M.Sc. [Zoology]
II - Semester
350 23

MICROBIOLOGY
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## Microbiology

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Microbiology is the branch of biology that studies microorganisms and their effects on human beings and other living organisms; such organisms include Bacteria, Fungi, Protozoa and Viruses. Microbiology involves examining their growth, characteristics, morphology, advantages and disadvantages, and various other aspects. It is generally believed that only 1 per cent of microbes on Earth have been studied, and this field is still in its nascent stage as compared with other biological disciplines like zoology and botany. The importance of microbiology in medical science cannot be underestimated. It is useful in waste management, medicine, research, mining, food production as well as Biotechnology. There are millions of different organisms that inhabit the planet Earth. The most common forms of organisms are those which can be seen without the aid of any magnifying devices. On the other hand, there are some organisms which are smallest of the small and the simplest of the simple. These organisms perform incredible amount of activities, which cannot be even achieved by human beings. These minute agents are called microorganisms and are studied under the subject ‘Microbiology’. The structure and activities of these microorganisms can be studied only with the help of some specialized techniques until recently, bacteria were the only known type of prokaryotic cells. The discipline of biology related to their study is called Bacteriology. By the middle of the present century the affinities of the Bacteria were determined.

This book, *Microbiology*, is divided into four block, which is further divided into fourteen units which will help you understand basics of microbiology, classification (Haeckel’s three kingdom, Whittaker’s five kingdom, three domain by Carl Woese), classification of bacteria according to Bergey’s manual, Fungi - general characters and classification, industrial uses of yeast and moulds, Viruses - ICTV system of classification, general properties, morphology and ultra-structure of virus (RNA, DNA), simple and compound microscopes (dark field, phase contrast, fluorescent), electron microscopes, confocal microscopy, stains and staining techniques, growth and growth culture (axenic and synchronous, aerobic and anaerobic, culture media and nutritional types, factors influencing microbial growth), preservation methods of microbes (storage, sterilization, disinfection), prokaryotic cell structure and organization, classification of algae, general characters and classification of Protozoa (structural characteristics, classification and reproduction), molecular taxonomy, phylogenetic trees, metagenomics, bacterial and viral diseases.

The book follows the Self-Instruction Mode (SIM) format wherein each unit begins with an ‘Introduction’ to the topic followed by an outline of the ‘Objectives’. The content is presented in a simple and structured form interspersed with ‘Check Your Progress’ questions and answers for better understanding. A list of ‘Key Words’ along with a ‘Summary’ and a set of ‘Self Assessment Questions and Exercises’ is provided at the end of the each unit for effective recapitulation.
Microbiology: An Introduction

UNIT 1  MICROBIOLOGY: AN INTRODUCTION

Structure

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1.0 INTRODUCTION

Microbiology is the study of all living organisms that are too small to be visible with the naked eye. This includes Bacteria, Archaea, Viruses, Fungi, Prions, Protozoa and Algae, collectively known as ‘Microbes’. These microbes play key roles in nutrient cycling, biodegradation/biodeterioration, climate change, food spoilage, the cause and control of disease, and biotechnology. Thanks to their versatility, microbes can be put to work in many ways, like making life-saving drugs, the manufacture of biofuels, cleaning up pollution, and producing/processing food and drink.

The existence of microorganisms was predicted many centuries before they were first observed, for example by the Jains in India and by Marcus Terentius Varro in Ancient Rome. The first recorded microscope observation was of the fruiting bodies of moulds, by Robert Hooke in 1666, but the Jesuit priest Athanasius Kircher was likely the first to see microbes, which he mentioned observing in milk and putrid material in 1658. Antonie van Leeuwenhoek is considered a father of microbiology as he observed and experimented with microscopic organisms in 1676, using simple microscopes of his own design. Scientific microbiology developed in the 19th century through the work of Louis Pasteur and in medical microbiology Robert Koch.
In this unit, you will study about microbiology, Haeckel’s three-kingdom concept, Whittaker’s five kingdom concept, three domain concept of Carl Woese in detail.

1.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand what microbiology is
- Discuss about Haeckel’s three kingdom concept
- Explain Whittaker’s five kingdom concept
- Describe the three domain concept of Carl Woese

1.2 MICROBIOLOGY: AN INTRODUCTION

Microbiology is the study of all living organisms that are too small to be visible with the naked eye. This includes Bacteria, Archaea, Viruses, Fungi, Prions, Protozoa and Algae, collectively known as ‘Microbes’. Microbiology is the study of organisms and agents that generally exist a single cells or cell clusters, too small to be seen clearly by the naked eye, for example, microscopic viruses, diverse group of Bacteria, Algae, Fungi and Protozoa (Refer Figure 1.1). Microbiology as a separate branch of study was established by Louis Pasteur and Robert Koch. It can be applied to other branches, such as:

As Koch’s postulates, a microorganism can be accepted as the causative agent of an infectious disease only if the following conditions are satisfied:

- The bacterium should be constantly associated with the lesions of the disease.
- It should be possible to isolate the bacterium in pure culture from the lesions.
- Inoculation of pure culture into suitable laboratory animals should reproduce the lesions of the disease.
- Possibility of isolating the bacterium in pure culture from the lesions produced in the experimental animals.
- Microbiology has been defined in various ways. The following are some of the definitions of microbiology:
  - Microbiology is the study of living organisms of microscopic size.
  - Microbiology is a branch of biology dealing especially with microscopic forms of life.
  - Microbiology is the scientific study of microorganisms, and deals with the structure, function and classification of these organisms and the ways in which they can be controlled and used.
Microbiology is the branch of biology dealing with microorganisms and their effects on other living organisms.

**Fig. 1.1 Classification of Microorganisms**

### Historical Development of Microbiology

Before really getting into the detailed study it is very essential that you have a broad historical overview. In ancient times, epidemic and even endemic diseases were believed to be supernatural in origin, sent by the gods as punishment for the sins of human kind. The concept of contagion was also known because people were aware that certain diseases spread by direct or indirect contact. Another ancient belief was that these diseases were produced by invisible living creatures.

So in order to probe into the causative agents of such diseases which was a mystery to the human kind, in more recent times a branch of science developed which was known as ‘Medical Microbiology’. This is the subdivision of microbiology concerned with the finding of the causative agents of infectious diseases of man, the response of the host to infection and various methods of diagnosis, treatment and prevention. The period from 1854 to 1914 is referred to as the Golden Age of Microbiology because in this era microbiologists achieved the following:

- Studied the chemical activities of microorganisms.
- Improved the techniques for performing microscopy.
- Progressed in the techniques of culturing microorganisms.
- Developed vaccines and surgical techniques.

Antony von Leeuwenhoek (1632–1723) used a simple microscope to observe and report bacterial organisms for the first time in 1673. He described various types of bacteria accurately and communicated these
He made the first microscope that consisted of a single biconvex lens that magnified about 200 times, and found microorganisms in materials, such as water, mud, saliva and the intestinal contents of healthy subjects and called them ‘Animalcules’.

Louis Pasteur (1822–1895) proved conclusively that all forms of life, even microbes, arose only from their like and not De Novo, De Novo is a Latin expression meaning ‘from the beginning’, ‘afresh’, ‘anew’, ‘beginning again’.

**History of Microbiology**

**Discovering the Organisms**

- 1676: A. Leeuwenhoek: First to observe and describe Microbes accurately.
- 1884: C. Chamberland: Constructed a bacterial filter that allowed the identification of Viruses.
- 1898: Loeffler and Frosch: Identified filterable infectious agents as cause of foot and mouth disease in cattle.
- 1898-1900: M. Beijerinck: Identified tobacco mosaic virus.

**Disapproving Spontaneous Generation that Living Organism could Develop from Non-Living Matter**

- 1688: F. Redi: First to propose theory of spontaneous generation conflict by showing that if raw meat was protected from flies the formation of maggots was prevented.
- 1776: L. Spallanzani: Challenged spontaneous generation by proposing that air carried germs to the culture medium supporting the growth.
- 1861: L. Pasteur: Rigorously disproved spontaneous generation.
- Filtered air, showed that air contained microbial organisms.
- Constructed flasks with curved neck that allowed air into the flasks while dust, etc., remained in the neck, placed broth into the flasks and boiled, showed that no microbial growth resulted unless flasks were tipped to allow the broth into the neck.

**The Germ Theory of Disease**

- Previously, it was believed that the disease was a punishment due to poisonous vapours or an imbalance of the four humors.
- First proponents of the idea that invisible organisms caused disease were Lucretius and Fracastoro (1546).
• 1835: A. Bassi showed that silkworm disease was a fungus.
• 1867: J. Lister showed that antiseptic surgical reduced the frequency of wound infection.
• 1876/1884: R. Koch proved that *Bacillus anthracis* caused the disease ‘Anthrax’ in cows and *Mycobacterium tuberculosis* caused the disease Tuberculosis using Koch’s postulates.
• The suspected pathogen should be present in ALL cases of the disease and NOT present in healthy animals.
• The suspected pathogen should grow *in-vitro* in pure culture.
• Cells from a pure culture of the putative pathogen should cause disease in healthy animals.
• The putative pathogen should be isolated from the infected animal.

**Preventing Diseases by Vaccination**

• E. Jenner inoculated people with Cowpox to protect against Smallpox.
• 1885: Pasteur developed the Rabies vaccine.
• 1890: Emil von Behring and Kitasato produced antibodies to purified toxins as a protection against Diphtheria and Tetanus.
• 1884: E. Metchnik explained Phagocytosis of Bacteria.

**Discovering the Effect of Microbes on Organic and Inorganic Matter**

• 1856: Pasteur explained lactic acid fermentation and manufacture of wine in industry.
• 1887-1900: S. Winogradsky and M. Beijerinck studied soil microbes and their role in biochemical cycle of Carbon, Nitrogen and Sulphur.

**Recent History of Microbiology - The 20th Century**

• **Infectious Diseases:** The etiological agent of most infectious diseases has been ascertained. Efforts are being made to understand the molecular mechanisms by which disease is caused.
• **Chemotherapy:** Discovery of antibiotics, antibiotic resistance.
• **Immunology:** Develops as a science to study the immune system.
• **Physiology and Biochemistry:** Using microbes as a model, many physiological and biochemical processes have been elucidated.
• **Genetics:** Many of the advances in molecular genetics were made using bacteria as models, such as:
  o 1941: Beadle and Tatum – 1 Gene = 1 Enzyme.
  o 1943: Luria and Delbruck – Mutations are spontaneous in occurrence.
Molecular Biology: Many of the advances in molecular biology were made using Bacteria as models, such as:

- 1970: Restriction enzymes discovered.
- 1979: Insulin synthesised using recombinant techniques.
- 1990: Gene therapy trials begin.
- 1995: The nucleotide sequence of the first free-living organism (Haemophilus influenzae) published.

Scope of Microbiology

Microbiology includes disciplines of Virology, Mycology and Bacteriology. Eukaryotic microorganisms exhibit cell organelles and include Fungi, Protists and Algae. Prokaryotes are conventionally classified as lacking organelles and include Eubacteria, Archaeabacteria.

Viruses have not been classified as organisms as they have been identified either as very simple or very complex. Prions are misfolded proteins which characterize several fatal neurodegenerative diseases in humans and many other animals. It is not known what causes the normal protein to misfold; the abnormal three-dimensional structure is suspected of conferring infectious properties. The word ‘Prion’ is derived from
Prions composed of the Prion Protein (PrP) are hypothesized as the cause of Transmissible Spongiform Encephalopathies (TSEs), including Scrapie in Sheep, Chronic Wasting Disease (CWD) in Deer, Bovine Spongiform Encephalopathy (BSE) in Cattle, commonly termed as ‘Mad Cow Disease’, and Creutzfeldt-Jakob Disease (CJD) in Humans.

Microbiology is linked to following scientific disciplines:

- Pure Microbiology
- Applied Microbiology

**Pure Microbiology**

Branches of pure microbiology are those in which scientists study a particular group of microorganisms for the sole purpose of understanding them better, and not to a specific end, such as learning how to use one bacteria to prevent the spread of another. Fields of pure microbiology include Mycology, which is the study of Fungi. Virology is the study of Viruses. Immunology is the study of the Immune System. Phycologists study Microscopic Algae. Protozoologists study Protozoa. Parásitologists study Parasitic Microorganisms. Bacteriologists study Bacteria Strains, and Nematologists study Nematodes.

Some other fields are as given below:

- **Bacteriology**: Study of Bacteria.
- **General Microbiology**: Study of Microscopic Organisms, which are defined as any living organism that is either a single cell (unicellular), a cell cluster, or has no cells at all (acellular). This includes Eukaryotes, such as Fungi and Protists, and Prokaryotes.
- **Medical Microbiology**: Microbes that cause human disease.
- **Public Health and Epidemiology**: Studies and controls transmission, frequency, and distribution of disease.
- **Immunology**: The study of immune system.
- **Agricultural Microbiology**: Impact of microbes on agriculture.
- **Microbial Ecology**: Relationships between microbes and their habitats.
- **Food Microbiology**: Prevention of food borne disease; microbes that make food and drink.
- **Industrial Microbiology**: Commercial use of microbes to produce products.
- **Biotechnology**: Manipulation of organisms to form useful products. Biotechnology can be defined as the scientific manipulation of living organisms, especially at the molecular and genetic level to produce useful products.


**Applied Microbiology**

Applied microbiology is the study of Microorganisms for the sake of using them, or controlling them in a way that aids humanity, for example, Medical Microbiologists study how microorganisms, such as Bacteria and Viruses that cause diseases in humans. Other branches of applied microbiology include industrial microbiology, in which scientists use microorganisms to develop products. In food microbiology, for example, they might develop new bacterial cultures for dairy products. Other fields of applied microbiology include Pharmaceutical Microbiology and Microbial Biotechnology.

- Veterinary Microbiology: Study of microbes of veterinary importance, such as probiotics and pathogens which are related to veterinary medicine.
- Pharmaceutical Microbiology: Study of microorganisms which are related to the production of antibiotics, enzymes, vitamins, biologics and other pharmaceutical products.
- Microbial Biotechnology: Manipulation of microorganisms at genetics and molecular level, for example generation of insulin from *E. Coli*.
- Microbial Genetics: Study of regulation of microbe genes in relation to their function.
- Microbial Cytology: Study of microscopic details of cellular organization of microbes.

**The Scope and Relevance of Microbes**

- L. Pasteur quotes: ‘The role of the infinitely small in nature is infinitely large’.
- Can survive in diverse environments.
- Constitutes the largest components of biomass.
- Fundamental to the ecosystem.
- In abundance compared to macro-organisms.

Microbiology because in this era microbiologists achieved the following:

- Metabolic chemical activities of microorganisms elucidated.
- Improved the techniques for performing microscopy.
- Progressed in culturing techniques of microorganisms.
- Developed vaccines and surgical techniques.

**Concept of Micrometry**

The method of measuring a microscopic organism under a microscope with the help of a calibrated scale is known as micrometry. Devices like the eyepiece micrometer and stage graticule is used in the measurement and calibration...
processes. The eyepiece reticulated in combination with stage micrometers are applied where measurements fall into the size range of 0.2 micrometers to 25 millimeters. Measurement of any Specimens with linear, area and volume dimensions at high magnifications have been inferred with micrometry or morphometrics. With the help of microscopy and microbiology the biggest challenge met is the spread of diseases that appear without showing their signs of presence. The genomic tools can be used to explore the characteristic features of microbial communities that are not cultured.

**Check Your Progress**

1. What is microbiology?
2. Define immunology.
3. Differentiate between Eukaryotic and Prokaryotes microorganisms.
4. Explain the term Prions.
5. What does pharmaceutical microbiology do?

### 1.3 HAECKEL’S THREE KINGDOM CONCEPT

The prehistoric opinion became formalised in scientific terms by Lennaeus recognised two primary kingdoms, the Plantae and Animalia (Refer Figure 1.2). This was on the basis of various structural and functional characters that cannot be maintained on logical grounds. When explorations of microbial world got underway in the 18th and 19th centuries like microalgae, microfungi, multicellular invertebrates and unicellular protozoa and bacteria came into knowledge.

![Fig. 1.2 Difference between Kingdom Plantae and Animalia](image)
The science of taxonomy involves not naming organisms, but grouping them with other organisms that share common properties. In the early days classification was with all living things apparently fitting into one of two kingdoms. In the mid-19th century, Ernst Haeckel proposed a third kingdom the Protista to include Bacteria, Fungi, Protozoans and Algae. Figure 1.3 illustrates the three kingdom system.

Ernst Haeckel, suggested a third kingdom Protista with features common to both or unique to its own for all unicellular and multicellular organisms without tissue, i.e., Fungi, Algae and Slime Moulds (Refer Figure 1.4). He made the first attempt at constructing a phylogenetic classification as the nature of bacteria became clearer. However Fungi were still classified in the Plant kingdom and separated into four classes. Based on the morphology of sexual organs these traditional groups of Fungi. For a long time biologists were content to classify living things as either plants or animals with fungi and lichens classified under algae in a group called Thallophyta. This is ironic as true fungi are closely related to animals than they are to plants.

The Protists are believed to have evolved from prokaryotic Monerans and the precursors from which higher Eukaryotic kingdoms: Plantae, Fungi and Animalia have evolved.

- These have a typical Eukaryotic cell organisation and possess nucleus, mitochondria, endoplasmic reticulum, plastids.
- Locomotion takes place with the help of cilia and flagella. The flagella or cilia have 9 fused internal microtubular structure.
- These exhibit diverse life forms, i.e., photosynthetic, parasitic or saprophytic.
- This system however was not appropriate as it included both Prokaryotes and Eukaryotic, chlorophyllous and non-chlorophyllous organisms together.
6. Who prosed kingdom Protista and when?
7. How are the Protists evolved?
8. Give the characteristic features of Protists.

1.4 WHITTAKER’S FIVE KINGDOM SYSTEM

An increased focus on the cellular and molecular similarities and dissimilarities between organisms led to proposals for further revelations to the three kingdom system. One of the most widely accepted of these has been the five kingdom system proposed by Robert Whittaker in 1969 (Refer Figure 1.5).

![Whittaker’s Five Kingdom Classification](image)

The system coined by R.H. Whittaker is the most frequently adopted modified by Lynn Margulis. It is recognised that the Prokaryotes were under
kingdom Monera and divides the Eukaryotes into three kingdoms, i.e., higher kingdoms of Plants, Fungi, and Animals transferred from the lower kingdom of Protista as three stocks making it more heterogenous. In the classification scheme, red and brown algae were placed near the base of stock of plants, green algae were placed both in the Protista kingdom and the base of the monophyletic plant kingdom. Myxomycetes were positioned near the base of the stock of fungi. The arrangement was on the basis of morphological complexities and tissue system, division of labour and mode of nutrition. Organisms lacking morphological complexities, tissue system, division of labour, and enjoying diversified modes of nutrition were segregated under Protista. Organisms having the above features were bifurcated into two categories. Figure 1.6 shows the five kingdom classification.

The levels of cellular organisation:

- Prokaryotic → Unicellular Eukaryotic → Multicellular Eukaryotic.
- The principal mode of nutrition includes photosynthesis, absorption, ingestion,
- The basis of classification was modified to consider genetics along with cellular organisation and mode of nutrition.
- Kingdom Monera: It includes Prokaryotic organisms. They are unicellular.

![Five Kingdom Classification](image)

**Fig. 1.6 Five Kingdom Classification**

It takes into account the fundamental difference in cell structure between Prokaryotes and Eukaryotes. Accordingly place Prokaryotes (Bacteria) in their own kingdom. The Monera separate form single celled Eukaryotes. Based on their nutrition fungi were assigned their own kingdom. Molecular
studies revealed that the Archaea differed from all other Bacteria in their 16S rRNA sequences, as well as in their cell wall structure, membrane lipids. Archaeobacteria can survive in the extreme environment. Eubacteria are known as Modern Bacteria and also called as True Bacteria. Kingdom Protista is the most Ancient Eukaryotic kingdom. The kingdom Protista include a variety of Eukaryotic forms with cell walls usually absent. It may be impregnated with silica (diatoms). Locomotory structures are present for motility.

A system based on phylogenetic relationships amongst organisms cannot be established until all the distinct evolutionary tendencies are taken into account. Five kingdom system does not satisfy in this respect, for example, photosynthetic Bacteria under kingdom Monera resemble certain green algae that both obtain hydrogen from sources other than water. Whittaker’s system of organism’s classification is based on evolutionary relationship of phenotypic characteristics in which three levels of cellular organization are thought to have evolved along three different lines of nutritional strategies: photosynthesis, absorption and ingestion.

The phenotypic characteristics taken into account to raise five kingdoms by Whittaker are:

- Cell Type: Prokaryotic or Eukaryotic.
- Level of Organisation: Solitary and Colonial Unicellular Organisation or Multicellular.
- Nutritional Type.

The brown algae are not closely related to the plants even though they belong to kingdom Plantae and Monera; Protista contain both walled and wall less organisms, photosynthetic and non-photosynthetic organisms and unicellular and filamentous or mycelia organisms.

Whittaker’s classification retained the basic Prokaryote – Eukaryote Distinction. Thus the kingdom Monera contains the Prokaryotes. The Eukaryotes are classified into four remaining kingdoms. The multicellular organisms are split into three kingdoms on the basis of mode of nutrition and other fundamental differences in organisation. Kingdom Fungi includes Moulds and Yeasts. The non-chordates and chordates make up Kingdom Animalia. Most of these forms ingest their food and digest them internally although some parasitic forms are absorptive. The Protists are believed to have given rise to all the multicellular organisms, which have evolved independently.

Whittaker’s thinking was influenced by Lynn Margulis’s theory of endosymbiosis. Contrary to the view that Protists had gradually evolved from Prokaryotic Bacteria, Margulis claimed that all Eukaryotic cells were derived from multiple symbiotic partnerships among Prokaryotic cells. In the process of the cellular evolution some of the specialised organelles of Eukaryotic cells had originated as free living bacteria. Endosymbiosis further strengthened Whittaker’s five kingdom system into three well defined evolutionary levels.
NOTES

In this five kingdom a dichotomy was recognised that gave a distinction between Eukaryotes and Prokaryotes. This central dogma explains the narrow boundary between kingdom Monera and Protista. Carl Woese explored organisms that have fundamentally different molecular, biochemical, ecologically different characteristics other than Bacteria. The Archaebacteria are intriguing as they occur in extreme habitats with high temperatures, high salinity, and high acidity, therefore, there was a need for restructuring into three domains: Archaea, Bacteria and Eucarya.

General Characteristic of Whittaker’s Five Kingdom

Kingdom Monera (Archaebacteria, Bacteria, and Cyanobacteria)

- Monerans are all the Prokaryotes smallest micro-organisms.
- The nuclei not organised within the nuclear membrane ‘incipient nuclei’ without nucleoli and chromatin fibers.
- They lack membrane bound organelles.
- The photosynthetic pigment present in the form of chromatophores compared to a single lamella of a granum.
- The respiratory enzymes are present along the infoldings of plasma membrane called mesosomes.
- Nutrition absorptive, chemosynthetic, photoautotrophic.
- Peptidoglycan is the chief constituent of the Moneran cell wall.
- Flagella 8 stranded lacking 9+2 arrangement.
- Ribosomes 70S with single DNA as nuclear genetic material.
- Reproduce asexually with amitosis and no precision in distribution of genetic material among daughter cells.

Kingdom Protista (Phytoplanktonic Algae, Protozoa, and Slime Moulds)

- Eukaryotic with solitary or colonial unicellular organisation without any differentiation into tissue and organs.
- Mostly absorptive nutrition, photosynthetic with chlorophyll pigment.
- The cellular organisation is of two envelop type: beside plasma membrane internal membrane occur around certain organelles.
- Genetic material is organised in the form of true nucleus.
- DNA associated with histone proteins.

Kingdom Fungi (The Fungi)

- They are versatile by virtue of their high degree of adaptability.
• The nuclear division is intra nuclear with resistant nuclear membrane and stages of division complete within it through karyochorisis.
• Heterotrophic with extracellular digestion.
• Branched vegetative body the filament called hyphae surrounded by a cell wall.
• Lack of transport vessels internally with no tissue differentiation.
• Chitinous cell wall commonly called fungal cellulose.
• Reserve food material in the form of glycogen.

**Kingdom Plantae (Macroalgae and Plants)**
• Either autotrophic, heterotrophic, or saprophytic.
• Multicellular organism except few Alga, thin walled and vacuolated Eukaryotic cells.
• Autotrophism by means of photosynthesis.
• Haploid and diploid phases of reproduction with alternation of generation.
• Food reserves in the form of starch and fats.
• Growth is usually indefinite.
• Development proceeds from embryo except in Algal groups.

**Kingdom Animalia (Invertebrate and Vertebrate Animals)**
• Individuals multicellular with wall less Eukaryotic cells.
• Multi cellularity accompanied with cellular tissue and organ system level of organisation with complex cell junctions.
• Nutrition ingestive with an internal cavity.
• Sexual reproduction with meiosis forming gametes.
• Zygotes develop into embryo.
• Centrioles occur in cells.
• Muscle cells present for mobility and nerve cells for impulse conduction.

