetically engineered cells to be used for the production of medicinally important proteins, **e.g.,** monoclonal antibodies, insulin, hormones, etc. Cell cultures even make use of cells as replacement tissues and organs, **e.g.**, artificial skin is used for treating burns and ulcers. Still studies on artificial organs such as pancreas, liver, and kidney are under process. The research work being carried with embryonic and adult stem cells may prove to be a potential supply of replacement cells and tissues. These cells can differentiate into different cell types. The knowledge about controlling the development of these cells may offer new treatment approaches for many medical conditions.
6) **Genetic Counselling:** Amniocentesis is a diagnostic technique used for removing and culturing foetal cells from pregnant women, thus aiding the doctors in early diagnosis of any foetal disorder. The cultured cells are then studied for detecting any chromosomal and genetic abnormalities using karyotyping, chromosome painting, and other molecular techniques.
7) **Genetic Engineering:** The ability to transfect or re-programme the cultured cells using new genetic material (DNA and genes) helps the molecular biologists in studying the cellular effects of gene expression (new proteins).
8) **Drug Screening and Development:** Cell-based assays are significantly used in pharmaceutical industries for cytotoxicity testing and for high throughput screening of compounds to be used as drugs. These cell culture tests were initially carried out using 96 well plates, but now 384 and 1536 well plates are used.
9) **Gene Therapy:** Various experimental techniques are used in Modern Molecular Biology, **e.g.,** cloned/altered genes are inserted into cells with the aid of r-DNA technology for replacing the defective genes (resulting in genetic abnormalities) or for preventing the potential disorders.

13.2. SUMMARY

The details given in chapter can be summarised as follows:

1) The process of removal of cells form an animal or plant and their subsequent growth in a favourable artificial environment is referred as **cell culture**.
2) Following types of cells are involved in the animal cell culture:
   i) **Primary cells** are derived from animal tissues, grow limitedly in culture, and give rise to a cell strain.
   ii) **Transformed cells** are derived from animal tumours or arise from primary rodent cells, and grow indefinitely in culture.
   iii) **Cultured cells** are treated with certain viruses or polyethylene glycol to be fused into heterokaryons (hybrids between cells of different species, which lose the chromosomes of one species on dividing).
3) A cell culture medium is comprised of the following two parts:
   i) **Basal Medium:** It supports the cellular requirements for nutrients, salts, and pH control.
   ii) **Set of Supplement:** It supports other cellular requirements and allows the cells to grow in the basal medium.
4) The conditions need to be maintained for the growth of animal cells are:
   i) Balanced Salt Solution (BSS),
   ii) Buffering systems,
   iii) pH,
   iv) Energy sources,
   v) Amino acids,
   vi) Vitamins,
   vii) Hormones and growth factors,
   viii) Proteins and peptides,
   ix) Fatty acids and lipids, and
   x) Accessory factors.
5) The process of cell culture can be discussed under the following heads:
   i) Preparation and sterilisation of the substrate and culture vessel,
   ii) Preparation and sterilisation of the medium,
   iii) Isolation of cells and tissues,
   iv) Disaggregation of tissues,
   v) Primary cell culture,
   vi) Sub-culture,
   vii) Cloning of cell lines, and
   viii) Contamination in cell culture.
6) Tissue disaggregation can be done by the following three methods:
   i) Physical disaggregation,
   ii) Enzymatic disaggregation, and
   iii) Treatment with chelating agents.
7) Physical disaggregation method is a **force based technique** which involves slicing of tissue and collection of spill out cells.
8) The primary viable cells of primary cell culture are called **adherent cells** and the culture is called **adherent culture**.
9) Enzymatic disaggregation of tissues commonly employs **collagenase** and **trypsin** enzymes (trypsin being more commonly used).
10) **Collagenase enzyme** is used for disaggregation of embryonic, normal, and malignant tissues.