**Limitation of Five Kingdom Classification**

The three kingdom classification failed to accommodate properly Prokaryotic forms. The limitation of the four kingdom classification was that despite creation of kingdom Monera, it failed to account for the integration between Protista and Animalia. Despite exclusion of Protozoa from the Animal kingdom, Protozoa share many characteristics with Metazoan animals. Thus it can be inferred that Metazoans have evolved from Protozoan groups. The interrelationship of various groups remain unexplained. Among non-chordates the grouping collectively called minor phyla has unsolved problem of phylogenetic placement. Despite its inadequacies this kingdom classification is most acceptable.
Shortcomings of Five Kingdom Classification

- Unicellular algae are kept in kingdom Protista, whereas Algae like multicellular organisms are kept in kingdom Plantae.
- There is diversity in kingdom Protista. Dissimilar organisms must not be kept in same group.
- Organisms that do not belong to Monerans are polyphyletic in origin.
- Viruses have no place in five kingdom classification.
- Unicellular and multicellular Algae are kept away from each other.
- It lacks distinction between Archebacteria and Eubacteria.
- The ill-defined lines between kingdom Monera, Protista, Fungi and Plantae.

Merits of Five Kingdom System

- Placement of Prokaryotes separately in the kingdom Monera. The organisms of the Prokaryotes differ from Eukaryotes in their cellular, physiological and reproductive organisation.
- Transitional forms of unicellular Eukaryotes included both amongst plants and animals. Placement of these unicellular Eukaryotes into kingdom Protista has removed this anomaly.
- Fungi with their unique physiological, biochemical and structural characteristics have never been related to plants. Their placement in the form of a separate kingdom was long overdue.
- Creation of five kingdom is based on level of organisation and nutritional strategy which evolved early but got incorporated later and currently existing.
- The animal and plant kingdom appears more homogenous than they were in two–kingdom system.
- Five kingdom has brought out the phylogenetic relationships even amongst the primitive forms.

Check Your Progress

9. Who proposed five kingdom classification and when?
10. What is the basis of Whittaker’s system of organism’s classification?
11. List the phenotypic characteristics that are accounted for Whittaker’s five kingdoms classification.
12. What are the three evolutionary levels of Whittaker’s five kingdom system?
1.5 THREE DOMAIN CONCEPT OF CARL WOESE

In 1970, Carl Woese, concluded that a group of Prokaryotic microorganisms called Archaebacteria are separate from other Monerans. Thus kingdom was split into two separate kingdoms – Eubacteria and Archebacteria. It shows how the major Prokaryotic groups are inter related, based on 16S rRNA data. Archebacteria are unicellular that do not require oxygen or light to live. Eubacteria are unicellular, Prokaryotic that are heterotrophic, autotrophic and chemotrophic, for example, Bacteria, Cyanobacteria. Developed by Carl Woese is a system of classifying biological organisms. The work of Woese revealed one group of prokaryotes differed from all others.

- **Archaeabacteria**: Unicellular forms that are Prokaryotic and exists in extreme climates. They do not need oxygen or light to live, for example, Methanogens, extreme Thermophiles, Halophiles.

- **Eubacteria**: Unicellular forms that are prokaryotic, heterotrophic, autotrophic and chemotrophic, for example, Bacteria, Cyanobacteria.

With advancement in genetic sequencing a new way of analysing relationships between organisms gave a system adapting the classification of living organisms that recognizes three domains based on differences in ribosomal RNA structure. Ribosomal RNA is a molecular building block for ribosomes. The organisms are classified under three domains and six kingdoms. The domains are Archaea, Bacteria, and Eukarya. The kingdoms are Archaeabacteria (Ancient Bacteria), Eubacteria (True Bacteria), Protista, Fungi, Plantae, and Animalia. Figure 1.7 illustrates the three domains based on Carl Woese rRNA sequence analysis.

![Fig. 1.7 Three Domains Based on Carl Woese rRNA Sequence Analysis](image-url)
Archaea Domain

The term *archae* (Greek, *archaio*, ancient) refers to the ancient origin of this group that are single celled, for example, Bacteria, which seem to have diverged very early from the Eubacteria. They possess genes that are similar to both Bacteria and Eukaryotes. They are inhabited mostly in extreme environments. The Archaebacteria are grouped (based primarily on the environments in which they live) into three general categories as Methanogens, Extremophiles and non-extreme Archaebacteria.

Archaea are extreme organisms that live under some of the most extreme environmental conditions. This includes within hydrothermal vents, acidic springs, and under Arctic ice. Archaea are divided into three main phyla: Crenarchaeota, Euryarchaeota, and Korarchaeota. Table 1.1 shows the classification of Archaea.

- Crenarchaeota include many organisms that are Hyperthermophiles and Thermoacidophiles. These Archaea thrive in environments with great temperature extremes (Hyperthermophiles) and in extremely hot and acidic environments (Thermoacidophiles).
- Archaea known as Methanogens are of the Euryarchaeota phylum. They produce methane as a byproduct of metabolism and require an oxygen free environment.
- Little is known about Korarchaeota. Of this Archaea only few species have been found living in places, such as hot springs, hydrothermal vents, and obsidian pools.

*Table 1.1 Classification of Archaea*

<table>
<thead>
<tr>
<th></th>
<th>Archaea</th>
<th>Bacteria</th>
<th>Eucarya</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main genetic material</strong></td>
<td>Single closed circle of dsDNA</td>
<td>Single closed circle of dsDNA</td>
<td>True nucleus with multiple linear chromosomes</td>
</tr>
<tr>
<td>Histones</td>
<td>Present</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Gene structure</td>
<td>Intron absent</td>
<td>Intron absent</td>
<td>Intron present</td>
</tr>
<tr>
<td>Plasmids</td>
<td>Common</td>
<td>Common</td>
<td>Rare</td>
</tr>
<tr>
<td>Polycistronic mRNA</td>
<td>Present</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Ribosomes</td>
<td>70S</td>
<td>70S</td>
<td>80S</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>Not sensitive to streptomycin, chloramphenicol</td>
<td>Sensitive to streptomycin, chloramphenicol</td>
<td>Not sensitive to streptomycin, chloramphenicol</td>
</tr>
<tr>
<td>Initiator tRNA</td>
<td>Methionine</td>
<td>N-formyl methionine</td>
<td>Methionine</td>
</tr>
<tr>
<td>Membrane fatty acids</td>
<td>Ether-linked, branched</td>
<td>Ester-linked, straight chain</td>
<td>Ester-linked, straight chain</td>
</tr>
<tr>
<td>Internal organelles</td>
<td>Absent</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Site of energy generation</td>
<td>Cytoplasmic membrane</td>
<td>Cytoplasmic membrane</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Cell wall</td>
<td>Muramic acid absent</td>
<td>Muramic acid present</td>
<td>Muramic acid absent</td>
</tr>
</tbody>
</table>
Bacteria Domain

Bacteria are classified under the Bacteria Domain. These organisms are generally feared because some are pathogenic and capable of causing disease. However, Bacteria are essential to life as some are part of the Human Microbiota. These bacteria preform vital functions, such as enabling us to properly digest and absorb nutrients from the foods we eat. Bacteria living on the skin prevents pathogenic microbes from colonizing the area and also help in the activation of the immune system. Bacteria are also crucial for the recycling of nutrients in the global ecosystem as they are primary decomposers.

Bacteria have unique cell wall composition and rRNA type. They are grouped into five main categories:

- **Protobacteria:** Diverse group of organisms morphologically and physiologically. This is due to the fact that their 16S rRNA indicates a common ancestor, Photosynthetic Gram Negative. Since classification is based on molecular aspect rather than shared phenotypic traits, few properties resemble all members of each class. Thus they have been defined in terms of their phenotypic characteristics rather than attempt to group them phylogenetically.
- **Cyanobacteria:** They are capable of photosynthesis also known as Blue Green Algae.
- **Firmicutes:** These are gram positive bacteria like Bacillus, Clostridium, Mycoplasma (bacteria without cell wall).
- **Chlamydiae:** They are patristic forms surviving inside the host cells, for example, *Chlamydia trachomatis* and *Chlamydophila pneumonia*.
- **Spirochates:** They are cock-screw shaped in appearance with a unique twisting motion. *Borrelia burgdorferi* causing Lyme disease and *Treponema pallidium* causing Syphilis.

Eukarya Domain

The Eubacteria are the most abundant organisms on the Earth. It plays critical roles like cycling carbon and sulphur. Much of the world’s photosynthesis is carried out by Eubacteria. However, certain groups of eubacteria are also responsible for many forms of disease.

The Eukarya domain includes Eukaryotes or organisms that have a membrane bound Nucleus. It consists of four kingdoms. The first of which is Protista, mostly unicellular organism like Amoeba. The other three kingdoms are Plants, Fungi, Animals. Multicellularity and sexuality are the two unique characters that differentiate from Prokaryote and Eukaryotes. They have RNA distinct from Bacteria and Archaeans. Plant and Fungi have a cell
Wall different to Bacteria in composition. They are resistant to antibacterial antibiotic, for example, Protists, Fungi, Plants and Animals.

**Limitations**

Today microbes often live in mixed communities that are capable of rapid attachment to surfaces as a biofilm. Biofilms are essential part of our environment, including extreme environment. Woese’s studies called to question many beliefs about evolutionary relationships bringing order to biological diversity. The three domains were thought to have diverged from some universal ancestor. It was inferred that the modern Eukaryotes use RNA to catalyse intron splicing and stable RNA processing from the molecular fossils with extremely primitive features, from the RNA world that has been the first step in the origin of life before the evolution of protein catalysis. The present day forms had a mix of selective features combined in different ways during the evolution.

Unfortunately, as the evolutionary clock is not constant in different lineages the time of occurrence of evolutionary events in the tree of life cannot be taken from rRNA gene sequence alone.

- Be universally distributed across the group chosen for study.
- Be functionally homologous.
- Change in sequence at a rate proportionate with the evolutionary distance to be measured.

Protein sequences offer several advantages over DNA sequences for some phylogenetic studies because homology, i.e., similarities of characteristics due to shared ancestry, is more easily distinguished from analogy similarity in structure or function that evolved through different pathways and different ancestries, a convergent evolution. Further, large shifts occur in gene coding frames that they are fatal. The root of the universal tree of life remains controversial. Evidence for ancient lateral transfer of genes obey the ancestry of Archaea makes the distant origins of the major lineages uncertain for ancient relationships. Pairs of SSU rRNA gene sequences from different organisms were aligned, used to infer phylogenetic trees to represents the evolutionary paths revealing the present day lineages. These pairs are free from mutational changes generated by convergent evolution.

### Check Your Progress

13. What does Carl Woese classification state?
14. Differentiate between Archaebacteria and Eubacteria.
15. What are Archaea?
16. Explain about Crenarchaeota and Methanogens Archaea.
17. Define the features of Eubacteria.
1.6 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Microbiology is the study of organisms and agents that generally exist a single cells or cell clusters, too small to be seen clearly by the naked eye, for example, microscopic viruses, diverse group of Bacteria, Algae, Fungi and Protozoa. Microbiology is the scientific study of microorganisms, and deals with the structure, function and classification of these organisms and the ways in which they can be controlled and used.

2. Immunology is defined as the immune system that protects against infections and attempts to understand many phenomena responsible for both acquired and innate immunity, in addition to the study of antibody–antigen reactions in the laboratory.

3. Eukaryotic microorganisms exhibit cell organelles and include Fungi, Protists and Algae. Prokaryotes are conventionally classified as lacking organelles and include Eubacteria, Archaebacteria.

4. Prions are misfolded proteins which characterize several fatal neurodegenerative diseases in humans and many other animals. It is not known what causes the normal protein to misfold; the abnormal three-dimensional structure is suspected of conferring infectious properties. The word ‘Prion’ is derived from ‘proteinaceous infectious particle’.

5. Pharmaceutical microbiology is the study of microorganisms which are related to the production of antibiotics, enzymes, vitamins, biologics and other pharmaceutical products.

6. In the mid-19th century, Ernst Haeckel proposed a third kingdom the Protista to include Bacteria, Fungi, Protozoans and Algae. As per Ernst Haeckel, the kingdom Protista has features common to both or unique to its own for all unicellular and multicellular organisms without tissue, i.e., Fungi, Algae and Slime Moulds.

7. The Protists are believed to have evolved from prokaryotic Monerans and the precursors from which higher Eukaryotic kingdoms: Plantae, Fungi and Animalia have evolved.

8. Following are the characteristic features of Protists:
   - These have a typical Eukaryotic cell organisation and possess nucleus, mitochondria, endoplasmic reticulum, plastids.
   - Locomotion takes place with the help of cilia and flagella. The flagella or cilia have 9 fused internal microtubular structure.
   - These exhibit diverse life forms, i.e., photosynthetic, parasitic or saprophytic.
9. The most widely accepted classification has been the five kingdom classification system proposed by Robert Whittaker (1969). Five kingdom classification comprises of Kingdoms - Monera, Protista, Fungi, Plantae and Animalia.

10. Whittaker’s system of organism’s classification is based on evolutionary relationship of phenotypic characteristics in which three levels of cellular organization are thought to have evolved along three different lines of nutritional strategies: photosynthesis, absorption and ingestion.

11. The phenotypic characteristics taken into account to raise five kingdoms by Whittaker are:
   - Cell Type: Prokaryotic or Eukaryotic.
   - Level of Organisation: Solitary and Colonial Unicellular Organisation or Multicellular.
   - Nutritional Type.

12. Endosymbiosis further strengthened Whittaker’s five kingdom system into three well defined evolutionary levels.
   - Prokaryotic Organisms
   - Eukaryotic Unicellular Organisms
   - Eukaryotic Multicellular Organisms

13. In 1970, Carl Woese, concluded that a group of Prokaryotic microorganisms called Archaebacteria are separate from other Monerans. Thus kingdom was split into two separate kingdoms – Eubacteria and Archebacteria.

14. Archaebacteria: Unicellular forms that are Prokaryotic and exists in extreme climates. They do not need oxygen or light to live, for example, Methanogens, extreme Thermophilles, Halophiles.

   Eubacteria: Unicellular forms that are prokaryotic, heterotrophic, autotrophic and chemotrophic, for example, Bacteria, Cyanobacteria.

15. Archaea are extreme organisms that live under some of the most extreme environmental conditions. This includes within hydrothermal vents, acidic springs, and under Arctic ice. Archaea are divided into three main phyla: Crenarchaeota, Euryarchaeota, and Korarchaeota.

16. Crenarchaeota include many organisms that are Hyperthermophiles and Thermoacidophiles. These Archaea thrive in environments with great temperature extremes (Hyperthermophiles) and in extremely hot and acidic environments (Thermoacidophiles).

   Archaea known as Methanogens are of the Euryarchaeota phylum. They produce methane as a byproduct of metabolism and require an oxygen free environment.
17. The Eubacteria are the most abundant organisms on the Earth. It plays critical roles like cycling carbon and sulphur. Much of the world’s photosynthesis is carried out by Eubacteria. However, certain groups of eubacteria are also responsible for many forms of disease.

1.7 SUMMARY

- Microbiology is the study of all living organisms that are too small to be visible with the naked eye. This includes Bacteria, Archaea, Viruses, Fungi, Prions, Protozoa and Algae, collectively known as ‘Microbes’.
- Microbiology is the study of organisms and agents that generally exist a single cells or cell clusters, too small to be seen clearly by the naked eye, for example, microscopic viruses, diverse group of Bacteria, Algae, Fungi and Protozoa.
- Microbiology as a separate branch of study was established by Louis Pasteur and Robert Koch.
- Possibility of isolating the bacterium in pure culture from the lesions produced in the experimental animals.
- Microbiology is the scientific study of microorganisms, and deals with the structure, function and classification of these organisms and the ways in which they can be controlled and used.
- In ancient times, epidemic and even endemic diseases were believed to be supernatural in origin, sent by the gods as punishment for the sins of human kind.
- The concept of contagion was also known because people were aware that certain diseases spread by direct or indirect contact.
- In order to probe into the causative agents of such diseases which was a mystery to the human kind, in more recent times a branch of science developed which was known as Medical Microbiology.
- Antony von Leeuwenhoek (1632–1723) used a simple microscope to observe and report bacterial organisms for the first time in 1673. He described various types of bacteria accurately and communicated these descriptions to the Royal Society of London.
- Antony von Leeuwenhoek made the first microscope that consisted of a single biconvex lens that magnified about 200 times, and found microorganisms in materials such as water, mud, saliva and the intestinal contents of healthy subjects and called them ‘Animalcules’.
- Microbiology includes disciplines of Virology, Mycology and Bacteriology.
• Eukaryotic microorganisms exhibit cell organelles and include Fungi, Protists and Algae.
• Prokaryotes are conventionally classified as lacking organelles and include Eubacteria, Archaebacteria.
• Viruses have not been classified as organisms as they have been identified either as very simple or very complex.
• Prions are misfolded proteins which characterize several fatal neurodegenerative diseases in humans and many other animals. It is not known what causes the normal protein to misfold; the abnormal three-dimensional structure is suspected of conferring infectious properties.
• The word prion is derived from ‘proteinaceous infectious particle’. Prions composed of the Prion Protein (PrP) are hypothesized as the cause of Transmissible Spongiform Encephalopathies (TSEs), including Scrapie in Sheep, Chronic Wasting Disease (CWD) in Deer, Bovine Spongiform Encephalopathy (BSE) in Cattle, commonly termed as ‘Mad Cow Disease’, and Creutzfeldt-Jakob Disease (CJD) in Humans.
• Fields of pure microbiology include Mycology, which is the study of Fungi. Virology is the study of Viruses. Immunology is the study of the Immune System. Phycologists study Microscopic Algae. Protozoologists study Protoza. Parositologists study Parasitic Microorganisms. Bacteriologists study Bacteria Strains, and Nematologists study Nematodes.
• Applied microbiology is the study of microorganisms for the sake of using them, or controlling them in a way that aids humanity, for example, medical microbiologists study how microorganisms, such as bacteria and viruses that cause diseases in humans.
• Veterinary microbiology is the study of microbes of veterinary importance, such as probiotics and pathogens which are related to veterinary medicine.
• Pharmaceutical microbiology is the study of microorganisms which are related to the production of antibiotics, enzymes, vitamins, biologics and other pharmaceutical products.
• Microbial biotechnology is the manipulation of microorganisms at genetics and molecular level, for example generation of insulin from *E. Coli*.
• Microbial genetics is the study of regulation of microbe genes in relation to their function.
• Microbial cytology is the study of microscopic details of cellular organization of microbes.
• The method of measuring a microscopic organism under a microscope with the help of a calibrated scale is known as micrometry.

• Devices like the eyepiece micrometer and stage graticule is used in the measurement and calibration processes.

• The eyepiece reticulated in combination with stage micrometers are applied where measurements fall into the size range of 0.2 micrometers to 25 millimeters.

• Measurement of any Specimens with linear, area and volume dimensions at high magnifications have been inferred with micrometry or morphometrics.

• With the help of microscopy and microbiology the biggest challenge met is the spread of diseases that appear without showing their signs of presence.

• The genomic tools can be used to explore the characteristic features of microbial communities that are not cultured.

• The prehistoric opinion became formalised in scientific terms by Lennaeus who recognised two primary kingdoms, the Plantae and Animalia.

• The science of taxonomy involves not naming organisms, but grouping them with other organisms that share common properties.

• Ernst Haeckel, suggested a third kingdom Protista with features common to both or unique to its own for all unicellular and multicellular organisms without tissue, i.e., Fungi, Algae and Slime Moulds.

• An increased focus on the cellular and molecular similarities and dissimilarities between organisms led to proposals for further revelations to the three kingdom system.

• One of the most widely accepted classification system has been the five kingdom system proposed by Robert Whittaker 1969.

• The system coined by R.H. Whittaker is the most frequently adopted and modified by Lynn Margulis.

• Archaebacteria can survive in the extreme environment. Eubacteria are known as Modern Bacteria and also called as True Bacteria.

• Kingdom Protista is the most Ancient Eukaryotic kingdom, Protista include a variety of Eukaryotic forms with cell walls usually absent.

• A system based on phylogenetic relationships amongst organisms cannot be established until all the distinct evolutionary tendencies are taken into account.
Five kingdom system does not satisfy in this respect, for example, photosynthetic bacteria under kingdom Monera resemble certain green algae that both obtain hydrogen from sources other than water.

Whittaker’s system of organism’s classification is based on evolutionary relationship of phenotypic characteristics in which three levels of cellular organization are thought to have evolved along three different lines of nutritional strategies: photosynthesis, absorption and ingestion.

The brown algae are not closely related to the plants even though they belong to kingdom Plantae and Monera; Protista contain both walled and wall less organisms, photosynthetic and non-photosynthetic organisms and unicellular and filamentous or mycelia organisms.

Whittaker’s classification retained the basic Prokaryote – Eukaryote Distinction. Thus the kingdom Monera contains the Prokaryotes.

The Eukaryotes are classified into four remaining kingdoms. The multicellular organisms are split into three kingdoms on the basis of mode of nutrition and other fundamental differences in organisation.

Kingdom Fungi includes Moulds and Yeasts. The non-chordates and chordates make up Kingdom Animalia.

The three kingdom classification failed to accommodate properly Prokaryotic forms. The limitation of the four kingdom classification was that despite creation of kingdom Monera, it failed to account for the integration between Protista and Animalia.

Despite exclusion of Protozoa from the Animal kingdom, Protozoa share many characteristics with Metazoan animals. Thus it can be inferred that Metazoans have evolved from Protozoan groups.

The interrelationship of various groups remain unexplained. Among non-chordates the grouping collectively called minor phyla has unsolved problem of phylogenetic placement.

In 1970, Carl Woese, concluded that a group of Prokaryotic microorganisms called Archaebacteria are separate from other Monerans. Thus kingdom was split into two separate kingdoms – Eubacteria and Archebacteria.

The term *archae* (Greek, *archaio*, ancient) refers to the ancient origin of this group that are single celled, for example, Bacteria, which seem to have diverged very early from the Eubacteria.

Crenarchaeota include many organisms that are Hyperthermophiles and Thermoacidophiles.

Bacteria are classified under the Bacteria Domain. These organisms are generally feared because some are pathogenic and capable of causing disease.
• The Eubacteria are the most abundant organisms on the Earth. It plays critical roles like cycling carbon and sulphur.

1.8 KEY WORDS

• **Microbiology**: Microbiology is the branch of biology which deals with the scientific study of microorganisms; their structure, function and classification and the ways in which they can be controlled and used.
• **Bacteriology**: Bacteriology is the study of Bacteria.
• **Mycology**: Mycology is the study of fungi.
• **Virology**: Virology is the study of viruses.
• **Microbial ecology**: Study of interrelationships between microbes and the environment.
• **Microbial physiology**: Study of metabolism of microbes at the cellular and molecular levels.
• **Molecular biology**: Advanced study of the genetic material, i.e., DNA, RNA and protein synthesis.
• **Micrometry**: Method of measuring a microscopic organism under a microscope with the help of a calibrated scale.
• **Pharmaceutical microbiology**: Study of microorganisms which are related to the production of antibiotics, enzymes, vitamins, biologics and other pharmaceutical products.
• **Microbial genetics**: Study of regulation of microbe genes in relation to their function.
• **Archaebacteria**: Unicellular forms that are Prokaryotic and exists in extreme climates.
• **Eubacteria**: Unicellular forms that are prokaryotic, heterotrophic, autotrophic and chemotrophic, for example, Bacteria, Cyanobacteria.
• **Cyanobacteria**: They are capable of photosynthesis, also known as Blue Green Algae.

1.9 SELF ASSESSMENT QUESTIONS AND EXERCISES

**Short Answer Questions**

1. Describe the evolution and scope of microbiology.
2. Differentiate between pure and applied microbiology.
3. Define the concept of micrometry.
NOTES

4. List the areas of microbiological studies.
5. Explain the three kingdom classification system.
6. What are the five kingdom classifications proposed by Whittaker?
7. Brief about three domain concept of Carl Woese.

Long Answer Questions

1. Discuss about the science of microbiology and the methods used in the study of microorganisms giving appropriate examples.
2. Briefly explain the historical development of microbiology.
3. Discuss about the distribution of microorganisms among the various kingdoms or domains giving appropriate examples.
4. Explain how an unexpected link can be established between a suspected microorganism and a disease based on Koch’s postulates.
5. Detail a note on Haeckel’s three kingdom classification and Whittaker’s five kingdom classification including their limitations and implications.
6. Discuss three domain concept of Carl Woese.

1.10 FURTHER READINGS

UNIT 2 MICROBIAL DIVERSITY

Structure
2.0 Introduction
2.1 Objectives
2.2 Classification of Bacteria According to Bergey’s Manual
2.3 Fungi: General Characteristics
2.4 Industrial Uses of Yeasts and Moulds
2.5 Lichens: Structural Organisation and Their Properties
2.6 Answers to Check Your Progress Questions
2.7 Summary
2.8 Key Words
2.9 Self Assessment Questions and Exercises
2.10 Further Readings

2.0 INTRODUCTION

Fungi are the members of a large group of eukaryotic organisms including microorganisms, such as yeasts, moulds and mushrooms. One major difference between plant and fungal cells is that the wall of the latter consists of chitin, whereas that of the former is made of cellulose. Like animals, Fungi have the ability to form hydrolytic enzymes that break down complex molecules, which can be absorbed for nutrition. Fungi live with their own food supply and simply grow into new food as the local environment becomes nutrient depleted. They have high protein content but low fat and carbohydrate contents. They are also rich in vitamins and minerals.

The baking and brewing industries are dependent on metabolic activities of yeast. Other industries use fungal species in the production of organic compounds and vitamins. Many enzymes are produced from fungal sources. The fine flavour of certain cheese, such as Roquefort is due to the fungus called Penicillium. Mycorrhizal Fungi help in the growth of trees. Predacious Fungi help in biological control of pestilence. The Fungi present in soil destroy parasitic nematodes. Many Fungi, particularly moulds and yeasts, are largely responsible for food spoilage. Moulds like Aspergillus secrete compounds called ‘Aflatoxins’ that are toxic for both man and animal.

A lichen is a composite organism consisting of a fungus living in intimate association with one or more photosynthetic partners that may be either a Green Alga or a Cyanobacterium. The fungal member of the lichen is called mycobiont and the photosynthetic partner is called photobiont. The two organisms live in close association with each other so much so that they appear to be a single organism. Normally, only one species of photobiont is found in association with each species of lichen fungus; there are some lichens which contain both an Algal and Cyanobacterial Photobionts.
In this unit, you will study about classification of Bacteria according to Bergey’s Manual, general characteristics of Fungi, industrial uses of yeasts and moulds, structural organisation of lichens and their properties in detail.

### 2.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand the characteristic features of Bacteria
- Classify Bacteria according to Bergey’s Manual
- Analyse the general characteristics of Fungi
- Discuss industrial uses of yeasts and moulds
- Explain the structural organisation of lichens and their properties

### 2.2 CLASSIFICATION OF BACTERIA ACCORDING TO BERGEY’S MANUAL

In 1750, Carl Linnaeus devised the first classification system, placing plants and animals in separate systems. He placed all microorganisms in one genus which was named Chaos. The first attempt to classify microscopic plants and animals was apparently undertaken by Müller in 1773. He placed all of them in a group known as the ‘Infusoria’. Later classifications were proposed by Ehrenberg (1838), Dujardin (1841), Perty (1852), Cohn (1854), Zopf (1885) and Migula (1897). Cohn believed that Bacteria showed more of the characteristics of plants than of animals and suggested that they should be classified in the plant kingdom. Nägeli (1857) appeared to be the first to suggest the use of the term ‘Schizomycetes’ (Fission Fungi) to include all organisms classified as Bacteria. Cohn (1872) believed that it was a difficult task to classify Bacteria into genera and species. He pointed out that so many species looked alike that a classification based on morphology alone was insufficient. Zopf (1879) did not agree with Cohn’s idea of fixity of species; however he classified Bacteria into following four groups on the basis of morphology. The classification was as follows:

- **Coccaceae**: Spherical forms.
- **Bacteriaceae**: Spherical, rod and filamentous forms, with no distinction between base and apex.
- **Leptotrichiae**: Spherical, rod and filamentous forms, the latter with distinction between base and apex.
- **Cladotrichiae**: Spherical, rod and spirals, and showing dichotomy.

The three kingdom system was given by Haeckel, in 1866. He was the first scientist to create a natural kingdom for the microbes, which had
been discovered nearly two centuries before by Antony van Leeuwenhoek. Haeckel placed all unicellular (microscopic) organisms in a new kingdom, called ‘Protista’, on the level with the existing kingdoms for plants (Plantae) and animals (Animalia), which are multicellular (macroscopic) organisms, followed by creation of four kingdom system (1950), where four kingdoms were created. The cells lacking the intracellular compartment of nucleus were shifted to fourth kingdom called Monera. Protista remained as a kingdom of Eukaryotic microorganisms. Later in 1967, Whittaker, a botanist at the University of California, refined the system into five kingdoms, by identifying the Fungi as a separate multicellular Eukaryotic kingdom of organisms, distinguished by their absorptive mode of nutrition. The developments of various molecular techniques resulted in changes in classification of Bacteria as well. Phylogenetic analysis of all forms of cellular life based on comparison of nucleotide sequences of the small subunit ribosomal RNA (ssrRNA) that is contained in all organisms was done by Carl Woese in 1970s.

He found that rRNA found in all cells, it could be analyzed to determine the exact sequence of nucleotide bases in its makeup. His analysis of RNA molecules from different types of cells revealed a new dichotomy, among the Prokaryotes. There exist two types of Prokaryotes, as fundamentally unrelated to one another as they are to Eukaryotes. Thus, Woese defined three cellular domains of life: Eukaryotes, EuBacteria and ArchaeBacteria. Whittaker’s Plant, Animal and Fungi kingdoms (all of the multicellular Eukaryotes) are at branch tips of the Eukaryote Domain, while other Eukaryote branches lead to Protists (unicellular Algae and Protozoa). Although the definitive difference between Woese’s Archaea and Bacteria is based on fundamental differences in the nucleotide base sequence in the ssrRNA, there are many biochemical and phenotypic differences between the two groups of Prokaryotes. Archaea are more closely related to Eukaryotes than Bacteria.

Thus, on the basis of small subunit ribosomal RNA (rRNA), the cell’s membrane lipid structure and its sensitivity to antibiotics the Woese’s classification forms a tree of life; this has three branches: Archaea, Bacteria and Eukaryotes. Bacteria and Archaea share the Prokaryotic type of configuration while Eukaryotes consists of all Eukaryotic cell types, including Protista, Fungi, Plants and Animals.

**A Revolution in Bacterial Taxonomy:** The term Bacterial taxonomy refers to a natural system of classification based on phylogenetics characters. In certain cases morphological character, such as shape, mode of cell division, lack of cell wall have no phylogenetic connection and can lead to ambiguity. Taxonomy refers to the academic discipline of defining groups of biological organisms on the basis of shared characteristics and giving names to those groups. Each group is given a rank and groups of a given rank can be aggregated to form a super group of higher rank and thus create a hierarchical classification. Bacterial taxonomy is, therefore, the rank-based
scientific classification of Bacteria. In the scientific classification established by Carl von Linné, each distinct species is assigned to a genus using a two-part binary name, for example, *Homo sapiens*. This distinct species is then placed within a hierarchy of ranks. These ranks range in ascending scale from family to suborder, and upward to order, subclass, class, division/phyla, kingdom and domain.

At present, as per the accepted scientific classification, there are three domains of microorganisms: the Eukaryotes, Bacteria and Archaea.

**Road Map to Bergey’s Classification**

With the rapid advances in molecular sequencing of highly conserved regions of the Prokaryotic genome, most notably genes coding for the RNA of the small ribosomal subunit, have led to their arrangement on the basis of some physical or metabolic similarities. Due to bewildering diversity of microorganisms it is desirable to classify them on the basis of their evolutionary history. Being a heterogeneous group based on differences in cellular organisation and metabolism kingdom Protista has been divided into two groups, namely Prokaryotes and Eukaryotes. Classification of Bacteria based on metabolic requirements allow us to make predictions on different cell components like DNA, fatty acids, antigens, etc.

The classification system however did not give a clarity whether the variations were between distinct species or same species. This could be accounted for lack of distinctive structures in Bacteria as well as lateral gene transfer between unrelated species. This would result in different morphologies and metabolisms in closely related Bacteria. The term ‘Bacteria’ was traditionally applied to all microscopic, single cell Prokaryotes. Molecular systematic showed Prokaryotic life that have evolved into two separate domains Bacteria and Archaea from Gram-Positive Bacteria. This is possible with the introduction of molecular systematic and a rapid increase in the number of genome sequences.

**Classification of Bacteria According to Bergey’s Manual**

The *Bergey's Manual of Systematic Bacteriology* is the key resource used to determine the identity of Bacteria species by taking into account each and every characterizing feature. David Hendricks Bergey has first published this classification system in 1923. Since then it is used to classify Bacteria based on their structural and functional features/attributes by arranging them into specific familial orders.

It was published in following four volumes.

Volume 1 included information on all types of Gram-Negative Bacteria that were considered to have ‘Medical and Industrial Importance’.
Volume 2 included information on all types of Gram-Positive Bacteria.

Volume 3 deals with all of the remaining, slightly different Gram-Negative Bacteria, along with the Archaea.

Volume 4 has information on filamentous Actinomycetes and other similar Bacteria.

Thus the Bergey’s Manual of Systematic Bacteriology is considered as a comprehensive Bacterial classification manuals which broadly classified as follows.

- **Bergey’s Division I:** The Cyanobacteria (formerly the Blue Green Alga). These Bacteria can use light as their energy source under aerobic conditions. They use carbon dioxide and produce oxygen.

- **Bergey’s Division II:** The Bacteria (includes the Photo-Bacteria and all other Classical Bacteria). Refer the 19 divisions of Bacteria that are listed below.

- **ArcheoBacteria:** In the 8th Edition (1974) the ArcheoBacteria were mixed within the 19 divisions.

The Bergey Classification of Bacteria into 19 Divisions

- **Phototrophic Bacteria:** Rhodospirillum - Rhodopseudomonas - Chromatium

- **Gliding Bacteria:** Myxococcus - Beggiatoa - Simonsiella - Leucothrix

- **Sheathed Bacteria:** Sphaerotilus - Leptothrix

- **Budding/Appended Bacteria:** Caulobacter - Gallionella

- **Spirochetes:** Spirochaeta - Treponema - Borrelia

- **Spiral and Curved Bacteria:** Spirillum - Auqaspirillum - Oceanospirillum - Bdellovibrio

- **Gram-Negative Aerobic Rods and Cocci:** Pseudomonas - Xanthanomonas - Zoogloea - Gluconobacter - Azotobacter - Rhizobium - Agrobacterium - Halobacterium - Acetobacter

- **Gram-Negative Facultative Anaerobic Rods and Cocci:** Escherichia - Citrobacter - Salmonella - Shigella - Klebsiella - Enterobacter - Serratia - Proteus - Yersinia - Erwinia - Vibrio - Aeromonas - Zymomonas - Chromobacterium - Flavobacterium

- **Gram-Negative Anaerobes:** Bacteriodes - Fusobacterium - Desulfovibrio - Succinimonas

- **Gram-Negative Cocci:** Nisseria - Branhamella - Acinetobacter - Paracoccus

Microbial Diversity
• **Gram-Negative Anaerobic Cocci:** Veillonella - Acidaminococcus  
• **Gram-Negative Chemolithotrophic:** Nitrobacter - Thiobacillus - Siderocapsa  
• **Methane Producing Bacteria:** Methylosinus, Methylocystis, Methanomonas, Methylococcus, Methanobacter, Methylococcus  
• **Gram-Positive Cocci:** Micrococcus - Staphylococcus - Streptococcus - Leuconostoc - Pediococcus - Aerococcus - Peptococcus - Ruminococcus - Sarcina  
• **Endospore-Forming Rods and Cocci:** Bacillus - Clostridium - Sporosarcina  
• **Gram-Positive, Non-Sporing Rods:** Lactobacillus - Listeria - Erysipelothrix - Caryophanon  
• **Actinomycetes and Related:** Corynebacterium - Arthobacter - Brevibacterium - Cellumonas - Kurthia - Propionibacterium - Eubacterium - Actinomyces - Archina - Bifidiobacterium - Rothia - Mycobacterium - Frankia - Streptosporangi - Nocardia - Streptomyces - Streptovericillium - Micromonaspora  
• **Rickettsias:** Rickettsia - Erhlichia - Wollbachia - Bartonella - Chlamydia  
• **Mycoplasmas:** Mycoplasma - Acoleplasma - Thermoplasma - Spiroplasma

Figure 2.1 shows the Bergey’s classification of Bacteria and Table 2.1 illustrates the common morphological, physiological and metabolic features used for the identification of Bacteria.
Consequently, the need to identify human pathogens was a major impetus for the development to techniques to identify Bacteria. The Gram Stain, characterises Bacteria based on the structure of their cell walls. The thick layers of peptidoglycan in the gram positive cell wall stain purple while the thin gram negative cell wall appears pink.

**Basic Concept of Taxonomy**

The naming or classifying organisms is called taxonomy. The interrelated disciplines involved are:

- **Identification**: Characterizing Organisms
- **Classification**: Arranging into Similar Groups
- **Nomenclature**: Naming Organisms

Organizing larger organisms based on morphology is often quite simple, such as fins, legs, fur, etc. But with Prokaryotes, it is not all that simple. Prokaryote classification involves technologies used to characterized and identify Prokaryotes.
Classification of Bacteria

There is no ‘official’ classification of Bacteria. The classification scheme which is accepted by most microbiologists is followed. This is according to Bergey’s manual of determinative bacteriology which comes closest to providing a concise and accurate system of classifying Bacteria. A classification that is of little use to microbiologists will soon be ignored or modified. The classification of Bacteria has two purposes. One is purely for descriptive reasons while the other is to assess evolutionary descent by phylogenetic analysis. In the descriptive or determinative analysis the organisms are grouped into one common pool or group and to describe the basis on which these groups are made. The second purpose is to provide a natural phylogenetic classification aimed at reflecting the evolutionary descent. Here organisms are grouped into successive levels of ordering called ‘Taxons’ (Refer Table 2.2) in a hierarchical family tree of species, genus, family, tribe and order.

<table>
<thead>
<tr>
<th>Formal rank</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kingdom</td>
<td>Procaryotae</td>
</tr>
<tr>
<td>Division</td>
<td>Gracilicutes</td>
</tr>
<tr>
<td>Class</td>
<td>Scotobacteria</td>
</tr>
<tr>
<td>Order</td>
<td>Spirochaetales</td>
</tr>
<tr>
<td>Family</td>
<td>Spirochaetaceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Treponema</td>
</tr>
<tr>
<td>Species</td>
<td>Treponema pallidum</td>
</tr>
</tbody>
</table>

Table 2.2 Taxonomic Ranks of Bacteria Explained with Example

I. The Archaea (Archaeabacteria)

The Archaea possess the following characteristics:

- Archaea are Prokaryotic cells.
- Unlike the Bacteria and the Eukarya, the Archaea have membranes composed of branched hydrocarbon chains, many also containing rings within the hydrocarbon chains attached to glycerol by either linkages.

The cell walls of Archaea contain no peptidoglycan. Archaea are not sensitive to some antibiotics that affect the Bacteria, but are sensitive to some antibiotics that the Eukarya. Archaea contain rRNA that is unique to the Archaea are indicated by the presence molecular region distinctly different from the rRNA of Bacteria and Eukarya.

Membrane lipids of Archaeabacteria and Eukarya. The Bacteria and Eukarya have membranes composed of unbranched fatty chains. Archae have composed of hydrocarbon branches (Refer Figure 2.2).
II. The Bacteria (Eubacteria)  Also called True Bacteria are Prokaryotic cells. Eubacteria can be found almost everywhere. The Bacteria possess the following characteristics:

- Bacteria are Prokaryotic cells.
- Like the Eukarya, they have membranes composed of unbranched fatty acid chains attached to glycerol by ester linkages.
- The cell walls of Bacteria, unlike the Archaea and the Eukarya contain peptidoglycan.
- Bacteria are sensitive to traditional antibacterial antibiotics but are resistant to most that affect Eukarya.
- Bacteria contain rRNA that is unique to the Bacteria as indicated by the presence of molecular regions distinctly different from the rRNA of Archaea and Eukarya.

Bacteria include Mycoplasmas, Cynobacteria, Gram-Positive Bacteria and Gram- Negative Bacteria.

III. The Eukarya (Eukaryotes)

Eukarya possess the following characteristics:

- Eukarya have Eukaryotic cells.
- Like the Bacteria, they have membranes composed of unbranched fatty acid chains attached to glycerol by ester linkages.
- Not all Eukarya possess cells with a cell wall, but for those Eukarya having a cell wall contains no peptidoglycan.
- Eukarya are resistant to traditional antibiotics but sensitive to the ones that affect Eukaryotic cells.
- Eukarya contains rRNA that is unique to the Eukarya as indicated by the presence of molecular regions different from Archaea and Bacteria.

Bergey’s Manual is based on following three primary features:

- Cell wall (or lack of cell wall), Gram reaction +ve or –ve.
- Cell morphology (shape) Bacillus, Coccus, Spirillum, Vibrio.
- Biochemical characteristics: Sugars they ferment, Enzymes like catalase and oxidase, decarboxylase, etc.

Table 2.3 shows a comparative account of the characteristic features of Bacteria, Archaea and Eucarya and Table 2.4 shows the classification of Bacteria following Bergey’s Manual of Systematic Bacteriology.

Table 2.3  A Comparative Account of the Characteristic Features of Bacteria, Archaea and Eucarya

<table>
<thead>
<tr>
<th>Property</th>
<th>Bacteria</th>
<th>Archaea</th>
<th>Eucarya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus with Nucleolus</td>
<td>Absent</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Membranous Cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organelles</td>
<td>Absent</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Cell Wall</td>
<td>Peptidoglycan</td>
<td>Various Types without Muramic Acid</td>
<td>No Muramic Acid</td>
</tr>
<tr>
<td></td>
<td>containing Muramic Acid</td>
<td></td>
<td>Acid</td>
</tr>
<tr>
<td>Membrane Lipids</td>
<td>Esterlinked</td>
<td>Esterlinked</td>
<td>Esterlinked</td>
</tr>
<tr>
<td></td>
<td>Straight</td>
<td>Branched</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chain Fatty Acids</td>
<td>Chain Fatty Acids</td>
<td>Straight Chain Fatty Acids</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fatty Acids</td>
</tr>
<tr>
<td>Gas Vesicles</td>
<td>Present</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>t – RNA</td>
<td>with Thymine and</td>
<td>No Thymine and</td>
<td>Thymine and</td>
</tr>
<tr>
<td></td>
<td>Initiation with N-</td>
<td>Initiation with</td>
<td>Initiation with</td>
</tr>
<tr>
<td></td>
<td>Formylmethionine</td>
<td>Methionine</td>
<td>Methionine</td>
</tr>
<tr>
<td>Polysytronic m-RNA</td>
<td>Present</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>m-RNA introns</td>
<td>Absent</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Ribosomes</td>
<td>70S</td>
<td>70S</td>
<td>80S</td>
</tr>
<tr>
<td>DNA Dependent RNA</td>
<td>4 Subunits</td>
<td>8-12 Subunits</td>
<td>12-14 Subunits</td>
</tr>
<tr>
<td>Polymerase Structure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATPase Similarity</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Methanogenesis</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Nitrogen Fixation</td>
<td>Present</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Photosynthesis</td>
<td>Present</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Chemolithotrophy</td>
<td>Present</td>
<td>Present</td>
<td>Absent</td>
</tr>
</tbody>
</table>

Source: Prescott et al. (2005).
Table 2.4 Classification of Bacteria Following Bergey's Manual of Systematic Bacteriology

<table>
<thead>
<tr>
<th>Taxonomic Rank</th>
<th>Representative Genera</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Volume 1. The Archaea and the Deeply Branching and Phototrophic Bacteria</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Domain Archaea</strong></td>
<td></td>
</tr>
<tr>
<td>Phylum Crenarchaeota</td>
<td>Thermoproteus, Pyrodictium, Sulfolobus</td>
</tr>
<tr>
<td>Phylum Euryarchaeota</td>
<td></td>
</tr>
<tr>
<td>Class I. MethanoBacteria</td>
<td>Methanobacterium</td>
</tr>
<tr>
<td>Class II. Methanococci</td>
<td>Methanococcus</td>
</tr>
<tr>
<td>Class III. Halobacteria</td>
<td>Halobacterium, Halococcus</td>
</tr>
<tr>
<td>Class IV. Thermoplasmata</td>
<td>Thermoplasma, Picrophilus</td>
</tr>
<tr>
<td>Class V. Thermococci</td>
<td>Thermococcus, Pyrococcus</td>
</tr>
<tr>
<td>Class VI. Archaeoglobi</td>
<td>Archaeoglobus</td>
</tr>
<tr>
<td>Class VII. Methanopyri</td>
<td>Methanopyrus</td>
</tr>
<tr>
<td><strong>Domain Bacteria</strong></td>
<td></td>
</tr>
<tr>
<td>Phylum Aquificae</td>
<td>Aquifex, Hydrogenobacter</td>
</tr>
<tr>
<td>Phylum Thermotogae</td>
<td>Thermotoga, Geotoga</td>
</tr>
<tr>
<td>Phylum ThermodesulfoBacteria</td>
<td>Thermodesulfobacterium</td>
</tr>
<tr>
<td>Phylum &quot;Deinococcus-Thermus&quot;</td>
<td>Deinococcus, Thermus</td>
</tr>
<tr>
<td>Phylum Chrysochromulina</td>
<td>Chrysochromulina</td>
</tr>
<tr>
<td>Phylum Chloroflexi</td>
<td>Chloroflexus, Herpetosiphon</td>
</tr>
<tr>
<td>Phylum Thermomicrobia</td>
<td>Thermomicrobium</td>
</tr>
<tr>
<td>Phylum Nitrospira</td>
<td>Nitrospira</td>
</tr>
<tr>
<td>Phylum Deferribacteres</td>
<td>Geovibrio</td>
</tr>
<tr>
<td>Phylum CyanoBacteria</td>
<td>Synechococcus, Pleurocapsa, Anabaena</td>
</tr>
<tr>
<td>Phylum Chlorobi</td>
<td>Chlorobium, Pelodictyon</td>
</tr>
<tr>
<td><strong>Volume 2. The ProteoBacteria</strong></td>
<td></td>
</tr>
<tr>
<td>Phylum ProteoBacteria</td>
<td></td>
</tr>
<tr>
<td>Class I. AlphaproteoBacteria</td>
<td>Rhodospirillum, Caulobacter, Rhizobium, Methylobacterium, Hyphomicrobium</td>
</tr>
<tr>
<td>Class II. BetaproteoBacteria</td>
<td>Neisseria, Alcaligenes, Comamonas, Nitrosonomas, Methylphilus</td>
</tr>
<tr>
<td>Class III. GammaproteoBacteria</td>
<td>Chromatium, Pseudomonas, AzotoBacter, Escherichia, Klebsiella, Proteus, Salmonella</td>
</tr>
<tr>
<td>Class IV. DeltaproteoBacteria</td>
<td>Desulfovibrio, Bdellovibrio, Myxococcus</td>
</tr>
<tr>
<td>Class V. EpsilonproteoBacteria</td>
<td>Campylobacter, Helicobacter</td>
</tr>
<tr>
<td><strong>Volume 3. The Low G + C Gram-Positive Bacteria</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
</tr>
<tr>
<td>Phylum Firmicutes</td>
<td></td>
</tr>
<tr>
<td>Class I. Clostridia</td>
<td>Clostridium, Eubacterium, HelioBacterium</td>
</tr>
<tr>
<td>Class II. Mollicutes</td>
<td>Mycoplasma, Ureaplasma, Spiroplasma</td>
</tr>
</tbody>
</table>
Bacteria are Prokaryotes, evolved first on living Earth. They do not contain membrane bound nucleus and organelles. Circular genomes and extrachromosomal DNA are present which helps in surviving different environment. Bacteria reproduce by prokaryotic fission, resulting in two genetically identical daughter cells. Bacterial cell walls are made of peptidoglycan contains sugar moieties (N-Acetylglucosamine, and N-Acetylmuramic acid cross linked with Pentapeptide (D-Amino Acids). Gram Stain is used for identification based on the constituents of cell wall.

**Gram-Positive Bacteria** have simple cell walls with large amounts of peptidoglycan that retains crystal violet. **Gram-Negative Bacteria** have more complex cell walls with less peptidoglycan that retains saffron. These are causative agents of most infectious human diseases. The unique lipid layer of the gram negative Bacteria are often toxic and saves pathogen from host defensive mechanisms.

**Classification Based on Nutrition and Metabolism**

Organisms can be categorized based on their nutrition, i.e., on how they obtain energy and carbon to build the organic molecules that make up their cells.

- **Autotrophs**: Obtain carbon source from inorganic compound.
- **Phototrophs**: Obtain energy from light.
- **Chemotrophs**: Obtain energy from chemicals.
- **Heterotrophs**: Obtain their carbon source from glucose as the organic nutrient.
• **Obligate Aerobes:** Requires oxygen for respiration.
• **Facultative Anaerobes:** Can grow both aerobically and anaerobically.

**Bacterial Taxonomy**

Prokaryotic taxonomy based on the criteria, such as shape, motility, nutritional mode and gram staining, etc., were important for culturing Bacteria and identifying them. But they did not reflect evolutionary relationships. For this reason molecular data was applied for investigation of Prokaryotic phylogeny and a comparison of sequences of Prokaryotic genes Carl Woese and his colleagues used ribosomal RNA as a marker for evolutionary relationship.


• **Crenarchaeota:** Diverse kingdom that contains Thermophilic and Hyperthermophilic.
• **Euryarchaeota:** Contains primarily Methanogenic and Halophilic Bacteria and Eubacteria – complex with several small groups of phototrophs, Cyanobacteria and deeply branching Eubacteria.