11) Tissue disaggregation with trypsin enzyme is termed **trypsinisation**. Depending on the temperature of trypsin, trypsinisation may be of following two types:
    i) Cold trypsinisation, and
    ii) Warm trypsinisation.
12) **Perfusion** is a type of enzymatic digestion in which a proteolytic solution is pumped through the tissue and the cells are collected in the enzyme solution. This technique is used for isolation of cells from liver.
13) The surgically removed cells of an organism attach, divide, and grow when placed into a suitable culture environment. The resultant culture is known as **primary culture**.
14) A group of cells obtained from a primary culture at the first sub-culture is termed as **cell line**.
15) Primary culture can be done by the following **two ways**:
    i) Explant cultures ii) Enzymatic dissociation
16) In single cell suspension, the cells mixture is used as a **primary** or **starter culture** which is sub-cultured by transferring into special growth nutrient medium contained in culture vessels at optimal growth conditions.
17) The cell culture may get contaminated by:
    i) Chemical contamination, and
    ii) Biological contamination.
18) For growing cells, two basic culture systems are used. These culture systems are mainly based on the ability of the cells to either grow on attached glass or treated plastic substrate (**Monolayer Culture Systems**) or floating free in the culture medium (**Suspension Culture Systems**).
19) The cultured cells are described on the basis of their morphology (shape and appearance) or their functional characteristics as follows:
    i) Epithelial-like cells,
    ii) Lymphoblast-like cells, and
    iii) Fibroblast-like cells.
20) The cultured cells may also be of the following types:
    i) Precursor or stem cells,
    ii) Undifferentiated but committed cells, and
    iii) Mature differentiated cells.
21) **Transformation** means a phenotypic change which depends on the uptake of new genetic material. Transformation of cells results in the following changes:
    i) Genetic instability,
    ii) Immortalisation,
    iii) Aberrant growth control, and
    iv) Malignancy.

13.3. EXERCISE

13.3.1. True or False

1) Primary cells are derived from animal tissues, grow limitedly in culture, and give rise to a cell strain.
2) Transformed cells derived from a multiple parental cell are known as cell lines.
3) The constituents of culture media and the reagents used for culturing should be sterilised by either autoclaving or filtration.
4) Enzymatic disaggregation involves slicing of the tissue into pieces and collecting the cells which spill out.

5) A cryopreservation agent (glycerol) is used for increase cell injuries during freezing and thawing.
6) Transformation means a phenotypic change which depends on the uptake of new genetic material.
7) Perfusion is a type of enzymatic digestion in which a proteolytic solution is pumped through the tissue and the cells are collected in the enzyme solution.

13.3.2. Fill in the Blanks

8) The process of removal of cells form an animal or plant and their subsequent growth in a favourable artificial environment is referred as_____.
9) A group of cells obtained from a _____ at the first sub-culture is termed as cell line.
10) Tissue disaggregation with _____ enzyme is termed trypsinisation.
11) A population of cells derived from a _____ parental cell is called a_____.
12) The primary viable cells of primary cell culture are called _____ and the culture is called_____.
13) The physical disaggregation method is a _____ which involves slicing of tissue and collection of spill out cells.

Answers:

1) True 2) False 3) True 4) False 5) False
6) True 7) True 8) Cell culture 9) Primary culture
10) Trypsin 11) Single, clone 12) Adherent cells, adherent culture
13) Force based technique

13.3.3. Very Short Answer Type Questions

1) Give types of cultured cells.
2) Define following terms:
   i) Trypsinisation
   ii) Cell strain
   iii) Totipotent
3) What is cell culture?
4) Write the conditions need to be maintained for the growth of animal cells.
5) Give definition of:
   i) Transformed cells
   ii) Primary cells
   iii) Explant cultures
   iv) Continuous cell lines
6) What is cold trypsinisation?

13.3.4. Short Answer Type Questions

1) What is primary cell culture?
2) Give the applications of cell cultures in pharmaceutical industry and research.
3) What is sub-culture?
4) Write a note on cell culture system.
5) What is transformed cell culture?
6) Give a short note on mechanical disaggregation.

13.3.5. Long Answer Type Questions

1) Give the general procedure of cell culture.
2) Write a note on growth of animal cells in culture.
3) Write a note on types of cultured cells. Give advantages and disadvantages of animal cell culture.
4) Give a detail note on disaggregation of tissues.