**Based on Bergey’s Manual Domain Bacteria Contains following Six Phyla in Volume One:**

• Phylum *Aquificiae:* Earliest branch of Bacteria that contain autotrophs which utilize hydrogen for energy production.
• Phylum *Thermotogae:* Anaerobic, thermophilic and fermentative Gram- Negative Bacteria.
• Phylum ‘Deinococcus Thermus:* Radiation resistance Bacteria.
• Phylum *Chloroflexi:* Green non-sulphur Bacteria that carries out anoxygenic photosynthesis.
• Phylum *Cyanobacteria:* Oxygenic photosynthetic Bacteria.
• Phylum *Chlorobil:* Green sulphur Bacteria that carry out anoxygenic photosynthesis.

DNA based tools used as diagnostics, for example, PCR are increasingly acceptable due to their specificity and speed compared to culture based methods. These methods allow the detection of viable but non-culturable cells that are though not dividing but metabolically active. Still even with advance in technology the total number of Bacterial species remain obscure.
2.3 FUNGI: GENERAL CHARACTERISTICS

Fungi belongs to domain Eukarya. They are unicellular (Yeast) as well as multicellular organisms (filamentous Fungi). Multicellular Fungi are composed of filaments called hyphae. Hyphae may be branched with internal cross walls, called septa which divide the hyphae into separate cells. They are saprophytic in nature and absorb nutrients after degrading the organic matter and heterotrophs. They have cell walls composed of chitin. Reproduction in them takes place through production of spores.

The fusion of hyphae from two individual is called plasmogamy. The fused hyphae contains haploid nuclei from two individuals are heterokaryotic. In some plasmogamy results in cells with one nucleus from each individual. This condition is called dikaryotic. Classification is done based on the mode of reproduction.

Structure of Fungal Cell: It consists of strong rigid cell wall enclosing the protoplast except for moulds. It protects the cell from lysis and metabolites from other organisms. It also acts the binding site of some enzymes. Chemical composition not all the same chitin is characteristically present in all the fungal cells.

The Fungal Tissue: It is a mass of hyphae interwoven loosely to form a network. During life cycle this becomes organised into a woven tissue called the parenchyma.

Specialised Somatic Structures

- **Rhizoids**: It is a short root like filamentous branch of the thallus present in both unicellular as well as mycelia thalli. These function as anchoring and absorbing units.

- **Rhizomorphs**: There are Fungi whose hyphae aggregate together to form a root like strand like a hard cortex and develop like a root tip. It mainly serves to function for absorption. Its growth may be renewed with the return of favourable conditions.
Fungal Taxonomy

Historically, classification is based on certain observable characteristics, i.e., the phenotypic approach. Morphological characteristics differ greatly depending on species and may not be reproduced under artificial conditions. The characteristics typical to Fungi have arisen polyphetically and there seems to be definite trend towards asexual reproduction, reducing the spectrum towards morphological characteristics. Out of the true biodiversity of Kingdom Fungi, which has been estimated at around 1.5 million species, with about 5% of these having been formally classified. The inability to identify plant pathogens from culture-based morphological techniques has led to the development of culture independent molecular approaches. Species identification hindered by unstable nature of phenotypical methods have been replaced by phylogenetic approaches.

The molecular revolution in fungal taxonomy with PCR amplifies rRNA genes have allowed the analysis of small number of fungal cells or even single spores. The selection of universal oligonucleotide primers specific to Fungi has been used as reference points for evolutionary divergence studies. The parts of the rDNA are conserved nucleotide sequences used in modern systematic. The new concept has now known to be appropriate for fungal groups in which no sexual reproduction has been observed (deuteromycetes).

Lower Fungi

Fungi under this family have non-septate walls and spores contained in small sporangia. These classes of Fungi include three groups, i.e., Chytridiomycota, Oomycota, Myxomycota.

Higher Fungi or True Fungi

- Fungi belongs to this family are having septate cross walls.
- Spores contained in complex structures.
- Include three groups: Zygomycota, Ascomycota, Basidiomycota.

Asexual Spores

Produced by mitosis and cell division.

Sporangiophore

- Spores form sac called sporangiophore.
- Sporangium forms at the end of aerial hyphae called sporangiophore. Hundreds of sporangiospores in a single sporangium.
Conidiospore

- Spores produced at the end of an aerial hyphae is called conidiophore.
- Conidia: Chains of conidiospores on conidiophores (Refer Figure 2.3).

Sexual Spores

Sexual spores formed by the fusion of two haploid nuclei into a diploid zygote. The zygote would then undergo meiosis to generate haploid spores (multiples of four usually).

- Zygosores: One thick spore between two parent hyphae.
- Ascospores: Four spores in a sac called an ascus at the end of one hyphae.
- Basidiospores: Four spores on the end of a basidium.
- Fungi organised into three phyla based on the type of sexual spore.

Classification of Fungi Proposed by Alexopoulos

The organisms of the fungal lineage include mushrooms, rusts, smuts, puffballs, truffles, morels, moulds and yeast. The kingdom Fungi includes some of the most important organisms with main ecological and economical roles. By breaking down the organic matter, they continue the cycle of nutrients through ecosystem.
C.J. Alexopoulos (1962) placed all Fungi along with slime moulds in a separate division Mycota, and was divided into two sub-divisions *Myxomycotina* and *Eumycotina* on the basis of absence and presence of cell wall, respectively.

Slime moulds were included in the subdivision *Myxomycotina* and the True Fungi in *Eumycotina*.

**Division Mycota:** Microscopic thallus, unicellular or filamentous, nucleus with a distinct nuclear membrane and nucleolus, cell wall chitinous or cellulosic, reproduction by asexual means.

**Sub-Division 1: Eumycotina**

Vegetative phase is represented by unicellular or branched siphonaceous mycelium, cell possesses distinct cell wall: hyphae are aseptate and multinucleate or septate. Cells are uninucleate or multinucleate, reproduction by spores or gametes. Sub-division Eumycotina is further divided into following classes.

**Class I: Trichomycetes:** Thallus is simple or branched and multinucleate; often parasitic on arthropods.

**Class II: Oomycetes:** Mycelium well developed and multinucleate during vegetative phase; motile cells biflagellate (one flagellum whiplash and the outer tinsel type). Flagella are arranged in opposite directions.

**Class III: Ascomycetes:** In this, hyphae are septate; ascospores produced endogenously in specialised sporangium, known as ascus.

**Class IV: Deuteromycetes:** In this, hyphae septate, reproduce only by asexual spores, sexual phase lacking.

**Class V: Chytridiomycetes:** They have motile cells with solitary posterior whiplash flagellum.

**Class VI: Zygomycetes:** They are parasitic or saprophytic Fungi, having a well-developed mycelium and multi-nucleate, motile structures absent.

**Class VII: Basidiomycetes:** In this, hyphae are septate, basidiospores exogenously on basidium.

**Class VIII: Hyphochytridiomycetes:** They have motile cells with solitary anterior tinsel flagellum, includes aquatic Fungi.

**Class IX: Plasmodiophoromycetes:** They are parasitic Fungi, they do not have a cell wall, multinucleate thallus remains inside the host tissue, and motile cells with two unequal anterior tinsel flagella.

**Sub-Division 2: Myxomycotina**

Plant body in the form of naked protoplast known as plasmodium.
**Myxomycetes**: Vegetative phase is represented by a solitary large multinucleate naked protoplast plasmodium; reproduction by minute multinucleate walled spores.

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## 2.4 INDUSTRIAL USES OF YEASTS AND MOULDS

Yeast are single celled Fungi. As Fungi, they are related to the other Fungi that people are more familiar with. These include edible mushrooms available at the supermarket, common baker’s yeast used to leaven bread, molds that ripen blue cheese and the moulds that produce antibiotics for medical and veterinary use.

A mould is a fungus that grows in the form of multicellular filaments called hyphae. In contrast, Fungi that can adopt a single celled growth habit are called yeasts. Moulds are a large and taxonomically diverse number of fungal species in which the growth of hyphae results in discoloration and a fuzzy appearance, especially on food. The network of these tubular branching hyphae, called a mycelium, is considered a single organism. The hyphae are generally transparent, so the mycelium appears like very fine, fluffy white threads over the surface. Cross-walls (septa) may delimit connected compartments along the hyphae, each containing one or multiple, genetically identical nuclei. The dusty texture of many molds is caused by profuse production of asexual spores (conidia) formed by differentiation at the ends of hyphae. The mode of formation and shape of these spores is traditionally used to classify molds. Many of these spores are colored, making the fungus much more obvious to the human eye at this stage in its life-cycle. Yeast and moulds are widely used in the industries, few of the industrial uses of yeast and moulds is discussed here.

**Yeast**

The yeast have a lot of economic value to mankind, which are as follows:

- **Bread Making**: Yeast is used for the manufacture of bread. Ethyl alcohol and carbon dioxide are formed. The bread becomes spongy due to pressure exerted by accumulation of carbon dioxide.
- **Saccharomyces cerevisiae** (yeast) popularly known as Baker’s Yeast in baking industry.
**Production of Alcohol:** Ethyl alcohol is manufactured by the fermentation of potatoes, cereals, etc.

\[
\text{C}_6\text{H}_{12}\text{O}_6 + \text{Yeast} \rightarrow \text{zymase} \quad 2\text{C}_2\text{H}_5\text{OH} + 2\text{CO}_2
\]

(Glucose) + Fermentation \quad (Ethyl Alcohol) + (Carbon Dioxide)

**Industrial use of Yeast:** The breakdown of carbohydrates - starch and sugar into carbon dioxide and ethyl alcohol in the absence of oxygen is called alcoholic fermentation. Alcohol is the major ingredient in the brewing industry.

- Yeast as a Source of Food: Dried yeast cells contain protein. Some have considerable amount of fat.
- As a source of Vitamin B complex and Vitamin D. Also used in medicine as a source of enzyme.
- Cheese Making: *Penicillium camemberti* and *Penicillium roquefortie* are used in cheese making. These moulds add a special flavour to the cheese.
- Role of Fungi in Medicine: More than 700 fungal species secrete antifungal and anti-Bacterial substances called antibiotics.
- *S. cerevisiae var ellipsoideus* is a high alcohol yielding variety used to produce industrial alcohol, wines and distilled liquors.
- Yeasts belonging to the genus Kluyveromyces reproduce by multilateral budding, and ascospores are liberated upon maturity.
- Yeasts belonging to the genus Zygosaccharomyces are notable for their ability to grow in high concentrations of sugar, hence they are termed osmophilic, and are involved in the spoilage of honey, syrups, and molasses and in the fermentation of soy sauce and some wines. *Z. nussbaumeri* grows in honey.
- Yeasts belonging to the genus Pichia are oval to cylindrical yeasts and may form pseudomycelia. Ascospores are round or hat-shaped, and there are one to four per ascus. A pellicle is formed on liquids; for example, *P. Membranae faciens* grows a pellicle on beers or wines.
- Yeasts of genus Hansenula resemble Pichia in appearance but are usually more fermentative, although some species form pellicles. Ascospores are hat or Saturn-shaped.
- Yeasts of genus Debaryomyces are round or oval yeasts form pellicles on meat brines. Ascospores have a warty surface. *D. kloeckeri* grows on cheese and sausages.
- Genus Hanseniaspora comprises lemon-shaped (apiculate) yeasts which grow in fruit juices. Nadsonia yeasts are large and lemon-shaped.
- Genus Torulopsis comprises round to oval fermentative yeasts with multilateral budding which cause spoilage in breweries and food.
Microbial Diversity

NOTES

industry. *T. sphaerica* ferments lactose and may spoil milk products. Other species can spoil sweetened condensed milk, fruit-juice concentrates, and acid foods.

- Genus Candida comprises yeasts which form pseudohyphae or true hyphae, with abundant budding cells or blastospores, and may form chlamydospores. Many form films and can spoil foods high in acid and salt. *C. utilis* is grown for food and feed. *C. krusei* has been grown with dairy starter cultures to maintain the activity and increase the longevity of the lactic acid Bacteria. *C. lipolytica* can spoil butter and oleomargarine.

- Yeasts belonging to the genus Brettanomyces are arch-shaped yeasts which produce high amounts of acids and are involved in the late fermentation of Belgian lambic Beer and English Beer. They also are found in French Wines. *B. bruxellensis* and *B. lambicus* are typical species.

- Yeasts belonging to the genus Kloeckera are imperfect apiculate or lemon-shape yeasts. *K. apiculata* is common on fruits and flowers and in the soil.

- Genus Trichosporon includes yeasts that bud and form arthrospores. They grow best at low temperatures and are found in breweries and on chilled beef. *T. pullulans* is a common species.

- Yeasts belonging to the genus Rhodotorula form coloured spots on meats or pink areas in sauerkraut. These red, pink, or yellow yeasts may cause discolourations on foods.

Slime Moulds

Slime moulds have both the properties of Fungi and Amoeba, and mostly related to Amoebazoa. They are the parasites of Bacteria and Fungi and produce spores in unfavourable conditions. It has been divided into two phyla, cellular slime moulds and plasmodial slime molds:

- **Cellular Slime Moulds:** In favourable conditions, they exist as unicellular amoeba and in unfavourable conditions, they form as aggregate of multicellular mushroom like structure to generate spores. When return to favourable conditions, spores germinate into unicellular Amoeba.

- **Plasmodial Slime Moulds:** In favourable conditions, they exist as plasmodium containing multinucleated mass of protoplasm. They have Amoeba like movement. Under unfavourable conditions, they form mycelium, which in turn produces spores on aerial hyphae. When it returns to the favourable conditions, spores germinate and undergo rapid cell division to form new plasmodium.
Following are some of the Industrial Uses of Moulds:

- Selected strains of *Aspergillus niger* are used for the industrial production of citric and gluconic acids.

- *Mucor racemosus* and *Mucor rouxii* are utilized in the preparation of sugars by the process of saccharification. Saccharification involves the breakdown of complex saccharides, such as starch into simpler products.

- Fungi, due to their characteristic pungent flavour form an important part of mediterranian and oriental cuisines. A large number of edible moulds are found in Japan, Mexico, China and Korea.

- Moulds have an important role in the cheese industry. Moulds impart the characteristic flavour to the cheese. Denmark which is called the country of cheese extensively uses the beneficial property of moulds to develop a wide variety of cheese, such as Stilton, Gruere, Parmesean, Moserella, Roquefort, Danish Blue Cheese, Soft Cheese, Hard Cheese and Semi-Hard Cheese.

- Bread mould is extensively used in bakery products. Bread moulds also cause spoilage of fruits, vegetables, processed sauce and ketchups. So extensive studies are still going on to control its propagation which starts just when the food crosses its shelf life.

- Rhizopus mould present in the chicken meat and fish that causes a whiskey flavour to develop and thereby causing the food to spoil.

- *Afflatus oryzae*, a variant of mould is essential in the preparation of the Oriental cuisine by producing the favourable enzymes which marinates and softens the hard meat. This species is found in many forms and colours.

- *Penicillium notatum* is the mold which is used extensively for the production of Penicillin vaccine. *Penicillium notatum* is also referred to as *P. chrysogenum*.

- Moulds cause extensive spoilage of fruits. Blue mould causes rotting of fruits whereas grayish conidia are helpful in the ripening of fruits. A few species with ascospores have the tendency to spoil canned foods with acidic base.

- Pink mould is very common; it can be seen on rotten wooden surfaces, on moistened paper, inside and outside fruit peels, such as that of apples and peaches and to some extend in vegetables, such as cucumber and cantaloupes.

- Red bread mould is pink in colour and grows on bread which has crossed its shelf life. There are also instances when we find this variety of mould in the sugarcane crops. This to some extend causes reduction
in the crop productivity. So proper Fungicide is used to prevent its spread.

- White spot, which is very common in vine crops, is caused due to *Botrytis cinera*. This mould is also sometimes found in chilled meat and fish items. It can be detected by looking for white spots in them, as otherwise eating them lead to food poisoning.

- *C. herbarum* is a species which can cause black spot to be formed on fruits and especially on wheat or cereals which have been kept in humid condition for long duration of time.

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### Check Your Progress

7. How is bread formed?
8. What is Pichia?
9. What is *Penicillium notatum*?

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### 2.5 LICHENS: STRUCTURAL ORGANISATION AND THEIR PROPERTIES

A lichen is a composite organism that consists of a fungus living in intimate association with one or more photosynthetic partners that may be either a green alga or a cyanobacterium. The fungal member of the lichen is called **mycobiont** and the photosynthetic partner (Algae or Cyanobacterium) is called **photobiont**. The two organisms live in close association with each other so much so that they appear to be a single organism. Normally, only one species of photobiont is found in association with each species of Lichen Fungus; there are some lichens which contain both an algal and Cyanobacterial Photobionts. Lichens are known where a single photobiont is in association with two fungal partners.

The association of a fungus and an alga and/or cyanobacterium in the lichen is generally believed to be symbiotic in which each partner of the association derives something essential for its survival. Apparently the alga or cyanobacterium provides the fungus with food, particularly carbohydrates produced by photosynthesis, and possibly with vitamins. The fungus probably absorbs, stores, and supplies water and minerals required by the alga or cyanobacterium. The remarkable aspect of the association is that its form is so constant that lichens are classified into families, genera and species, and their taxonomy is based exclusively on fungal characteristics.

Lichens are the result of a fungus and an algae living together. The mycobiont, a fungus is mostly ascomycetes, but some basidiomycetes. It provides a suitable environment and minerals to algae. The Photobiont, an
Algae-Green or Cynobacterium. It provides carbohydrates and nitrogen compounds to fungus. Symbiosis allows for them to live in harsh environments like rock surfaces, tree trunks. The ability to survive is related to ability to dehydrate quickly. Fungal surface blocks UV light. Figure 2.4 shows the cross-section of a lichen thallus.

![Fig. 2.4 Cross-Section of a Lichen Thallus](image)

The above Figure 2.4 illustrates the cross-section of a lichen thallus shows the (a) Upper Cortex of Fungal Hyphae, which provides protection; the (b) Algal Zone where Photosynthesis occurs, the (c) Medulla of Fungal Hyphae, and the (d) Lower Cortex, which also provides Protection and may have (e) Rhizines to Anchor the Thallus to the Substrate.

**Structure of Lichens**

On the basis of their overall habit, lichens are classified into three main morphological groups: Crustose, Foliose and Fruticose Lichens.

- **Crustose Lichens**: Crustose lichens are tightly attached to their substrate that often they cannot be separated from it.
- **Foliose Lichens**: They are leaf like thalli and are partially attached to their substrate by hair like rhizinae.
- **Fruticose Lichens**: They are erect, shrubby or pendent and hair like or strap-shaped structures attached only at their base.
Loosely organised fungal hyphae and algal component growing as powdery patches constitute the simplest type of lichen thallus known as leprose.

**Internal Structure**

The internal structure of lichens contains the following parts:

- **Upper Cortex:** It is composed of more or less compactly, interwoven, thick-walled and heavily gelatinised fungal haphae, usually without intercellular spaces.
- **Algal Layer:** In this layer, the algal cells are surrounded by thin-walled loosely packed fungal hyphae.
- **Medulla:** It makes up the bulk of the thallus and consists of loosely-packed and thick-walled fungal hyphae.
- **Lower Cortex:** In some, especially foliose forms, there may be a lower cortex consisting of closely packed, dark coloured hyphae. From the lower cortex, arise attaching and absorbing rhizinae.

**Special Structures of Lichen Thallus**

Below are the special structures of lichens thallus:

- **Cyphellae and Pseudocyphellae:** Cyphellae and Pseudocyphellae are the pores or cracks found on the upper or lower cortical layers.
- **Cephalodia:** In many lichens, two photobionts (usually a Green Alga and a Cyanobacterium) are associated with a fungus. In such cases, Cyanobacterial cells are restricted to dark coloured, peculiar gall like swellings known as cephalodia on the upper surface of the lichen thallus.
- **Soredia:** Soredia are minute, rounded structures forming a powdery granule on the surface of the lichen thallus. They consist of a few algal cells closely surrounded by fungal hyphae.
- **Isidia:** Isidia are minute, coral like structures consisting of both fungal hyphae and algal cells. They are found on the upper surface or margins of the lobes of lichens.

**Physiological Characteristics of Lichens**

Following are some physiological features of lichen:

- **Growth Rates:** Lichens grow very slowly, which enables them to tolerate habitats where the water and nutrients supply is very slow.
- **Adaptations to Fluctuating Moisture Conditions:** Lichens desiccate rapidly in dry conditions and with the onset of a rain shower, they absorb water quickly. They are also capable of absorbing water vapour.
from humid atmospheres; and for desert species dew is a crucially important source of water.

- **Photosynthesis and Respiration**: The rate of photosynthesis and respiration depends critically upon water content. Due to the intimate association of the fungus and the autotroph some of the water becomes available to the autotroph, allowing it to photosynthesize at lower water potentials.

- **Absorption of Nutrients**: Lichens can absorb large amounts of substances from solution. The fungal host is usually considered to be primarily responsible for the massive uptake.

**Properties of Lichens**

- Lichens are regarded as the pioneers of vegetation as they are capable of colonising bare rocks.
- These plants play a very important role in the ecological formation of soil.
- The organic acid secreted by lichens gradually dissolve and disintegrate the rocks into soil particles over which they grow. Mosses are the successors of the crustaceous rocks.
- When the lichens die and decay they contribute organic matter to the soil, improve the soil fertility, so that other plants can grow on it.
- Lichens can enrich the soil by trapping water to support an active life over long dry spell.

**Economic Importance of Lichens**

Following are the economic importance of lichens:

- **Pioneers of Vegetation**: The lichens are considered to be ‘pioneer’ organisms because they may make a region suitable for other plants through the weathering of rocks and the accumulation of organic debris.

- **Use in Perfumery**: Several species of lichens are used in the preparation of dhup and hawan samagris, and other perfumes. *Evernia prunastri* and *Pseudovernia furfuracea* (Tree Moss) are the source of an oily substance used in the perfume.

- **In Tanning Industry**: Some lichens, for example, *Lobaria pulmonaria* and *Cetraria islandica* are used as tanning agents in tanning industry.

- **Harmful Properties of Lichen Substances**: Various lichen substances are reported to cause contact dermatitis, a severe skin rash in humans.

- **Lichens as Food**: Numerous small creatures, for example mites, slugs, snails, caterpillars and termites are known to feed upon lichens like *Aspicilia calcarea*, and *Lecanora saxicola*.
• **Lichens as Source of Dyes:** Lichens were used as a source of dyestuff from the time of the ancient Greeks, but are of little economic importance today due to the use of synthetic dyes.

• **Application to Pharmacology and Medicine:** Several species of lichen were of use in earlier times in the treatment of various diseases, but today, they have been replaced by more effective drugs.

• **Lichen as Bio-Indicators or Pollution Indicators:** Lichen are able to survive in harsh climate but they are very sensitive to air pollution. Fruticose lichen is the most vulnerable and Crustose is the least vulnerable lichen type to air pollution. They are pollution sensitive so they can provide the valuable information about the environment. Lichens absorbs everything from air, and chemicals like carbon, sulphur, heavy metals into their thallus. Monitoring the intensity of air pollution toxins can be easily extracted. Abundance of lichen in a particular area is an indicator of non-polluted environment of the area whereas the declined growth is an early warning of air pollution.

• **Nitrogen Source:** Nitrogen an important nutrient are obtained by lichens convert nitrogen into nitrates into the soil.

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**Check Your Progress**

10. Give the structure of lichen.

11. Write any three properties of lichens.

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### 2.6 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. **Bacteriaceae** is spherical, rod and filamentous forms, with no distinction between base and apex, whereas **Leptotriacheae** is spherical, rod and filamentous forms, the latter with distinction between base and apex.

2. **Bergey’s Division I:** The **Cyanobacteria** (formerly the blue green alga). These Bacteria can use light as their energy source under aerobic conditions. They use carbon dioxide and produce oxygen.

   **Bergey’s Division II:** The **Bacteria** (includes the photo Bacteria and all other classical Bacteria).

3. **Basic concept of taxonomy:** The naming or classifying organisms is called taxonomy. The interrelated disciplines involved are:
   - Identification: Characterizing Organisms
   - Classification: Arranging into Similar Groups
   - Nomenclature: Naming Organisms
4. Rhizoids is a short root like filamentous branch of the thallus present in both unicellular as well as Mycelia Thalli. These function as anchoring and absorbing units.

5. Rhizomorphs are Fungi whose hyphae aggregate together to form a root like strand like a hard cortex and develop like a root tip. It mainly serves to function for absorption. Its growth may be renewed with the return of favourable conditions.

6. Fungal cell consists of strong rigid cell wall enclosing the protoplast except for moulds. It protects the cell from lysis and metabolites from other organisms. It also acts the binding site of some enzymes. Chemical composition not all the same chitin is characteristically present in all the fungal cells.

7. In bread making yeast is used for the manufacture of bread. Ethyl alcohol and carbon dioxide are formed. The bread becomes spongy due to pressure exerted by accumulation of carbon dioxide.

8. Yeasts belonging to the genus Pichia are oval to cylindrical yeasts and may form pseudomycelia. Ascospores are round or hat-shaped, and there are one to four per ascus. A pellicle is formed on liquids; for example, *P. Membranae faciens* grows a pellicle on beers or wines.

9. *Penicillium notatum* is the mould which is used extensively for the production of Penicillin vaccine. *Penicillium notatum* is also referred to as *P. chrysogenum*.

10. On the basis of their overall habit, lichens are classified into three main morphological groups as Crustose, Foliose and Fruticose Lichens.

    - Crustose Lichens: Crustose lichens are tightly attached to their substrate that often they cannot be separated from it.
    - Foliose Lichens: They are leaf like thalli and are partially attached to their substrate by hair like rhizinae.
    - Fruticose Lichens: They are erect, shrubby or pendent and hair like or strap-shaped structures attached only at their base.

11. Following are the properties of lichens:

    - Lichens are regarded as the, ppioneers of vegetationn as they are capable of colonising bare rocks.
    - These plants play a very important role in the ecological formation of soil.
    - The organic acid secreted by lichens gradually dissolve and disintegrate the rocks into soil particles over which they grow. Mosses are the successors of the crustaceous rocks.
2.7 SUMMARY

- In 1750, Carl Linnaeus devised the first classification system, placing plants and animals in separate systems. He placed all microorganisms in one genus which was named *Chaos*.
- The first attempt to classify microscopic plants and animals was apparently undertaken by Müller in 1773. He placed all of them in a group known as the ‘Infusoria’.
- Later classifications were proposed by Ehrenberg (1838), Dujardin (1841), Perty (1852), Cohn (1854), Zopf (1885) and Migula (1897).
- Cohn believed that Bacteria showed more of the characteristics of plants than of animals and suggested that they be classified in the plant kingdom.
- Nägeli (1857) appeared to be the first to suggest the use of the term ‘Schizomycetes’ (Fission Fungi) to include all organisms classified as Bacteria.
- Cohn (1872) believed that it was a difficult task to classify Bacteria into genera and species. He pointed out that so many species looked alike that a classification based on morphology alone was insufficient.
- Zopf (1879) did not agree with Cohn’s idea of fixity of species; nevertheless he classified Bacteria into four groups on the basis of morphology.
- The three kingdom system was given by Haeckel, in 1866. He was the first scientist to create a natural kingdom for the microbes, which had been discovered nearly two centuries before by Antony van Leeuwenhoek.
- Haeckel placed all unicellular (microscopic) organisms in a new kingdom, called ‘Protista’, on the level with the existing kingdoms for plants (Plantae) and animals (Animalia), which are multicellular (macroscopic) organisms, followed by creation of four kingdom system (1950), where four kingdoms were created.
- The cells lacking the intracellular compartment of nucleus were shifted to fourth kingdom called Monera. Protista remained as a kingdom of Eukaryotic microorganisms.
- Later in 1967 Whittaker, a botanist at the University of California, refined the system into five kingdoms, by identifying the Fungi as a separate multicellular Eukaryotic kingdom of organisms, distinguished by their absorptive mode of nutrition.
- The developments of various molecular techniques resulted in changes in classification of Bacteria as well.
• Phylogenetic analysis of all forms of cellular life based on comparison of nucleotide sequences of the small subunit ribosomal RNA (ssrRNA) that is contained in all organisms was done by Carl Woese in 1970s.

• With the rapid advances in molecular sequencing of highly conserved regions of the Prokaryotic genome, most notably genes coding for the RNA of the small ribosomal subunit, have led to their arrangement on the basis of some physical or metabolic similarities.

• Classification of Bacteria based on metabolic requirements allow us to make predictions on different cell components like DNA, fatty acids, antigens, etc.

• Consequently, the need to identify human pathogens was a major impetus for the development to techniques to identify Bacteria. The Gram Stain, characterises Bacteria based on the structure of their cell walls.

• The thick layers of peptidoglycan in the gram positive cell wall stain purple while the thin gram negative cell wall appears pink.

• Unlike the Bacteria and the Eukarya, the Archaea have membranes composed of branched hydrocarbon chains (many also containing rings within the hydrocarbon chains attached to glycerol by either linkages.

• The cell walls of Archaea contain no peptidoglycan. Archaea are not sensitive to some antibiotics that affect the Bacteria, but are sensitive to some antibiotics that the Eukarya.

• Archaea contain rRNA that is unique to the Archaea are indicated by the presence molecular region distinctly different from the rRNA of Bacteria and Eukarya.

• Bacteria are Prokaryotes, evolved first on living earth. They do not contain membrane bound nucleus and organelles.

• Circular genomes and extrachromosomal DNA are present which helps in surviving different environment.

• Bacteria reproduce by Prokaryotic fission, resulting in two genetically identical daughter cells.

• Bacterial cell walls are made of peptidoglycan contains sugar moieties (N-Acetylglucosamine, and N-Acetylmuramic acid cross linked with Pentapeptide (D-Aminoacids).

• Gram-Positive Bacteria have simple cell walls with large amounts of peptidoglycan that retains crystal violet.

• Gram-Negative Bacteria have more complex cell walls with less peptidoglycan that retains saffron.
• Prokaryotic taxonomy based on the criteria, such as shape, motility, nutritional mode and gram staining, etc., were important for culturing Bacteria and identifying them.

• DNA based tools used as diagnostics, for example, PCR are increasingly acceptable due to their specificity and speed compared to culture based methods. These methods allow the detection of viable but non-culturable cells that are though not dividing but metabolically active.

• Fungi belongs to domain Eukarya. They are unicellular (Yeast) as well as multicellular organisms (filamentous Fungi).

• Multicellular Fungi are composed of filaments called hyphae. Hyphae may be branched with internal cross walls, called septa which divide the hyphae into separate cells.

• The fusion of hyphae from two individual is called plasmogamy. The fused hyphae contains haploid nuclei from two individuals are heterokaryotic.

• In some plasmogamy results in cells with one nucleus from each individual. This condition is called dikaryotic.

• Structure of fungal cell consists of strong rigid cell wall enclosing the protoplast except for moulds.

• The organisms of the fungal lineage include mushrooms, rusts, smuts, puffballs, truffles, morels, moulds and yeast.

• The kingdom Fungi includes some of the most important organisms with main ecological and economical roles.

• C.J. Alexopoulos (1962) placed all Fungi along with slime moulds in a separate division Mycota, and was divided into two sub-divisions Myxomycotina and Eumycotina on the basis of absence and presence of cell wall, respectively.

• Slime moulds were included in the subdivision Myxomycotina and the True Fungi in Eumycotina.

• A mould is a fungus that grows in the form of multicellular filaments called hyphae.

• Moulds are a large and taxonomically diverse number of fungal species in which the growth of hyphae results in discoloration and a fuzzy appearance, especially on food.

• The hyphae are generally transparent, so the mycelium appears like very fine, fluffy white threads over the surface.

• Cross-walls (septa) may delimit connected compartments along the hyphae, each containing one or multiple, genetically identical nuclei.

• Bread mould is extensively used in bakery products. Bread moulds also cause spoilage of fruits, vegetables, processed sauce and ketchups.
• A lichen is a composite organism that consists of a fungus living in intimate association with one or more photosynthetic partners that may be either a green Alga or a Cyanobacterium.

• The fungal member of the lichen is called mycobiont and the photosynthetic partner (Algae or Cyanobacterium) is called photobiont.

• The two organisms live in close association with each other so much so that they appear to be a single organism.

• Lichens are the result of a fungus and an algae living together. The mycobiont, a fungus is mostly ascomycetes, but some basidiomycetes.

• Lichen are able to survive in harsh climate but they are very sensitive to air pollution.

• Fruticose lichen is the most vulnerable and Crustose is the least vulnerable lichen type to air pollution.

• Lichens absorbs everything from air, and chemicals like carbon, sulphur, heavy metals into their thallus.

• Abundance of lichen in a particular area is an indicator of non-polluted environment of the area whereas the declined growth is an early warning of air pollution.

2.8 **KEY WORDS**

• **Hyphae**: A mould is a fungus that grows in the form of multicellular filaments called hyphae.

• **Plasmogamy**: The fusion of hyphae from two individual is called plasmogamy.

• **Heterokaryotic**: The fused hyphae contains haploid nuclei from two individuals are heterokaryotic.

• **Dikaryotic**: In some plasmogamy results in cells with one nucleus from each individual called as dikaryotic.

• **Mycobiont**: The fungal member of the lichen is called mycobiont.

• **Photobiont**: The photosynthetic partner, i.e., Algae or Cyanobacterium is called photobiont.

2.9 **SELF ASSESSMENT QUESTIONS AND EXERCISES**

**Short Answer Questions**

1. What is taxonomy and what are the basic concepts of taxonomy?

2. Write a short note on classification of Bacteria.
3. Distinguish between Gram-Positive Bacteria and Gram-Negative Bacteria.

4. Write a short note on fungal taxonomy.

5. Distinguish between yeasts and moulds.

6. What are lichens?

7. Draw a well-labelled diagram to show the cross-section of lichen thallus.

8. Write about any three economic importance of lichens.

Long Answer Questions

1. Explain briefly about the characteristic features of Bacteria according to Bergey’s Manual.

2. Explain the common morphological, physiological and metabolic features used for the identification of Bacteria.

3. Discuss about the classification of Bacteria following Bergey’s Manual of Systematic Bacteriology with the help of appropriate examples.

4. Discuss about the general characteristics of Fungi.

5. List all the industrial uses of yeasts and moulds.

6. Explain the structural organisation of lichens and their properties.

2.10 FURTHER READINGS


UNIT 3 VIRUSES: CHARACTERISTICS AND CLASSIFICATION

Structure
3.0 Introduction
3.1 Objectives
3.2 Virus: An Introduction
3.3 Morphology and Ultra-Structure of Viruses
3.4 Classification of the Viruses
   3.4.1 Classification Systems
   3.4.2 Multiplication of Viruses
3.5 Answers to Check Your Progress Questions
3.6 Summary
3.7 Key Words
3.8 Self Assessment Questions and Exercises
3.9 Further Readings

3.0 INTRODUCTION

A virus is a small parasite that cannot reproduce by itself. Once it infects a susceptible cell, however, a virus can direct the cell machinery to produce more viruses. Most viruses have either RNA or DNA as their genetic material. The nucleic acid may be single or double-stranded. The infectious virus particle, called a virion, consists of the nucleic acid and an outer shell of protein. The simplest viruses contain only enough RNA or DNA to encode four proteins. The most complex can encode 100 – 200 proteins. Viruses are obligate intracellular parasites, which contain either DNA or RNA. They lack metabolic machinery and depend on the host cell to carry out vital functions. Viruses cannot multiply outside the host because they do not possess an enzyme system and protein synthesis machinery. Viruses do not have any cytoplasm and thus, the cytoplasmic organelles like mitochondria, Golgi bodies, lysosome, ribosome, etc., are absent. The origins of viruses in the evolutionary history of life are unclear; some may have evolved from plasmids, while others may have evolved from Bacteria.

Viruses are much smaller than Bacteria. It was their small size and 'filterability' that led to their recognition as a separate class of infectious agents. Hence, they were for a time known as 'Filterable Viruses'. As they were too small to be seen under the light microscope, they were called 'Ultramicroscopic'. Some of the larger viruses, such as Poxviruses, can be seen under the light microscope. The virus particles seen in this manner are known as 'elementary bodies'.
In this unit, you will study about viruses, ICTV system of classification of viruses, the general properties of viruses, and the morphology and ultra-structure of viruses (RNA-DNA) in detail.

3.1 OBJECTIVES

After going through this unit, you will be able to:

- Discuss about viruses and its general properties
- Explain the morphology and ultra-structure of viruses (RNA-DNA)
- Understand the ICTV system of classification

3.2 VIRUS: AN INTRODUCTION

A virus is a small infectious agent that replicates only inside the living cells of an organism. Viruses are obligate intracellular parasites, which contain either DNA or RNA. Viruses can infect all types of life forms, from animals and plants to microorganisms, including Bacteria and Archaea. Most viruses have either RNA or DNA as their genetic material. Nucleic acid is the genetic material, which is present inside the capsid. Capsid is the protein layer, which protects the genetic material (DNA, RNA). The nucleic acid may be single-or double-stranded. The entire infectious virus particle, called a virion, consists of the nucleic acid and an outer shell of protein. The simplest viruses contain only enough RNA or DNA to encode four proteins. The most complex can encode 100 – 200 proteins. Since Dmitri Ivanovsky’s 1892 article describing a non-bacterial pathogen infecting Tobacco plants, and the discovery of the Tobacco Mosaic Virus (TMV) by Martinus Beijerinck in 1898, about 5,000 virus species have been described in detail, although there are millions of types. Viruses are found in almost every ecosystem on Earth and are the most numerous type of biological entity. The study of viruses is known as virology, a sub-specialty of microbiology. The origins of viruses in the evolutionary history of life are unclear: some may have evolved from plasmid pieces of DNA that can move between cells while others may have evolved from Bacteria. In evolution, viruses are an important means of horizontal gene transfer, which increases genetic diversity. Viruses are considered by some to be a life form, because they carry genetic material, reproduce, and evolve through natural selection, but lack key characteristics (such as, cell structure) that are generally considered necessary to count as life. Because they possess some but not all such qualities, viruses have been described as ‘Organisms at the Edge of Life’, and as replicators.

Viruses spread in many ways; viruses in plants are often transmitted from plant to plant by insects that feed on plant sap, such as aphids; viruses in animals can be carried by blood-sucking insects. These disease bearing
organisms are known as vectors. Influenza viruses are spread by coughing and sneezing. Norovirus and Rotavirus, common causes of Viral Gastroenteritis, are transmitted by the fecal oral route and are passed from person to person by contact, entering the body in food or water. HIV (Human Immunodeficiency Viruses) is one of several viruses transmitted through sexual contact and by exposure to infected blood. The variety of host cells that a virus can infect is called its ‘Host Range’. This can be narrow, meaning a virus is capable of infecting few species, or broad, meaning it is capable of infecting many. Viral infections in animals provoke an immune response that usually eliminates the infecting virus. Immune responses can also be produced by vaccines, which confer an artificially acquired immunity to the specific viral infection. Some viruses, including those that cause AIDS (Acquired Immuno-Deficiency Syndrome) and Viral Hepatitis, evade these immune responses and result in chronic infections. Several antiviral drugs have been developed.

In 1935, Schlesinger determined the composition of a virus for the first time. He also showed that a Bacteriophage consists of only protein and DNA. In the same year, Wendell Stanley introduced the concept of chemical nature of viruses and Twort-d’Herelle coined the term Bacteriophage (Viruses that infect Bacteria). In 1983, LUC Montagnier discovered the HIV virus. In 1957, Lwoff defined virus as, ‘Strictly intracellular and potentially pathogenic entities with an infectious phase, and (1) Possessing only one type of nucleic acid, (2) Multiplying in the form of their genetic material, (3) Unable to grow and to undergo binary fission, (4) Devoid of a Lipmann system.’ In 1968, Luria and Darnell defined alternatively defined viruses as ‘Elements of genetic material that can determine in the cells in which they reproduce, the biosynthesis of a specific apparatus for their own transfer into other cells’. The famous Russian botanist, Dmitri Iwanowski presented a paper on viruses to the St. Petersburg Academy of Science in 1892. In this, he showed that extracts from Tobacco plants infected with Mosaic Virus could transmit disease to other plants after passing through ceramic filters which can easily filter Bacteria. This is generally recognized as the beginning of Virology. Virology is the study of viruses and virus-like agents; their structure, classification and evolution, their ways of infecting and exploiting cells for virus reproduction, the diseases they cause, the techniques of isolating and culture them, and their use in research and therapy. Virology is often considered a part of microbiology and even pathology.

**Size and Shape of Viruses**

**Size:** Viruses are much smaller than Bacteria. It was their small size and ‘Filterability’ (ability to pass through filters that can hold back Bacteria) that led to their recognition as a separate class of infectious agents. Hence, they were for a time known as ‘Filterable Viruses’. As they were too small to be seen under the light microscope, they were called ‘Ultramicroscopic’. Some of
Viruses: Characteristics and Classification

NOTES

the larger viruses, such as Poxviruses, can be seen under the light microscope. The virus particles seen in this manner are known as ‘Elementary Bodies’.

Viruses vary widely in size. The largest among them (for example, Poxviruses) measuring about 300 nm, are as large as the smallest Bacteria (Mycoplasma). The smallest viruses (for example, Parvovirus) measuring about 20 nm are nearly as small as the largest protein molecules, such as Haemocyanin. The earliest method of estimating the size of virus particles was by passing them through collodion membrane filters of graded porosity (gradocol membranes). The average pore diameter of the finest filter that permitted passage of the virion gave an estimate of its size. With the development of the ultracentrifuge, a second method became available. From the sedimentation of virus in the ultra-centrifuge, the particle size could be calculated using Stokes’ Law. The third and the most direct method of measuring virus size is ‘electron microscopy’. Purified preparations of virions may be examined under the electron microscope either unstained or stained. The extracellular infectious virus particle is called the **virion**. By this method, both the shape and size of virions can be studied.

**Shape and Composition**: The virion consists essentially of a nucleic acid surrounded by a **protein coat**, the **capsid**. The capsid with the enclosed **nucleic acid** is known as the **nucleocapsid**. The function of the capsid is to protect the nucleic acid from inactivation by nucleases and other deleterious agents in the environment. The capsid is composed of a large number of capsomeres with its morphological units. The chemical units of the capsid are polypeptide molecules, which are arranged symmetrically to form molecules, and symmetrically to form an impenetrable shell around the nucleic acid core. One of the major functions of the capsid is to introduce viral genome into host cells by adsorbing readily to cell surfaces.

**General Characteristics of Viruses**

- Viruses are much smaller than Prokaryotes and Eukaryotes.
- Unlike cells they have generally simple and static structure.
- They are obligate intracellular parasites.
- They have either DNA and RNA genomes but lack ribosomes or other factors needed for translation. Thus they are dependent on host cells for viral proteins.
- Their genome encode minimal information to insure the following:
  1. Genome Replication and Packaging.
  2. Production of Viral Proteins.
  3. Subvert Cellular Functions to Allow the Production of Virions.
- Viruses like Bacteriophages infect Prokaryotic cells while others infect Eukaryotic cells.
• Some viruses destroy cells producing disease other persist in existing cells.

• Viral protein, besides protecting the nucleic acid, also determines the antigenic specificity of the virus.

• Some viruses also contain small amounts of carbohydrate. Most viruses do not possess any enzymes for the synthesis of viral components or for energy production but some have other enzymes; for example, the Neuraminidase in the Influenza Virus.

• Retroviruses have a unique enzyme, RNA-dependent-DNA polymerase or ‘Transcriptase’, which can transcribe RNA into DNA.

• Viruses are inactivated within seconds at 56°C, within minutes at 37°C and within days at 4°C. They are stable at low temperatures.

• Lyophilized Virus can be stored for years and reconstituted when required by adding water. Some viruses (such as, Poliovirus) do not stand freeze drying.

• Viruses vary greatly in their resistance to acidity. For example, Enteroviruses are very resistant to acid pH while Rhinoviruses are very susceptible. All viruses are disrupted under alkaline conditions.

• Molar concentrations of certain salts (MgCl₂, Na₂SO₄) also protect some viruses (for example, Poliovirus) against heat inactivation.

• The most active antiviral disinfectants are oxidising agents, such as hydrogen peroxide, potassium permanganate and hypochlorites.

• Organic iodine compounds are actively virucidal. Chlorination of drinking water kills most viruses but its efficacy is greatly influenced by the presence of organic matter.

• The action of lipid solvents, such as ether, chloroform and bile salts is selective, the enveloped viruses being sensitive and the naked viruses resistant to them.

• The selective action is useful in the identification and classification of viruses.

• Antibiotics active against Bacteria are completely ineffective against viruses. This property is helpful in eliminating Bacteria from clinical specimens by antibiotic treatment before virus isolation.

**Structure and Function**

Viruses have no metabolic system of their own viewed as mobile genetic elements most probably as cellular origin and characterised by long co-evolution of virus and host. For propagation viruses depend on specialised host cells supplying the complex metabolic and biosynthetic host machinery. They have either DNA or RNA genomes but lack ribosomes and other factors.
Viruses: Characteristics and Classification

NOTES

Self-Instructional Material

needed for translation. Thus they are dependent on the host cells for the production of **Viral Proteins**. Viruses contain one kind of **nucleic acid-DNA** or **RNA genome** inside a **protein shell - capsid** which may be surrounded by a **lipid bilayer membrane** (Refer Figure 3.1).

![Fig. 3.1 Structure of a Virus](image)

**Check Your Progress**

1. Define virus.
2. How does a virus spread?

### 3.3 MORPHOLOGY AND ULTRA-STRUCTURE OF VIRUSES

Viruses are of different sizes, even much smaller than Bacteria. The largest virus, **Poxvirus** measures about 300 nm and are is large as the smallest Bacteria; the smallest virus, **Parvovirus** measuring about 20 nm is small as the largest protein molecules, such as Haemocyanin. Virus particles are also called as **virions** that consist of two or, in some cases, three parts:

- The Genetic Material made from either DNA or RNA.
- A Protein Coat that protects these Genes.
- In some cases an envelope of Lipids that surrounds the Protein Coat when they are outside a cell.

The shapes of viruses range from simple helical and icosahedral forms to more complex structures. The protein shell of the virus is called capsid, it protects the nucleic acid from inactivation by nucleosides and other deleterious agents in the environment. The capsid is composed of a large number of capsomeres which form its morphological units. Of the many functions a capsid performs, an important one is to introduce the viral genome into the host cells by absorbing readily to cell surfaces.

Capsids are made up of multiple copies of a single protein or association of several proteins. The capsid can take a variety of geometric shapes that
are characteristics of various virus families, these include, Icosahedral Naked or Enveloped: The number and faces of corners may vary according to the number and type of association among structural proteins, helical structure naked Tobacco Mosaic Virus (TMV) or enveloped (Rabies Virus). Figure 3.2 shows the non-enveloped virion with an icosahedral capsid, nucleic acid enclosed within the capsid.

![Fig. 3.2 Non-Enveloped Virion with an Icosahedral Capsid, Nucleic Acid Enclosed Within the Capsid](image1)

In them the protomers, i.e., the structural polypeptide chain are arranged in clusters capsomeres readily delineated forming closed capsid shell. The arrangement of capsomeres permits the classification of viruses by capsomeres numbers and pattern (Refer Figure 3.3).

![Fivefold axis](image2)

![Twofold axis](image3)

![Threefold axis](image4)

![Threefold](image5)

![Fivefold](image6)

![Twofold](image7)

**Fig. 3.3 Arrangement of Capsomeres Permitting the Classification of Viruses by Capsomere Numbers and Pattern**

Capsule can be covered by a host membrane, i.e., envelope acquired by budding of nucleocapsid through a cellular membrane can be any cell membrane but is virus specific (Refer Figure 3.4).
I. Physical and Chemical Properties of Viruses

To better understand the structure and morphology, as well as other pathological features of viruses, you need to study about their physical and chemical properties.

Nucleic Acid

The genetic material, which is present inside the capsid, is called the nucleic acid. Three different types of viruses, which contain different types of nucleic acids are DNA Viruses, RNA Viruses and DNA-RNA Viruses.

- **DNA Viruses**: These viruses contain DNA as the genetic material. Common examples include Poxviruses, the Coliphages T2, T4, T6, T3 and Lambda, and Papilloma Viruses. Adenoviruses consist of double stranded DNA. The DNA molecule may be cyclic, as in case of SV-40 and Papilloma viruses, and in case of Coliphage Lambda it is of linear shape. The T2, T4, T6 Coliphages contain an unusual base, 5-hydroxymethylcytosine, in place of cytosine. In case of Bacteriophage phi × 174, M–13, the DNA is circular.

- **RNA Viruses**: Viruses contain RNA as the genetic material. Double stranded RNA is found in the Reovirus and Wound Tumour Virus. Single stranded RNA is found in Tobacco Mosaic Virus, Influenza Virus, Poliomyelitis Virus and Leukaemia Virus. Plant Virus contains only RNA. The RNA of several viruses fold upon themselves to form helices in some regions.

- **DNA-RNA Viruses**: These viruses contain alternatively both RNA virus and DNA virus. An animal virus contains either DNA virus or RNA virus or rarely RNA-DNA virus.

Glycoprotein

The general features of the glycoproteins are as follows:

- Glycoproteins consist of oligosaccharide chains (glycans) covalently bonded to polypeptide side chains.

- In a co-translational or post-translational modification, carbohydrate is bound to the protein and this phenomenon is called glycosylation.
• The extracellular segments present in proteins are frequently glycosylated.
• They are also present in the cytosol, but their functions and the pathways producing these modifications are not completely understood.

**Viral Matrix Protein**

The general features of viral matrix protein are as follows:

• The envelope surrounding the viral particle is linked with the core region with the help of viral matrix proteins, which are not part of the viral particle.
• They play a major role in assembling the virus particle, and work together with the RNP complex as well as with the viral membrane.
• These proteins are located in Morbillivirus, Paramyxovirus and Pneumovirus.
• Viral matrix proteins are responsible for driving out the genetic material upon the entry of a virus into the cell.

**Viral Genome**

The huge variety of genomic structures can be seen among viral species; as a group, they hold more structural genomic diversity than Animals, Archaea, Plants or Bacteria. There are millions of different types of viruses, although only about 5,000 of them have been studied and explained. The huge majority of viruses have RNA genomes. Plant viruses tend to have single-stranded RNA genomes and bacteriophages tend to have double-stranded DNA genomes.

The extracellular fully assembled infectious virus particle is called a **virion**. The main function of the virion is to deliver its DNA and RNA into the host cell so that the genome can be expressed by the host cell. The protein coat or capsid of an individual virion (fully assembled virus particle) is composed of multiple copies of one or more type of proteins coded by viral genome. These proteins assemble, forming structural units called capsomeres. The simplest virions consist of two basic components. Nucleic acid DNA/ RNA and a protein coat which functions as a shell to protect the viral genome from nucleases and which during infection attaches the virion to specific receptors exposed on the prospective host cell.

Viral genomes have the following characteristics:

• Viral genomes are either circular or linear.
• The kind of nucleic acid is irrelevant to the shape of the genome.
• Genomes in RNA and DNA viruses are often segmented.
• In RNA viruses, each segment frequently codes for only one protein and they are usually found together in one capsid. However, all
segments are not needed to be in the same virion for the virus to be infectious, for example, Plant viruses, the Brome Mosaic Virus.

- Irrespective of nucleic acid type, a viral genome is always either single-stranded or double-stranded.
- Single-stranded genomes comprise an unpaired nucleic acid, analogous to one half of a ladder split down the middle.
- Double-stranded genomes comprise two complementary paired nucleic acids, analogous to a ladder.

The nucleoproteins together with genomes forms the nucleocapsid. In enveloped viruses the nucleocapsid is surrounded by a lipid bilayer derived from the modified host cell membrane studded with outer layer of virus envelope of glycoproteins. Capsid is formed of large number of capsomeres which forms its morphological units. The chemical units of the capsid are polypeptide molecules which are arranged symmetrically. They form a shell around the nucleic acid.

The capsid shows two kinds of symmetry-icosahedral (cubical) and helical. An icosahedrons is a polygon with 12 vertices and 20 facets or sides. Each facet is in the shape of an equilateral triangle. Two types of capsomeres are present in the icosahedral capsid. They are the pentagonal capsomeres at the vertices and the hexagonal capsomeres making up the facet, for example, Adenovirus and Herpes Simplex Virus. With the helical symmetry the capsomeres and nucleic acid are wound together to form a helical or spiral tube, for example TMV (Refer Figure 3.5).

**Fig. 3.5 Helical Virus, TMV**

**Helical Capsid:** Composed of a single type of capsomeres stacked around a central axis form a helical structure, which may have a central cavity. This arrangement results in rod-shaped or filamentous virions.

In replication of viruses with helical symmetry, identical protein subunits or protomers self-assemble into a helical symmetry, surrounding the nucleic acid, which follows a similar spiral path. Such nucleocapsids form rigid, highly elongated rods or flexible filaments.
Enveloped viruses contain an external membrane surrounding the nucleocapsid. The viral envelope derived from host cell membranes composed by lipid bilayer, with virus protein inserted on it, is derived from host cell membrane. Viruses are inert outside the host cell.

Virions are either enveloped or non-enveloped. The envelope of viruses is derived from the host cell membrane on releasing by budding protein subunits may be present as projecting spikes on the envelope surface known as peplomers, for example, influenza virus carries two different kinds of peplomers. Envelope is sensitive to the action of lipid solvents. Envelopes confer chemical, antigenic and biological properties on viruses.

Check Your Progress

3. What are capsids made of?
4. What is RNA virus?
5. What is DNA-RNA virus?

3.4 CLASSIFICATION OF THE VIRUSES

Viruses are not usually classified into conventional taxonomic groups but are usually grouped according to properties as type of nucleic acid they contain, the structure of the capsid and the number of protein subunits in it, host species and immunological characteristics. Like the relatively consistent classification systems seen for cellular organisms, virus classification is controversial due to pseudo-living nature of viruses. Thus, they do not fit into the established biological classification system for several reasons. Virus classification is based on phenotypic characteristics, including morphology, nucleic acid type, mode of replication and type of diseases they cause.

The genomic RNA strand of single-stranded RNA viruses is called sense (positive sense) in orientation if it can serve as mRNA, and antisense (negative sense) if a complimentary strand synthesised by a viral RNA transcriptase serves as mRNA. Also considered in viral classification is the site of capsid, in enveloped viruses, the site of envelopment.

Common Properties of Virus Classification

- Virion Morphology
- Nature of Genome in Virion
- Presence or Absence of Lipid Membrane (Envelope)
- Genome Organisation and Replication
- Antigenic Properties
- Biological Properties
Based on Morphology

On the basis of shared properties viruses are grouped on the basis mode of replication, configuration of nucleic acid and whether the genome consists of one molecule or is segmented. Helical morphology is seen in nucleocapsids of many filamentous and pleomorphic viruses. Helical nucleocapsids consist of a helical array of capsid assembly – or protomers in enveloped viruses, the site of nucleocapsid envelopment.

The number and arrangement of the capsomers (morphologic subunits of the icosahedron) are useful in identification and classification. Many viruses also have an outer envelope, which is the site of viral capsid assembly and, in enveloped viruses, the site of nucleocapsid envelopment.

Based on Chemical Composition and Mode of Replication

The genome of virus may consist of DNA or RNA, which may be single stranded or double stranded linear or circular. The entire genome may occupy either one nucleic acid molecule or several nucleic acid segments.

3.4.1 Classification Systems

Baltimore Classification: David Baltimore the Nobel Prize winner devised the classification system. The Baltimore Classification of viruses is based on the method of viral mRNA synthesis (Refer Figure 3.6). The International Committee on Taxonomy of Viruses (ICTV) classification system is used in conjunction with the Baltimore classification system in modern virus classification. Baltimore classification places viruses depending on a combination of their nucleic acid-DNA/RNA, strandedness, sense and method of replication. The Baltimore system of classification provides a guide with regard to various mechanisms of viral genome replication. According to this all viruses must generate positive strand mRNA from their genomes, in order to produce proteins and replicate. These various types of virus genome can be broken down into seven different groups replicating differently. By convention top strand of coding DNA/mRNA is +ve sense. The nature of the genome decides the replication strategy of virus. Viruses can be classified into following seven groups:

- **Double Stranded DNA**: Replicate in the nucleus using cellular proteins.
- **Single Stranded +ve Sense DNA**: Replication in nucleus involving the formation of a –ve sense strand, which serves as a template for +ve strand RNA and DNA synthesis.
- **Double Stranded RNA**: They have segmented genomes transcribed to produce monocistronic mRNA.
- **Single Stranded**: They have +ve sense RNA.
- **Single Stranded –ve Sense RNA**: They must have a virion particle RNA directed RNA polymerase.
- Single Stranded +ve Sense RNA with DNA Intermediate in Life Cycle: Genome is unique +ve sense diploid and serve as a template for reverse transcription.
- Double Stranded DNA with RNA Intermediate: Relies on reverse transcription but occurs inside the virus particle on maturation unlike Retroviruses.

**Fig. 3.6 Baltimore Classification of Virus**

**ICTV Classification**: The International Committee on Taxonomy of Viruses (ICTV) developed the current classification system and put a greater certain virus properties to maintain family uniformity. The general taxonomic structure is as follows. The ICTV is the only body charged by the International Union of Microbiological Societies (IUMS) with the task of developing, refining and maintaining a Universal Virus Taxonomy. There exists a similarity in taxon structure with the classification system of cellular organisms. On the basis of shared properties viruses are grouped at different hierarchical levels of order, family, subfamily, genus and species.

Grouping of viruses is based on its morphology can be shown as:

Order: Virales
Family: Viridae
Subfamily: Virinae
Genus: *Virus*
Species: *Virus*
In the current ICTV taxonomy, six orders have been established, the Caudovirales, Herpesvirales, Mononegavirales, Nidovirales, Picornaviruses, Tymovirales. A seventh order Ligamenvirales has also been proposed.

Virus Classification

Viral Core Structure: Viral genome consists of either DNA or RNA. The DNA genome may have their ends covalently linked to each other, for example circular and may be not linked as in linear genome.

DNA Viruses

Table 3.1 and Figure 3.7 shows the classification of DNA virus.

**Group I:** Viruses possess double stranded DNA.

**Group II:** Viruses possess single stranded DNA.

<table>
<thead>
<tr>
<th>Virus Family</th>
<th>Virus Genus</th>
<th>Virion- Naked/ Enveloped</th>
<th>Capsid Symmetry</th>
<th>Type of Nucleic Acid</th>
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<tbody>
<tr>
<td>1. Adenoviridae</td>
<td>Adenovirus</td>
<td>Naked</td>
<td>Icosahedral</td>
<td>ds</td>
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<td>2. Papovaviridae</td>
<td>Papillomavirus</td>
<td>Naked</td>
<td>Icosahedral</td>
<td>ds circular</td>
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<td>3. Paroviridae</td>
<td>B19 Virus</td>
<td>Naked</td>
<td>Icosahedral</td>
<td>ss</td>
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<td>4. Herpesviridae</td>
<td>Herpes Simplex Virus, Varicella Zoster Virus, Cytomegalovirus, Epstein Barr Virus</td>
<td>Enveloped</td>
<td>Icosahedral</td>
<td>ds</td>
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<td>5. Poxviridae</td>
<td>Smallpox Virus, Vaccinia Virus</td>
<td>Complex Coats</td>
<td>Complex</td>
<td>ds</td>
</tr>
<tr>
<td>6. Hepadnavirida</td>
<td>Hepatitis B Virus</td>
<td>Enveloped</td>
<td>Icosahedral</td>
<td>ds circular</td>
</tr>
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<td>7. Polyomavirida</td>
<td>Polyoma Virus (Progressive Multifocal Leucoencephalopathy)</td>
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</tbody>
</table>

In Table 3.1, the classification of DNA virus illustrates the type of nucleic acid is ‘ds’ and ‘ss’, where ss stands for single stranded and ds stands for double stranded.

**Fig. 3.7** Classification of DNA Virus
RNA Viruses

Table 3.2 shows the classification of RNA virus.

**Group III:** Viruses possess double stranded RNA genomes that are segmented.

**Group IV:** Viruses possess positive (+ve) sense single stranded RNA genomes.

**Group V:** Viruses possess negative (–ve) sense single stranded RNA genomes.

**Table 3.2 Classification of RNA Virus**

<table>
<thead>
<tr>
<th>Virus Family</th>
<th>Virus Genera</th>
<th>Virion-Naked/Enveloped</th>
<th>Capsid Symmetry</th>
<th>Types of Nucleic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reoviridae</td>
<td>Reovirus, Rotavirus</td>
<td>Naked</td>
<td>Icosahedral</td>
<td>ds</td>
</tr>
<tr>
<td>Picornaviridae</td>
<td>Enterovirus, Rhinovirus, Hepatovirus, Cardiovirus, Aphthovirus, Parechovirus, Erbovirus, Kobuvirus, Tescheovirus</td>
<td>Naked</td>
<td>Icosahedral</td>
<td>ss</td>
</tr>
<tr>
<td>Caliciviridae</td>
<td>Norwalk Virus, Hepatitis E Virus</td>
<td>Naked</td>
<td>Icosahedral</td>
<td>ss</td>
</tr>
<tr>
<td>Togaviridae</td>
<td>Rubella Virus</td>
<td>Enveloped</td>
<td>Icosahedral</td>
<td>ss</td>
</tr>
<tr>
<td>Arenaviridae</td>
<td>Lymphocytic Choriomeningitis Virus</td>
<td>Enveloped</td>
<td>Complex</td>
<td>ss</td>
</tr>
<tr>
<td>Retroviridae</td>
<td>HIV-1, HIV-2, HTLV-I</td>
<td>Enveloped</td>
<td>Complex</td>
<td>ss</td>
</tr>
<tr>
<td>Flaviviridae</td>
<td>Dengue Virus, Hepatitis C Virus, Yellow Fever Virus</td>
<td>Enveloped</td>
<td>Complex</td>
<td>ss</td>
</tr>
<tr>
<td>Orthomyxoviridae</td>
<td>Influenzavirus A, Influenzavirus B, Influenzavirus C, Isavirus, Thogotovirus</td>
<td>Enveloped</td>
<td>Helical</td>
<td>ss</td>
</tr>
<tr>
<td>Paramyxoviridae</td>
<td>Measles Virus, Mumps Virus, Respiratory Syncytial Virus</td>
<td>Enveloped</td>
<td>Helical</td>
<td>ss</td>
</tr>
<tr>
<td>Bunyaviridae</td>
<td>California Encephalitis Virus, Hantavirus</td>
<td>Enveloped</td>
<td>Helical</td>
<td>ss</td>
</tr>
<tr>
<td>Rhabdoviridae</td>
<td>Rabies Virus</td>
<td>Enveloped</td>
<td>Helical</td>
<td>ss</td>
</tr>
<tr>
<td>Filoviridae</td>
<td>Ebola Virus, Marburg Virus</td>
<td>Enveloped</td>
<td>Helical</td>
<td>ss</td>
</tr>
<tr>
<td>Coronaviridae</td>
<td>Corona Virus</td>
<td>Enveloped</td>
<td>Complex</td>
<td>ss</td>
</tr>
<tr>
<td>Astroviridae</td>
<td>Astrovirus</td>
<td>Naked</td>
<td>Icosahedral</td>
<td>ss</td>
</tr>
<tr>
<td>Bornaviridae</td>
<td>Borna Disease Virus</td>
<td>Enveloped</td>
<td>Helical</td>
<td>ss</td>
</tr>
</tbody>
</table>

In Table 3.2, the classification of RNA virus illustrates the type of nucleic acid is ‘ds’ and ‘ss’, where ss stands for single stranded and ds stands for double stranded.

**Reverse Transcribing Viruses**

**Group VI:** Virus possessing single stranded RNA genomes and replicate using reverse transcriptase. The retrovirus are included in this group, of which HIV is a member.

**Group VII:** Viruses possess double stranded DNA genomes and replicate using reverse transcriptase, the hepatitis B virus can be found in this group. Figure 3.8 shows the viral genomes.
Holmes Classification

Holmes (1948) used Carl Linnaeus’s system of binomial nomenclature to classify viruses into 3 groups under one order, Virales. They are placed as follows:

- **Group I: Phaginae** (Attacks Bacteria)
- **Group II: Phytophaginae** (Attacks Plants)
- **Group III: Zoophaginae** (Attacks Animals)

LHT System of Virus Classification

The LHT System of Virus Classification approved by Provisional Committee on Nomenclature of Virus is based on chemical and physical characters like nucleic acids - DNA and RNA, Symmetry - Helical or Icosahedral or Complex, presence of Envelope, diameter of Capsid, number of Capsomeres.

Phylum Vira (divided into 2 Subphyla)

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)
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 Tymovirales
  - 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

Subviral Agents

The following agents are smaller than viruses but have some of their properties.

Viroids

They are plant pathogens that consists of a short stretch of nucleobases of highly complementary, circular, single stranded RNA without the protein coat. The small cytoplasmic RNA associated with Rice Yellow Mottle is first
discovered. The human pathogen Hepatitis D is similar to viroids. Viroids RNA does not code for any protein. The replication mechanism involves RNA Polymerase II, an enzyme normally associated with synthesis of mRNA from DNA syntheseses of new RNA using the viroids RNA as template. Some viroids are ribozymes having catalytic properties which works for larger replication intermediates.

- **Family Pospiviroidae**
  - Genus *Pospiviroid*; type Species: *Potato spindle tuber viroid*
  - Genus *Hostuviroid*; type Species: *Hop stunt viroid*
  - Genus *Cocadviroid*; type Species: *Coconut cadang-cadang viroid*
  - Genus *Apscaviroid*; type Species: *Apple scar skin viroid*
  - Genus *Coleviroid*; type Species: *Coleus blumei viroid 1*

- **Family Avsunviroidae**
  - Genus *Avsunviroid*; type Species: *Avocado sunblotch viroid*
  - Genus *Pelamoviroid*; type Species: *Peach latent mosaic viroid*

**Satellites**

- **Satellite Viruses**
  - Single-Stranded RNA Satellite Viruses
    - Subgroup 1: *Chronic bee-paralysis satellite virus*
    - Subgroup 2: *Tobacco necrosis satellite virus*

- **Satellite Nucleic Acids**
  - Single-Stranded Satellite DNAs
  - Double-Stranded Satellite RNAs
  - Single-Stranded Satellite RNAs
    - Subgroup 1: Large Satellite RNAs
    - Subgroup 2: Small Linear Satellite RNAs
    - Subgroup 3: Circular Satellite RNAs

**3.4.2 Multiplication of Viruses**

Multiplication of viruses is called replication. Viruses contain the genetic information for their replication but they lack the enzymes. They depend on host cell machinery for their replication. The cycle can be divided into following six phases.

**Adsorption:** In this phase, the presence of certain receptors on the surface of the host cell help virus gets attached to the host cell. These receptors recognize viral surface components. The attachment to the host cell membrane is facilitated by host cell interaction.
Penetration: Bacteria have rigid cell wall. Infecting viruses have difficulty in penetrating into the bacterial cell. Only the nucleic acid of the virus enters the bacterial cell. Viruses that are enveloped have the host cell membrane of fuse with the envelope surface. Then the nucleocapsid is released into the cytoplasm.

Uncoating: Lysosomal enzymes of the host cell play a big role here. Sometime may be replaced by a viral un-coating enzyme. In this process the outer layers and capsid of the virus are removed. The viral nucleic acid is released into the cell.

Biosynthesis: In this phase, the viral nucleic acid and capsid are synthesised. The enzymes necessary in the various stage of viral synthesis, assembly and release are also synthesised. Certain regulator protein are synthesised restricting the metabolism of the host cell. They direct the production of viral components. In general most DNA viruses synthesise their nucleic acid in the host cell nucleus. They are DNA viruses, but they synthesise all their components in the host cell cytoplasm. Most RNA viruses synthesise all their components in the cytoplasm. They synthesise some components in the host cell nucleus. Biosynthesis consists essentially of the following steps:

- Transcription of messenger RNA (mRNA) from the viral nucleic acid.
- Replication of viral nucleic acid.
- Synthesis of late proteins or structural proteins. They are the components of daughter virion capsids.

The specific mRNAs must be transcribed from the viral nucleic acid for duplication of genetic information. With the help of other cell components viruses translate the mRNA. Different pathways are used depending on the structure of viral nucleic acid with the help of RNA polymerase. The negative strand viruses must supply their own mRNA polymerase, in due to absence of enzymes in cells.

The vast amount of variations in the gene expression is noticeably located among RNA containing viruses.

Viral proteins is made in the cytoplasm on polyribosomes with virus specific mRNA and ribosome present in the host cells. Contrary to viral genomic RNA is duplicated in cell cytoplasm while the viral DNA that is replicated in the nucleus.

Maturation: This is the assembly of daughter virions in the host cell following the synthesis of viral nucleic acid and proteins. Sometimes excess of viral components may accumulate and get incorporated in the cells. This would result in the death of the cells through self-destruction.

Release: Viruses which infect Bacteria, Bacteriophage (Refer Figure 3.9) are released by lysis of the Infected Bacterium. Icosahedral capsid condenses in the absence of nucleic acid whereas helical nucleocapsids cannot be formed in the absence of viral RNA.
Viruses: Characteristics and Classification

NOTES

Animal viruses are usually released without cell lysis. The host cell is unaffected. Daughter virions released may infect other cells. From the stage of penetration till the appearance of mature virions, the virus cannot be demonstrated inside the host cells. This is called the Eclipse phase. A single releases a large number of progeny virions.

Non-enveloped viruses accumulate in the infected cells. The cells finally lyse releasing the new virus particle. Whereas enveloped viruses mature by a budding process enveloped viruses are not infectious till they have acquired their envelope. Infectious virions do not accumulate within the infected cell.

In fact Adenoviruses first found mRNA dependent ‘splicing’ when codes on separated sequences in the template resulted in expressing a protein, along with noncoding intervening sequences spliced out of the transcript.

The gene expression models could well be elucidated by small animal RNA viruses and Bacteriophages. For example, the discovery of overlapping genes where some DNA sequences are utilized in the synthesis of two different polypeptides. Figure 3.10 shows the Apple Latent Spherical Virus, Cheravirus and Figure 3.11 shows the Voguish Virus.

Function of Virion Proteins

Protection of the Genome:

- Assembly of a stable, protective protein shell.
- Specific recognition and packaging of the nucleic acid genome.
- Interaction with host cell membranes to form the envelope.

Virions are Metastable

- Must protect the genome (stable).
- Must come apart quickly on infection (unstable).
- An infectious virion is a molecular machine.
Virus Particles are Not-Inert Structures

- Virions are metastable structures that have not attained free energy conformation.
- Unfavorable energy barrier must be surmounted.

![Apple Spherical Virus, Cheravirus](image)

Fig. 3.10  Apple Spherical Virus, Cheravirus

![Voguish Virus](image)

Fig. 3.11  Voguish Virus

Chemical Composition of Viruses

**Viral Protein**

- **Envelope Protein**: Specified by both viruses and host cells. Members of all classes differ in glycoproteins differing from virus to virus.
- **Nucleocapsid Protein**: Viral capsid are made up totally of protein of identical subunits (promoters).

- **Core Protein**: It is found in the nucleic acid and is called core protein.

**Viral Enzyme**: Many virions specific enzymes have been characterised in enveloped animal viruses, for example RNA, reverse transcriptase in Retrovirus.

The structural proteins present facilitate transfer of viral nucleic acid from one host cell to another. They help in protecting viral genome against inactivation by nucleases, participate in the attachment of virus particle and determine the antigenic characteristics.

- **Viral Nucleic Acids for Viral Replication**: Viruses contain either single or double stranded DNA or RNA molecules that encode the genetic information essential for viral replication.

- **Viral Envelope**: There are containing plant and animal viruses and Bacteriophages, which are surrounded by a thin membranous envelope. This is made up of protein, lipids and carbohydrates that combine to form glycoprotein. Lipid provide flexibility to the shape. The spikes attached to the outer surface of the envelope are made up of glycoproteins.

- **Viral Carbohydrates**: Amount of carbohydrates are specified by the host cells or viral genome is found in viral envelope, for example, galactose, mannose, glucose, glucosamine, galactosamine are found in Influenza Virus (Refer Figure 3.12), and Para-Influenza Virus.

![Fig. 3.12 Influenza Virus](image)

### Check Your Progress

6. What are the common properties of virus classification?
7. What are viroids?
8. Explain adsorption phase.
9. What happens in maturation phase?
3.5 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. A virus is a small infectious agent that replicates only inside the living cells of an organism. Viruses can infect all types of life forms, from animals and plants to microorganisms, including Bacteria and Archaea.

2. Viruses spread in many ways; viruses in plants are often transmitted from plant to plant by insects that feed on plant sap, such as aphids; viruses in animals can be carried by blood-sucking insects. These disease bearing organisms are known as vectors.

3. Capsids are made up of multiple copies of a single protein or association of several proteins.

4. RNA Viruses contain RNA as the genetic material. Double stranded RNA is found in the Reovirus and Wound Tumour Virus. Single stranded RNA is found in Tobacco Mosaic Virus, Influenza Virus, Poliomyelitis Virus and Leukaemia Virus. Plant Virus contains only RNA. The RNA of several viruses fold upon themselves to form helices in some regions.

5. DNA-RNA viruses contain alternatively both RNA and DNA. An Animal Virus contains either DNA or RNA or rarely DNA-RNA.

6. Common properties of virus classification are as follows:
   - Virion Morphology
   - Nature of Genome in Virion
   - Presence or Absence of Lipid Membrane(Envelope)
   - Genome Organisation and Replication
   - Antigenic Properties
   - Biological Properties

7. Viroids are plant pathogens that consists of a short stretch of nucleobases of highly complementary, circular, single stranded RNA without the protein coat.

8. In adsorption phase, the presence of certain receptors on the surface of the host cell help virus gets attached to the host cell. These receptors recognize viral surface components. The attachment to the host cell membrane is facilitated by host cell interaction.

9. Maturation is the assembly of daughter virions in the host cell following the synthesis of viral nucleic acid and proteins. Sometimes excess of viral components may accumulate and get incorporated in the cells. This would result in the death of the cells through self-destruction.
3.6 SUMMARY

- A virus is a small infectious agent that replicates only inside the living cells of an organism.
- Viruses can infect all types of life forms, from animals and plants to microorganisms, including Bacteria and Archaea.
- Most viruses have either RNA or DNA as their genetic material. The nucleic acid may be single- or double-stranded.
- The entire infectious virus particle, called a virion, consists of the nucleic acid and an outer shell of protein.
- The simplest viruses contain only enough RNA or DNA to encode four proteins. The most complex can encode 100 – 200 proteins.
- Since Dmitri Ivanovsky’s 1892 article describing a non-bacterial pathogen infecting tobacco plants, and the discovery of the tobacco mosaic virus by Martinus Beijerinck in 1898, about 5,000 virus species have been described in detail, although there are millions of types.
- Viruses are found in almost every ecosystem on Earth and are the most numerous type of biological entity.
- The study of viruses is known as virology, a sub-specialty of microbiology.
- The origins of viruses in the evolutionary history of life are unclear: some may have evolved from plasmid pieces of DNA that can move between cells while others may have evolved from Bacteria.
- In evolution, viruses are an important means of horizontal gene transfer, which increases genetic diversity.
- Viruses are considered by some to be a life form, because they carry genetic material, reproduce, and evolve through natural selection, but lack key.
- Viruses spread in many ways; viruses in plants are often transmitted from plant to plant by insects that feed on plant sap, such as aphids; viruses in animals can be carried by blood-sucking insects.
- The variety of host cells that a virus can infect is called its ‘host range’. This can be narrow, meaning a virus is capable of infecting few species, or broad, meaning it is capable of infecting many.
- Viral infections in animals provoke an immune response that usually eliminates the infecting virus.
- Immune responses can also be produced by vaccines, which confer an artificially acquired immunity to the specific viral infection.
- Some viruses, including those that cause AIDS (Acquired Immuno-Deficiency Syndrome) and Viral Hepatitis, evade these immune responses and result in chronic infections.
- Viruses are much smaller than Bacteria. It was their small size and ‘Filterability’ (ability to pass through filters that can hold back Bacteria) that led to their recognition as a separate class of infectious agents.
- The virion consists essentially of a nucleic acid surrounded by a protein coat, the capsid.
- The capsid with the enclosed nucleic acid is known as the nucleocapsid. The function of the capsid is to protect the nucleic acid from inactivation by nucleases and other deleterious agents in the environment.
- The capsid is composed of a large number of capsomeres with its morphological units.
- The chemical units of the capsid are polypeptide molecules, which are arranged symmetrically to form molecules, and symmetrically to form an impenetrable shell around the nucleic acid core.
- One of the major functions of the capsid is to introduce viral genome into host cells by adsorbing readily to cell surfaces.
- Viruses like Bacteriophages infect Prokaryotic cells while others infect Eukaryotic cells.
- Some virus destroy cells producing disease other persist in existing cells.
- Viral protein, besides protecting the nucleic acid, also determines the antigenic specificity of the virus.
- Some viruses also contain small amounts of carbohydrate. Most viruses do not possess any enzymes for the synthesis of viral components or for energy production but some have other enzymes; for example, the Neuraminidase in the Influenza Virus.
- Retroviruses have a unique enzyme, RNA-dependent-DNA polymerase or ‘transcriptase’, which can transcribe RNA into DNA.
- Viruses are inactivated within seconds at 56°C, within minutes at 37°C and within days at 4°C. They are stable at low temperatures.
- Viruses have no metabolic system of their own viewed as mobile genetic elements most probably as cellular origin and characterized by long co-evolution of virus and host.
- The shapes of viruses range from simple helical and icosahedral forms to more complex structures.
- The protein shell of the virus is called capsid, it protects the nucleic acid from inactivation by nucleosides and other deleterious agents in the environment.
• The genetic material, which is present inside the capsid, is called the nucleic acid. Three different types of viruses, which contain different types of nucleic acids are DNA viruses, RNA viruses and DNA-RNA viruses.

• A huge variety of genomic structures can be seen among viral species; as a group, they hold more structural genomic diversity than Animals, Archaea, Plants or Bacteria.

• The extracellular fully assembled infectious virus particle is called a virion.

• The main function of the virion is to deliver its DNA and RNA into the host cell so that the genome can be expressed by the host cell.

• The nucleoproteins together with genomes forms the nucleocapsid.

• In enveloped viruses the nucleocapsid is surrounded by a lipid bilayer derived from the modified host cell membrane studied with outer layer of virus envelope of glycoproteins.

• Viruses are not usually classified into conventional taxonomic groups but are usually grouped according to properties as type of nucleic acid they contain, the structure of the capsid and the number of protein subunits in it, host species and immunological characteristics.

• The genomic RNA strand of single stranded RNA viruses is called sense (positive sense) in orientation if it can serve as mRNA, and antisense (negative sense) if a complimentary strand synthesised by a viral RNA transcriptase serves as mRNA.

• The International Committee on Taxonomy of Viruses (ICTV) developed the current classification system and put a greater certain virus properties to maintain family uniformity.

• The ICTV is the only body charged by the International Union of Microbiological Societies (IUMS) with the task of developing, refining and maintaining a universal virus taxonomy. There exists a similarity in taxon structure with the classification system of cellular organisms.

• Viroids are plant pathogens that consists of a short stretch of nucleobases of highly complementary, circular, single stranded RNA without the protein coat.

• The small cytoplasmic RNA associated with Rice Yellow Mottle is first discovered.

• Viruses contain the genetic information for their replication but they lack the enzymes. They depend on host cell machinery for their replication. The cycle can be divided into six phases.

• The structural proteins present facilitate transfer of viral nucleic acid from one host cell to another. They help in protecting viral genome against inactivation by nucleases, participate in the attachment of virus particle and determine the antigenic characteristics.
3.7 KEY WORDS

- **Viruses**: These are obligate intracellular parasites, which contain either DNA or RNA.
- **Capsid**: It is the protein layer, which protects the genetic material (DNA, RNA).
- **Nucleic acid**: It is the genetic material, which is present inside the capsid.
- **Virion**: The extracellular fully assembled infectious virus particle is called a virion.
- **Core protein**: It is found in the nucleic acid and is called core protein.

3.8 SELF ASSESSMENT QUESTIONS AND EXERCISES

**Short Answer Questions**

1. What is a virus?
2. Write a short note on shape and size of a virus.
3. List any five general characteristics of viruses.
4. What is nucleic acid?
5. List the common properties of virus classification.
6. Explain the chemical composition of viruses.

**Long Answer Questions**

1. Discuss briefly about different types of viruses with the help of suitable diagrams.
2. Explain the morphology and ultra-structure of viruses.
3. Differentiate between DNA, RNA and DNA-RNA viruses.
4. Briefly explain the features and structure of viral genome.
5. Explain the various classification of viruses giving appropriate examples.
6. Discuss the classification system of DNA viruses.
7. Explain the classification system of RNA viruses.
8. Write a detailed note on multiplication of viruses.
3.9 FURTHER READINGS


4.0 INTRODUCTION

Microbiology is the study of microorganisms that are not visible to naked eyes, except some parasitic worms. To view and observe these microorganisms, special equipments are required, which magnify their images several times the actual size. Microscopes are an arrangement of lenses that perform this function. While a simple microscope has only one lens, the compound microscope has a minimum of two lenses, which sequentially magnify the image. The magnified image has to be sharp and clear. The resolution and the numerical aperture of the microscope determine this feature. To be a good
microbiologist, you have to be a good microscopist. Hence, it is important to learn the handling and maintenance of the microscope very carefully.

In this unit, you will study about the basic components of a compound microscope, dark field, fluorescent, electron microscope, etc, their characteristics and functions. This unit also discusses the methods of using a microscope properly and the care and maintenance measures that must be taken while using it.

### 4.1 OBJECTIVES

After going through this unit, you will be able to:

- Discuss the various parts of a compound microscope and their functions
- Understand what the numerical aperture of a lens means
- Use a light microscope
- Analyse how to care for and maintain a compound microscope
- Classify the various types of microscopes
- Explain about confocal microscopy, its principle, advantages and disadvantages

### 4.2 MICROSCOPE

A microscope is an optical instrument which is used to see highly magnified images of tiny objects, such as bacteria, cells, viruses and protozoans, etc., because these tiny objects cannot be seen by naked eyes. The optical microscope, often referred to as the light microscope, is a type of microscope that uses visible light and a system of lenses to magnify images of small subjects. There are two basic types of optical microscopes, namely the Simple Microscopes and Compound Microscopes.

The microscope is an essential instrument for the diagnosis of disease. It is a precision instrument and requires careful maintenance to prevent damage to the mechanical and ocular parts and also to stop fungi from obscuring the lenses. Figure 4.1 shows the two different types of microscope, simple and complex.

#### 4.2.1 Components of a Microscope

The various components of the microscope can be classified into four systems:

1. The Support System
2. The Magnification System
3. The Illumination System
4. The Adjustment System

1. **Support System**
The support system consists of the following:

- The Foot
- The Limb
- The Revolving Nose Piece Corrective Changer
- The Stage
- The Mechanical Stage which gives Selective Controlled Movements to the Slide

2. Magnification System

This consists of a system of lenses. The lenses of the microscope are mounted in two groups:

- The first group of lenses is at the bottom of the tube, just above the preparation under examination (the object) and is called the ‘Objective Piece’.
- The second group of lenses is at the top of the tube and is called the ‘Eye Piece’.

(i) Objective Piece

The magnification power of each objective is shown by a figure engraved on the sleeve of the lens:

- The x 10 objective magnifies 10 times.
- The x 40 objective magnifies 40 times.
- The x 100 objective magnifies 100 times.
• The x 100 objective is usually marked with a red ring to show that it must be used with the immersion oil.

Some microscopes are fitted with x3 or x5 objective instead of x10 objective.

(a) **Numerical Aperture**

The numerical aperture is also engraved on the sleeve, next to the magnification, for example:

- 0.30 on the x10 objective.
- 0.65 on the x40 objective.
- 1.30 on the x100 objective.

The greater the numerical aperture, the greater the resolving power. Moreover, the greater the numerical aperture, the smaller the front lens mounted at the base of the objective. The front lens of the x 10 objective is the size of pinhead, so handle it with care.

(b) **Working Distance**

The working distance of an objective is the distance between the front lens of the objective and the object slide when the image is in focus. The greater the magnifying power of the objective, the smaller the working distance:

- x 10 objective the working distance is 5-6 mm.
- x 40 objective the working distance is 0.5-1.5 mm.
- x100 objective the working distance is 0.15-0.20 mm.

(c) **Resolving Power**

The resolving power of an object is its ability to reveal closely adjacent details as separate and distinct. The greater the resolving power of the objective, the clearer the image. The maximum resolving power of a good medical laboratory microscope is about 0.25µ (the resolving power of the normal human eye is about 0.25 mm).

Immersion oil increases the resolving power by conserving many light rays than would be lost by refraction if a dry objective were used.

(ii) **Eye Piece**

The magnification power of the eyepiece is marked on it.

- x 5 eyepiece magnifies the image produced by the objective five times.
- x 10 eyepiece magnifies the image 10 times.

If the object is magnified 40 times by the x 40 objective, then by five times by the x 5 eyepiece, the total magnification is: 5 x 40 = 200. To calculate the total magnification of the object observed, multiply the magnifying power of the objective by that of the eyepiece. Microscopes
used in the medical laboratories have a magnifying power of between x50 and x1000.

**Binocular Microscopes**

Binocular microscopes (two pieces but using only one objective at a time) are generally recommended. They are less tiring for the eyes than monocular microscopes when long examinations have to be made. Electric illumination is however essential for using the x100 objective.

3. **Illumination System**

(i) **Light Source**

An electric light source is preferable, since it is easy to adjust. It is provided either by a lamp built into microscope beneath the stage, or by an external lamp placed in front of the microscope.

(ii) **Mirror**

The mirror reflects rays from the light source onto the object. One side has a plane surface, the other a concave surface. The concave side forms a low power condenser and is not intended to be used if the microscope already has a condenser.

(iii) **Condenser**

The condenser brings the rays of light to a common focus on the object to be examined. It is situated between the mirror and the stage. The condenser can be raised (Maximum illumination) and lowered (Minimum illumination). It must be centered and adjusted correctly.

(iv) **Diaphragm**

The diaphragm which is inside the condenser, is used to reduce or increase the angle and therefore also the amount of light that passes into the condenser. The wider the diaphragm, the greater the numerical aperture and smaller the detail seen. But the contrast is correspondingly diminished.

(v) **Filters**

In some microscopes, coloured filters (particularly blue filters) are fitted below the condenser. These can be left in place or removed according to the type of preparation being examined.

4. **Adjustment System**

Adjustment system consists of the following:

- A coarse adjustment screw
- A fine adjustment screw
- A condenser adjustment screw
- Condenser centering screws
- An iris diaphragm lever
- Mechanical stage controls

**NOTES**

**Coarse Adjustment Screw:** This is the largest screw. It is used first to achieve an approximate focus.

**Fine Adjustment Screw:** This moves the objective more slowly. It is used to bring the object into perfect focus.

**Condenser Adjustment Screw:** This is used to raise the condenser for greater illumination or to lower it to reduce the illumination.

**Condenser Centering Screws:** There may be three screws placed around the condenser: one in front, one on the left and one on the right. They are used to centre the condenser exactly in relation to the objective.

**Iris Diaphragm Lever:** This small lever fixed to the condenser. It can be moved to close or open the diaphragm, thus reducing or increasing both the angle and the intensity of the light.

**Mechanical Stage Controls:** These are used to move the object slide on the stage: one screw moves it backward and forward and the other screw moves it left or right.

### 4.2.2 Microscope: Setting Up and Cleaning

When a new microscope is received in the laboratory, it is important to know how to set it up correctly.

Place it on a firm level bench of adequate size but not too high. The microscope must be placed in the shade away from the window. Place a square felt pad under the microscope. If no felt is available, use a piece of heavy cloth.

Microscopes must be used in a well ventilated and illuminated area and away from chemicals. Microscopes are used to investigate biological tissues and fluids and must therefore be decontaminated at regular intervals.

- Clean it with a piece of old cloth or a fine linen handkerchief.
- A small bottle of cleaning solution should be used.
- A plastic cover should be always wrapped over the microscope after the use.
- Never use ordinary paper to clean the lens.
- Never dip objective in Xylene as it may cause the lens to become detached.
- Never touch the lens with your fingers.
- Keep the mechanical stage clean.
- Never carry the microscope by the limb with one hand, use both the hands one under the foot and other holding the limb.
If the main voltage fluctuates excessively use the voltage stabilizers.

At the end of the day's work clean the microscope thoroughly.

4.2.3 Simple Microscope

A simple microscope is one which uses a single lens for magnification, such as a magnifying glass while a compound microscope uses several lenses to enhance the magnification of an object. Basically, a simple microscope uses a lens to enlarge an object through angular magnification alone, giving the viewer an erect enlarged virtual image.

The use of a single convex lens or groups of lenses is found in simple magnification devices, such as the magnifying glass, loupes, and eyepieces for telescopes and microscopes.

Therefore, a simple microscope is actually a convex lens of small focal length, which is used for seeing the magnified images of small objects.

**Principles of Simple Microscope**

A simple microscope works on the principle that when a tiny object is placed within its focus, a virtual, erect and magnified image of the object is formed at the least distance of distinct vision from the eye held close to the lens.

**Magnification of Simple Microscope**

The magnifying power of a simple microscope is given by:

\[ M = 1 + \frac{D}{F} \]

Where, \( D \) = Least Distance of Distinct Vision  
\( F \) = Focal Length of the Convex Lens

The focal length of the convex lens should be small because smaller the focal length of the lens, greater will be its magnifying power.

The maximum magnification of a simple microscope is about 10, which means that the object will appear 10 times larger by using the simple microscope of maximum magnification.

**Applications of Simple Microscope**

- It is typically used for studying the microscopic Algae, Fungi and biological specimen.
- It is commonly used by watch makers to see the magnified view of small parts of a watch.
- It is also used by the jewellers to see the magnified view of the fine parts of jewellery.
- It is used to see the enlarged image of letters of a book or threads of a cloth.
- It is used to see the magnified view of different particles of different types of soils.
4.3 COMPOUND MICROSCOPE

Compound microscope should more appropriately be called compound light microscope. Simply put, in a microscope, the object (specimen) to be observed is put on a glass slide and is scanned with a focused beam of light. The parts of the object, which are optically dense or are stained, form a potential image in the path of the light. This image is then magnified twice by using glass lenses and is seen by the observer.

Compound microscopes are either monocular or binocular, depending on whether they have a single eyepiece or two eyepieces. Monocular microscopes are useful for training of beginners, because they may find it difficult to fuse the two images generated in a binocular microscope. It is also useful for demonstration purposes to many viewers because the binocular head distance doesn’t have to be adjusted for each viewer. For photography too, it is better than a binocular microscope, though a monocular head may be temporarily fitted in place of binocular head for this purpose.

A binocular microscope is recommended for routine laboratory work because it reduces eye strain and fatigue. The various parts of a compound microscope are illustrated in the following figure (Refer Figure 4.2).

![Fig. 4.2 Compound Microscope](image)

The microscope rests on a heavy foot from which the fixed upright stand (5) arises. At the upper end of the upright stand is present an inclined binocular head (2). The head has two eyepieces (1) above and a revolving
nose piece (3) with several objectives below. Horizontal platform called the stage (6) is attached to the upright stand through a racking mechanism. This racking mechanism allows the stage to be moved up and down for focusing purposes. The stage has a central hole above which the slides for viewing are kept. The slide is secured in position by a spring mechanism. The stage can be moved up and down for focusing using the coarse (14) and fine adjustments (15). To move the slide on the stage for scanning different areas, the stage adjustments (11) can be used.

A built-in lamp (16) is present at the foot of the microscope. Light is passed upwards from it to the substage condenser (8). This substage condenser focuses the light on the specimen kept on the stage. The condenser has an aperture diaphragm (iris) (9), which is used to control the intensity of the light. The condenser can be moved up or down by condenser focusing adjustment (7). An electronic control is present on the side of the foot to increase or decrease the illumination.

While in the monocular microscope, the light passes straight from the objective to the eyepiece, in the binocular microscope it has to be directed into two separate eyepieces. This is achieved by dividing the light from the objective equally using half-silvered surfaces inclined at 45°, which permit half the light to pass through them while reflecting the rest at right angles. Prisms are used to direct each half of the light into its appropriate eyepiece.

The two eyepieces can be moved towards each other or apart from each other to allow the observer to adjust their positions to his/her interpupillary distance. The focusing of one of the two eyepieces can be adjusted to compensate for the difference in focusing power of each eye. Due to the inclined head of the binocular microscope, the eyepieces are positioned at an angle convenient for a seated observer. However, this inclined head may increase the magnifying power of the microscope by a certain factor (say, 1.5 times). This factor, if present, is engraved on the side of the microscope and, in such cases, eyepieces of lower magnification, i.e., 6X or 8X should be used instead of 10X.

4.3.1 Optical System of a Compound Microscope

The optical system of a compound microscope is defined by its magnifying power, numerical aperture, definition and resolution. These characteristics of a compound microscope are discussed in the following sections.

Magnification

Microscope is used to produce an enlarged and well-defined image of objects too small to be seen by the naked eye. The degree of enlargement is called ‘magnification’ or the ‘magnifying power’ of the microscope. The number of times the length, breadth or the diameter (not the area) of an object is increased is the magnifying power of the microscope.
Magnification by a microscope occurs in two stages; first is the magnification by the objective lens and second is the magnification by the eyepiece lenses. The overall magnifying power of the microscope is calculated by multiplying the magnifying power of the objective with that of the eyepiece. If the inclined binocular head increases the magnifying power of the microscope, then the magnifying powers of objective and eyepiece should be further multiplied by that factor.

While the magnifying power of the eyepieces is usually constant, objective lenses of differing magnifying power are incorporated into the nosepiece. The magnifying power of an objective is calculated by dividing the optical tube length with the focal length of the objective lens. The optical tube length is usually 160 mm while the focal length of the objectives differs from lens to lens. Thus, an oil-immersion lens of 2 mm focal length should give a magnification of $160/2 = 80$ (80X). However, the oil-immersion lenses marked as having a focal length of 2 mm, actually have a slightly shorter focal length and therefore, their magnification is 95 to 100 times (95X to 100X).

**Numerical Aperture**

The ability of an objective lens to gather the light and to resolve details of the object depends on the angle of aperture of the lens. Numerical aperture is defined as the ratio of the diameter of the lens to its focal length. Mathematically, Numerical Aperture (NA) is expressed by the formula:

$$NA = n \sin U$$

Here, ‘$n$’ is the refractive index of the medium present between the object and the objective (it is 1.0 for air and 1.5 for immersion oil). ‘$2U$’ is the angle of aperture (angle CAD is shown in Figure 4.3), i.e., it is the angle formed by the two most divergent rays starting from the centre of the object which are focused by the lens on the eye of the observer.

![Fig. 4.3 Numerical Aperture](image-url)
Theoretically, the angle of aperture, i.e., $2U$ can be maximum up to $180^\circ$ and thus $U$ can be maximum up to $90^\circ$. Thus, the greatest possible NA of a dry lens cannot exceed 1.0 because $\sin 90^\circ$ is 1, and the refractive index 'n' of air is also 1. However, practically, 0.95 is the highest possible NA of dry lenses.

For oil-immersion lenses, since the ‘n’ of immersion oil is 1.5, the highest possible NA is 1.5. However, the ordinary 2 mm lenses have a NA of 1.3, while the ‘apochromat’ objectives have a NA of 1.4.

### Resolution

Though it is possible to make microscopes which would give a magnification much more than 1000X (say 1,000,000X), beyond 1000X the image gets blurred and its sharpness is lost. The highly magnified but blurred images reveal no further detail and hence are of no use. Such magnification, which serves no purpose, is called ‘empty magnification’. This happens because the resolving power of the microscope depends upon the wavelength of the light. Since the wavelength of the light is a constant, so the resolving power too is limited.

The resolving power of a microscope can be defined as its ability to reveal closely adjacent structural details as separate and distinct from each other. Quantitatively, it is expressed as the ‘Limit of Resolution’ (LR), i.e., the minimum distance between two points at which they are seen as separate and not in contact with each other. Mathematically, LR is expressed by the formula:

$$LR = \frac{(0.61 \times W)}{NA}$$

Here, ‘W’ is the wavelength of the light and ‘NA’ is the numerical aperture of the lens.

Thus, the resolving power of a microscope depends on the wavelength of the light used and the numerical aperture of the lens. Let us consider an example where green light of wavelength 0.55 um is used along with the 2 mm apochromatic oil-immersion objective of NA 1.4. Using the formula for LR, you get:

$$LR = \frac{(0.61 \times 0.55)}{1.4}$$
$$= 0.24 \text{ um}.$$ 

Thus, the minimum distance at which this microscope can distinguish two points to be distinct from each other is 0.24 um, i.e., 0.00024 mm. This resolving power is enough to observe even the smallest of the bacteria but only large viruses, such as smallpox virus.

### Definition

The capability of an objective lens to render the outline of the image of an object as clear and distinct is called definition. It must not be confused with
resolution. Definition depends on the elimination or correction of optical aberrations found in the glass lenses. These optical aberrations are of two types, namely, spherical aberration and chromatic aberration.

Spherical aberration results from the rays, passing through the edge of the lens, not being brought to the same point of focus as the rays passing nearer to the centre of the lens.

Chromatic aberration results from the separation of white light into its component colours while passing through a lens. These components have different wavelengths and are refracted to different extents. Thus, they are not recombined at the same point of focus after passing through the glass lens.

The two aberrations lead to the formation of a hazy image fringed with different colours. Aberrations are usually corrected by using a combination of lenses which have different dispersive qualities. ‘Achromatic’ objectives are made using the combination of convex lenses of crown glass with concave lenses of flint glass. Crown glass convex lenses have low dispersive power while flint glass concave lenses have high dispersive powers. An assembly combining four or more such lenses unites most of the divergent rays of various colours to form white light at the point of focus. Achromatic lenses are used for almost all clinical microbiology work and for research purposes.

‘Apochromatic’ objectives are the best optical lenses. They are prepared from glass containing the mineral, fluorite. They have high degree of transparency, low refractive index and little dispersive power. Since apochromat lenses can have a numerical aperture as high as 1.4, they give almost complete colour correction with maximum resolving power. However, they are very costly, which limits their use for critical research work and photomicrography. Apochromatic lenses should always be used with compensating eyepieces and a properly centered condenser.

### 4.3.2 Types of Objectives and Eyepieces

There are two kinds of objectives; they are either ‘Dry Objective’ or ‘Oil-Immersion Objective’. The difference is the presence of a layer of oil between the object and the lens in oil-immersion objectives, while in dry objectives, air is present between the object and the lens. Most of the microbiology work is done by using the oil-immersion objectives.

The presence of oil between the object and the lens increases the angle of the cone of rays from the object that enter the objective, thus increasing the numerical aperture of the objective. The oil used has the same refractive index as that of glass, i.e., 1.5. Earlier ‘Cedar-Wood Oil’ was used for this purpose, but nowadays, synthetic immersion oil is used. Since the oil has the same refractive index as that of the glass slide and the lens (both are made of glass), the rays of light are not refracted outwards when they leave the glass slide but are passed straight into the lens.
It is illustrated in the Figure 4.4, where the path of rays through a dry lens is represented on the right and the path through an oil immersion lens is represented on the left. In the figure, the light ray ABCD is refracted out of the Lens (L) when no oil is used. The ray MBNP passes through oil on exiting the glass slide and hence reaches the lens. Thus, the angle of the cone of light rays, which enter an oil-immersion objective, is wider than the cone of the light rays entering a dry objective of same dimensions.

![Fig. 4.4 Objectives](image)

Eyepieces in a microscope form a magnified and virtual image of the real image formed by the objective. Three types of eyepieces are in general use. These are as follows:

**Huygenian Eyepieces**

Huygenian eyepieces are the most commonly used eyepieces. They consist of two plano-convex lenses with their plane sides facing upwards. A circular field diaphragm is present between the two lenses, which restricts the field of view to the central and flattest part of the image.

**Ramsden Eyepieces**

These eyepieces are used especially when micrometry is to be performed. They too consist of two plano-convex lenses, but here the convex sides face each other. The field diaphragm is placed below the lower lens. For micrometry, the scale is put on the diaphragm.

**Compensating Eyepieces**

These eyepieces are used to correct the chromatic difference of magnification present in apochromatic objectives. They should always be used along with apochromatic objectives. In these eyepieces, a triplet system of lenses
functions as the lower lens. They can be used with high power achromatic lenses also but should not be used with low power achromatic lenses.

**Condenser**

The light which emerges from the lamp is focused on the object by the condenser. Thus, the condenser is present below the stage. It has a rack and pinion mechanism for adjusting its focus. Centering screws are also present in the condensers, which help in centering the illumination for critical work.

A simple Abbe condenser, composed of two lenses which are not corrected for spherical and chromatic aberrations, is not suitable for microbiology work. For microbiology work, an achromatic condenser with a high numerical aperture (1.37) should be used. For low power objectives, the numerical aperture of the condenser can be reduced by using the iris diaphragm present in the condenser. Since the highest numerical aperture of a dry lens is 1.0, oil should be introduced between the top lens of the condenser and the glass slide to increase the numerical aperture of the condenser lens, particularly when critical work is done using the oil-immersion objective.

To ‘centre’ the condenser, close the iris diaphragm of the condenser to its limit. Next, rack down the condenser till the image of the iris appears in the field. Using the centering screws, the image of the iris should be brought to lie in the centre of the field. To check this, open the iris diaphragm slowly, till its margins come to lie just inside the margins of the field. If needed, the image can be centered further using the centering screws. The iris diaphragm is then opened completely and the condenser is racked up for use.

When oil-immersion objective is used, the condenser should be racked fully to provide maximum brightness. With lower power objectives, either the condenser is racked down or its top lens is swung out of the train to reduce illumination. Similar reduction of illumination is performed, when wet films are viewed using the high power objective. It helps by making the bacteria and cells appear more refractile, but the definition and resolution are decreased.

**Illumination**

In older microscopes, the light source is not provided in the foot. The light is provided from a lamp which is separate from the microscope. This light is then reflected upwards into the condenser by using a plane mirror fitted in the foot of the microscope. The angle of the mirror can be adjusted by tilting it. In such microscopes, the position of the lamp and the angle of the mirror have to be adjusted every time the microscope is used. In modern microscopes, the lamp and the mirror are fitted into the foot obviating the frequent adjustments.

The lamp used in microbiology microscopes is a high-intensity lamp. Usually, an electronic control is provided which is used to control the intensity of the illumination. The lamp intensity should always be reduced to dim by
using the control, before switching the lamp on or off. Using the lamp at full intensity for prolonged periods should be avoided. If the lamp is separate from the microscope, it should be kept at a distance of 20 to 25 cm from the mirror.

4.3.3 **Using a Light Microscope**

A light microscope should be placed horizontally on the plane surface of a bench. The height of the observer’s chair should be adjusted in such a way that his/her eyes should be level with and close to the eyepieces. The forearms and elbows should be rested on the bench. Generally, those observers who wear glasses don’t need them while working on a microscope because the microscope compensates for their defect. However, if the defect cannot be compensated, the observer should wear contact lenses. If spectacles have to be worn by the observer, then he/she should take care not to scratch the lenses of the eyepieces. There are several other precautions and measures that must be kept in mind while using a compound light microscope. These are enlisted as follows:

- The eyepieces and the objectives should be clear of dust and immersion oil. Dust and liquid immersion oil can be removed using a fresh lens tissue.
- If the immersion oil has hardened, then it should be removed by using only xylol or benzol.
- The condenser should be racked up fully, i.e., the surface of its top lens should be just 1 mm below the glass slide kept on the stage.
- If the condenser is too high or too low from the fully-racked up position, then its mounting should be adjusted to bring it to the right position.
- The filter carrier in the substage condenser should be in its correct position. Its rim should not obstruct the light.
- The iris diaphragms of the condenser and the lamp assembly (if present) should be fully opened.
- The lamp should be switched on in the low intensity setting. The intensity should then be increased to the desired level.
- The slide with the object to be viewed should be kept on the stage. It should be firmly held in position by the spring clamp.
- The slide with the object on it should be in close contact of the surface of the stage.
- The specimen should be first viewed with the low power (16 mm) dry objective.
- Coarse adjustment is used to bring the slide in focus of this objective. (Fine focus adjustment should be used only to sharpen the image focused by the coarse objective and to maintain focus when moving the slide from one area to another).
• An area of the slide with easily visible material should be moved to the centre of the field.

• The distance between the eyepieces should be adjusted to merge the two fields of the image into one image. This image can then be focused for one eye by using the fixed eyepiece only along with the fine adjustment.

• Next the image can be focused for the other eye by using the focusing adjustment present in the other eyepiece (the fine focus of the microscope should be left untouched). Thus, the image would be in sharp focus for both the eyes.

• If low power objective has to be used for observation, then the light should be diffused by either racking the condenser down or by swinging out its top lens.

• If illumination requires to be reduced, then it should be done by using the intensity control and not by closing the diaphragm.

• When high objective is used, the iris diaphragms should be open and the condenser should be completely racked up.

• The light should be properly centered.

• When an oil-immersion objective is to be used, the stage should first be racked down. A moderately large drop of immersion oil should be placed on the middle of the specimen in line with the centre of the light beam being focused by the condenser.

• A dropper should be used to apply the oil so that it doesn’t spill over the microscope.

To focus the oil-immersion objective, the observer should first bring his/her eyes at the level of the stage. Using the coarse focusing adjustment, the stage should be slowly raised till the oil on the slide makes contact with the oil immersion objective. As soon as this contact happens the oil appears to ‘light up’, i.e., the drop of oil becomes illuminated with light. When this happens, the stage should be raised little further (using the coarse adjustment) till the slide comes as close as possible to the objective, without touching the objective.

The eyes are now applied to the eyepieces and while observing the object, the stage is slowly lowered away from the objective using the coarse adjustment. The movement of the stage is stopped as soon as the focus is reached and the specimen is seen. Only now should fine adjustment be used to bring the image to a sharp focus as well as to maintain the focus while moving the specimen.

A failure to find focus is common while using the oil-immersion objectives. If the focus is missed, then it should not be focused again while looking through the eyepieces, so that the glass slide doesn’t touch the objective lens. The failure to find focus may be due to slide not being brought
close enough or the absence of any visible material on area within the field of the objective.

To focus again, the slide should be moved so that some visible (though out of focus) material is brought in the field of observation. Next with the eyes at the level of the stage, the glass slide should be raised using the coarse adjustment, to bring it as close to the objective as possible. Again the eyes are applied to the eyepieces and the stage is moved away from the objective using the coarse adjustment and the same procedure is followed to focus the image. The procedure may have to be repeated if focus is still not found.

The specimen is observed by moving the slide in an orderly way, using the adjustments of the mechanical stage. Fine focus adjustment is used to maintain the area being scanned in sharp focus.

After the completion of observation, the objective lens should be wiped clean of the immersion oil by using a clean lens tissue. If any oil has been split on the stage or any other part of the microscope, then it too should be wiped off. The light intensity control of the lamp should be turned down before switching off the light.

4.3.4 Common Problems in Microscopy

A beginner may encounter some problems while performing microscopic examinations. The following solutions may help in overcoming them.

1. Inability to Focus the Image

(i) The procedure for finding the focus as described in the previous section should be followed.

(ii) Check whether the glass slide has not been kept upside down, i.e., the side of the slide carrying the specimen should be facing up.

(iii) Check the lens of the objective for the presence of dirt or dried oil on it. If it is present, it should be removed as described in the previous section.

(iv) If the immersion oil is too sticky, the slide may stick to the objective and not move down when the stage is lowered for focusing. If so, then wipe off the old oil and use fresh immersion oil.

(v) A film of dried oil or dirt may be adhering to the specimen slide hampering the observation of the specimen. It should be removed using a lens paper moistened with xylol or benzol.

(vi) If a coverslip has been applied to the specimen, then it shouldn’t be so thick that it prevents the objective (this may happen in case of oil-immersion objective) lens to come close enough to the object for focusing.
(vii) A thick layer of mounting fluid may also hamper focusing. In such cases, the coverslip has to be removed and replaced with a thinner coverslip applied with a thin layer of mounting fluid.

(viii) If all of the above problems are absent, then the possibility of the objective being defective should be considered. The objective should be replaced with an objective taken from another microscope. If this objective gives a clear sharp image, then the original objective is defective and it should be replaced.

2. Dark Shadows Passing into the Image Blurring It

This problem is caused by the movement of an air bubble in the oil. Since air is lighter than the oil, the bubble would always float to the middle of the field because it is the highest point of the oil drop. To remove this bubble, move the stage downwards using the coarse adjustment. The contact between the oil and the objective has to be broken to let the bubble burst. The slide has to be refocused as described in the previous section. If this fails to remove the bubble, wipe off the oil on the slide and the objective and use a fresh drop of oil. Refocus the image and observe.

3. Poor Illumination

(i) The condenser may not be racked up completely. Sometimes, the condenser slips down in its mounting ring and has to be pressed up so that it can be within 1 mm below the glass slide carrying the specimen.

(ii) If the condenser has been provided with the swing out top lens, then it should be in its ‘swing in’ position, i.e., the top lens should be in line with the light beam.

(iii) If the substage iris diaphragm is not fully open, then enough light won’t be able to pass through to enable the use of oil-immersion objective. Open it completely if closed.

(iv) The condenser should be properly centered. If not, then use the centering screws to bring the illumination to centre.

(v) If the mirror used is moveable, then its flat (and not concave) surface should be facing the light source. The mirror should be adjusted so that maximum amount of light is focused on the condenser.

4.3.5 Care and Maintenance

The accuracy and precision of the microscope can be easily preserved by taking proper care of the instrument. Care must be taken to provide the proper environment for its storage and use. The optical parts should be maintained properly.
The microscope should not be exposed to direct sunlight or a source of heat. To move it from one place to another, it should be lifted using its fixed upright and the foot. It should not be lifted by any of its moving parts. Care must be taken to avoid jarring and mechanical damage to the microscope while moving it.

When the microscope is not being used, it should be protected from dust. This can be done either by keeping it in its box or by covering it with a plastic cover. The microscope should be cleaned at intervals and its working surfaces should be lightly smeared with soft paraffin oil. The component parts of the condenser and the objective should never be dismantled for cleaning.

If immersion oil is spilled and allowed to dry, it becomes quite troublesome. Dried oil present on an oil-immersion lens (or any other lens too) would interfere with its focusing. Even fresh oil, if it is spilled on a dry objective, would lead to focusing problems of the dry objective. If the oil is spilled on the stage, it would hamper the movement of the glass slide. Oil spilled and dried on the moving parts would interfere with their working.

Oil-immersion objectives should be cleaned after every use by wiping off the oil using a well-washed and clean cotton cloth or a fresh lens paper. The cloth or paper should not be reused because it may have grit on it, which may cause scratching of the surface of the lens.

Eyepieces may become contaminated internally with dust. This dust can be seen as specks in the field. To make sure that the dirt is located in the eyepiece (and not elsewhere), rotate the eyepiece in its mount. The specks would also be seen to rotate. To determine whether the dust particles are present on the upper lens or the lower lens of the eyepiece, lift the eyepiece a little with one hand and unscrew the upper lens with other hand. If the specks move, then the dirt is on the upper lens. Similarly, when the mount of the lower lens is moved by unscrewing, the specks would move too if the dust is on the lower lens.

Check Your Progress

1. What are the two kinds of compound microscopes?
2. Give any one point of difference between monocular microscope and binocular microscope.
3. What are the two stages of magnification that occur in a microscope?
4. What do you understand by definition of a compound microscope?
5. What is the resolving power of a microscope?
6. What are apochromatic objectives made of?
4.4 DARK FIELD

In dark field microscopy, the non-diffracted rays are removed altogether so that the image is composed solely of diffracted wave components. This technique is very sensitive because images based on small amounts of diffracted light from minute phase objects are seen clearly against a black or very dark background. Dark field microscopy is most commonly used for minute light-diffracting specimens such as diatoms, bacteria and bacterial flagella, isolated organelles and polymers such as cilia, flagella, microtubules and actin filaments, and silver grains and gold particles in histochemically labelled cells and tissues. An example is dark field image of labeled neurons. The number of scattering objects in the specimen is an important factor, because the scattering of light from too many objects may brighten the background and obscure fine details.

A. Theory and Optics

Dark field conditions are obtained by illuminating the specimen at an oblique angle such that direct, non-diffracted rays are not collected by the objective lens. The effect of dark field optics can be obtained quickly with bright-field optics by rotating the condenser turret so that rays illuminate the specimen obliquely. Only diffracted light from the specimen is captured by the objective, and the direct waves pass uncollected off to one side of the lens. The disadvantage of this technique is that unidirectional illumination of highly refractile objects can introduce large amounts of flare. Much better images are obtained with a special dark field condenser annulus, which is mounted in the condenser turret. Special oil-immersion dark field condensers must be used for oil immersion objectives. Dark field microscopy resembles phase-contrast microscopy in that the specimen is illuminated by rays originating at a transparent annulus in the condenser.

However, in dark field optics only diffracted rays are collected by the objective and contribute to the image; non-diffracted rays are pitched too steeply and do not enter the lens. Since non-diffracted background light is absent from the image, light-diffracting objects look bright against a dark field. The dark field microscope is shown in Figure 4.5.
Fig. 4.5  Dark Field Microscope

Source: http://en.wikipedia.org/wiki/Dark_field_microscopy—accessed on 21/02/13

B. Image Interpretation

The appearance of a dark field image is similar to one of self-luminous or fluorescent objects on a dark background, but with the difference that edges of extended, highly refractile objects diffract the greatest amount of light and dominate the image, sometimes obscuring the visibility of fainter, smaller objects. In addition, details in dark field images are broader and less distinct compared to other imaging modes such as phase contrast, because removal of one entire order of light information from the diffraction plane makes edge definition less distinct in the image. Further, if the NA of the objective selected is too restricted, many diffracted waves are also eliminated, resulting in a loss of definition of fine details in the specimen.

4.5 PHASE CONTRAST

In the case of stained, histological preparations or specimens with naturally occurring pigments, specific wavelengths are absorbed by dyes or pigments, allowing objects to appear in colour when illuminated with white light. With monochromatic illumination a colour filter complementary to the colour of the specimen is used. For example, a blue object examined through yellow filter-object rays is significantly reduced in amplitude, resulting in a high-contrast image. Such objects are called amplitude objects because they directly
Microscopy

produce amplitude differences in the image that are detected by the eye as differences in the intensity. Although most transparent biological specimens do not absorb light, they do diffract light and cause a phase shift in the rays of light passing through them; thus, they are called phase objects.

A. Theory

Phase contrast microscopes feature an optical design that transforms differences in the phase of object-diffracted waves to differences in the image, making objects appear as if they have been optically stained (Refer Figure 4.6).

Because the method is dependent on diffraction and scattering, phase-contrast optics also differentially enhance the visibility of the light scattering edges of extended objects and particles. The performance of modern phase-contrast microscopes is remarkable. Under favourable conditions and with electronic enhancement and image processing, objects containing just a few protein molecules can be detected.

![Phase Contrast Microscope Diagram](image)

**Fig. 4.6 Phase Contrast Microscope**

To form a phase contrast image, the rings of the annulus and phase plate must have matching diameters and be perfectly aligned. A multiple-position condenser with a rotating turret may contain two or three annuli intended for use with different phase contrast objectives. Small annuli are used for low-power dry objectives, whereas large annuli are employed with high-power, oil-immersion lenses. Since the objective lens is usually fixed, alignment is performed by moving the condenser annulus with special annulus positioning screws on the condenser. The annulus-adjustment screws, not to be confused with the condenser-centration screws, are either permanently mounted on the condenser turret or come as separate tools that must be inserted into the condenser for this purpose. After bringing the rings into sharp focus with the telescope focus, move the bright image of the annulus to exactly coincide with the dark ring on the phase plate. Improper alignment gives a bright, low
contrast image, because the bright background rays are not properly attenuated or advanced in phase as required by phase contrast theory.

**B. Interpreting the Phase Contrast Image**

Phase contrast images are easiest to interpret when the cells are thin and spread out on the substrate. When such specimens are examined in positive-contrast mode, which is the conventional mode of viewing, objects with a higher refractive index than the surrounding medium appear dark. Most notably, phase contrast optics differentially enhances the contrast of the edges of extended objects such as cell margins. Generally, positive phase contrast optics gives high-contrast images that we interpret as density maps. As an approximation, this interpretation is usually correct, because the amplitude and intensity in an object image are related to refractive index and optical path length. Thus, a series of objects of increasing density (such as, cytoplasm, nucleus and nucleolus) is typically seen as progressively darker objects. However, the size and orientation of asymmetric objects also affect intensity and contrast. Further, there are optical artifacts we need to recognize that are present in every phase contrast image.

### 4.6 FLUORESCENT MICROSCOPE

Molecules absorbing the energy of electromagnetic radiation (i.e., photons) will be elevated to a higher energy level, or excited state. These excited molecules will return to the ground state and some molecules will emit radiation on their return to the ground state. This phenomenon is known as fluorescence and fluorescent molecules are known as fluorochromes.

**A. Theory**

The fluorometer is similar to a spectrophotometer, except that the photomultiplier tube to detect the emitted light is located at a right angle from the path of the excitation light. The sample is exposed to UV light of the desired wavelength and emitted light of the appropriate wavelength range is detected by a photomultiplier tube. The intensity of the emitted light is quantified and usually expressed as relative fluorescence units.

Since many fluorescent molecules emit light in the visible range it is possible to view fluorescence in conjunction with microscopy. An UV light source is used to illuminate the sample through the objective lens using a beam-splitting mirror. The fluorescence emitted from the sample (epi-fluorescence) passes through this same mirror, but the UV light does not. Filters before the beam-splitting mirror will control the excitation wavelength and filters before the eyepiece (or camera) will control the wavelength of the emitted light.

Fluorescent probes can be used to identify individual cells or subcellular components. The location of fluorochromes associated with cells will appear
as light objects against a dark background. Immunofluorescence uses antibodies with a conjugated fluorescent molecule (for example fluorescein or rhodamine) to determine the location of a protein within a cell.

B. Image Interpretation

Numerous fluorescent dyes which bind to specific subcellular components are also available. For example, acridine orange fluoresces when it binds to either RNA or DNA. Acridine orange bound to RNA fluoresces a yellow to green color and acridine orange bound to DNA fluoresces orange to red. Other fluorescent probes which bind to specific subcellular compartments are also available. In addition, there are many fluorescent probes available that are sensitive to pH, divalent cations such as Ca$^{2+}$, and membrane potentials. Such probes permit physiological measurements in individual living cells. Figure 4.7 shows the fluorescence microscope.

Dual-labelling experiments in which fluorochromes with different emission spectra are co-incubated with the sample can also be carried out. The different fluorochromes will appear as different colours due to the differences in their emission spectra. If the emission spectra are far enough apart then it is possible to use filters so that the two fluorochromes are examined separately. Dual-labeling experiments are especially useful for determining whether two proteins (or other substances) are localized to the same subcellular compartment.

4.7 ELECTRON MICROSCOPES

Electron microscope is an expensive technique that uses a beam of electrons to create an image of the specimen (Refer Figure 4.8). It has greater resolving power and forms an image of higher magnification as compared to light microscope.
Electron Microscopes: SEM and TEM

Electron microscopes are specifically used to investigate the ultrastructure of a wide range of biological and inorganic specimens including microorganisms, cells, large molecules, biopsy samples, metals, and crystals. In the industries, the electron microscopes are frequently used for quality control and failure analysis. Modern electron microscopes produce electron micrographs using specialized digital cameras and frame grabbers to capture the images.

Transmission Electron Microscope (TEM) and Scanning Electron Microscope (SEM) are two types of electron microscopes. TEM uses electrons passed through the sample to build a picture of the sample internal structure, whereas SEM uses electrons scattered from the sample surface to build up a picture of the sample surface.

Transmission Electron Microscope (TEM)

The original form of the electron microscope, the Transmission Electron Microscope (TEM), uses a high voltage electron beam to illuminate the specimen and create an image. The electron beam is produced by an electron gun, commonly fitted with a tungsten filament cathode as the electron source. The electron beam is accelerated by an anode typically at +100 keV (40 to 400 keV) with respect to the cathode, focused by electrostatic and electromagnetic lenses, and transmitted through the specimen that is in part transparent to electrons and in part scatters them out of the beam. When it emerges from the specimen, the electron beam carries information about the structure of the specimen that is magnified by the objective lens system of the microscope. The spatial variation in this information, i.e., the ‘image’ may be viewed by projecting the magnified electron image onto a fluorescent viewing
screen coated with a phosphor or scintillator material, such as zinc sulphide. Alternatively, the image can be photographically recorded by exposing a photographic film or plate directly to the electron beam, or a high-resolution phosphor may be coupled by means of a lens optical system or a fibre optic light-guide to the sensor of a digital camera. The image detected by the digital camera may be displayed on a monitor or computer.

TEMs are often used in electron diffraction mode. The advantages of electron diffraction over X-ray crystallography are that the specimen need not be a single crystal or even a polycrystalline powder, and also that the Fourier transform reconstruction of the object’s magnified structure occurs physically and thus avoids the need for solving the phase problem faced by the X-ray crystallographers after obtaining their X-ray diffraction patterns.

One major disadvantage of the TEM is the need for extremely thin sections of the specimens, typically about 100 nanometers. Creating these thin sections for biological and materials specimens is technically very challenging. Biological tissue specimens are chemically fixed, dehydrated and embedded in a polymer resin to stabilize them sufficiently to allow ultrathin sectioning.

**Scanning Electron Microscope (SEM)**

The Scanning Electron Microscope (SEM) produces images by probing the specimen with a focused electron beam that is scanned across a rectangular area of the specimen (raster scanning). When the electron beam interacts with the specimen, it loses energy by a variety of mechanisms. The lost energy is converted into alternative forms such as heat, emission of low-energy secondary electrons and high-energy backscattered electrons, light emission (cathodoluminescence) or X-ray emission, all of which provide signals carrying information about the properties of the specimen surface, such as its topography and composition. The image displayed by an SEM maps the varying intensity of any of these signals into the image in a position corresponding to the position of the beam on the specimen when the signal was generated.

Generally, the image resolution of an SEM is lower in comparison to that of a TEM. However, because the SEM images the surface of a sample rather than its interior, the electrons do not have to travel through the sample. This reduces the need for extensive sample preparation to thin the specimen to electron transparency.

The SEM also has a great depth of field, and so can produce images that are good representations of the three-dimensional surface shape of the sample.

**Types of Specimens Used**

With most specimens it is not sufficient to move them only in the horizontal plane. Although the specimen is thin, there is nevertheless information in the
image coming from various depths within the specimen. This can be seen by tilting the specimen and taking 3D photographs.

In order to define the axis of tilt, it is necessary to be able to rotate the specimen. Crystalline specimens need to have a second tilt axis perpendicular to the first tilt axis in order to be able to orient a part of the specimen so as to obtain the required diffraction pattern. These requirements can be fulfilled in a device called a goniometer.

The goniometer is a specimen stage designed to provide, in addition to X and Y translation of the specimen, tilt about one or two axes and rotation, as well as Z movement (specimen height) parallel to the beam axis.

A TEM can be used in any branch of science and technology where it is desired to study the internal structure of specimens down to the atomic level. It must be possible to make the specimen stable and small enough (some 3 millimeters in diameter) to permit its introduction into the evacuated microscope column and thin enough (less than about 0.5 micrometers) to permit the passage of electrons. Every branch of research has its own specific methods of preparing the specimen for electron microscopy.

In biology for example, tissues are sometimes treated as follows:

- First there is a chemical treatment to remove water and preserve the tissue as much as possible in its original state.
- It is then embedded in a hardening resin.
- After the resin has hardened, slices (sections) with an average thickness of 0.5 micrometers are cut with an instrument called an ultramicrotome equipped with a glass or diamond knife.
- The tiny sections thus obtained are placed on a specimen carrier usually a 3 mm diameter copper specimen grid which has been coated with a structure-less carbon film 0.1 micrometer thick.

In metallurgy the following method of preparation is sometimes applied:

- A 3-mm diameter disc of material (thickness say 0.3 mm) is chemically treated in such a way that in the centre of the disc the material is fully etched away.
- Around this hole there will usually be areas that are sufficiently thin (approximately 0.1 micrometer) to permit electrons to pass through.
- In a semiconductor it is sometimes desired to cut out a section of material perpendicular to the surface in order to investigate a defect. This is done by ion-beam etching.
- A technique which is gaining importance in the field of structural biology is that of freezing the specimen and observing it in the frozen state.
Applications of Electron Microscopy

Electron microscopes and ion beam microscopes are both amazing and incredibly complex scientific instruments used by research laboratories, universities, nanotechnology centers, and companies worldwide. Although few of us will ever own or use an electron microscope, their impact is pervasive, impacting our lives in a variety of ways, from the clothes we wear, to the tools and devices we use, and the food we eat. The applications of these instruments are diverse, ranging from particle analysis to material characterization to industrial failure analysis and process control. In the electronics industry, for example, semiconductor and electronics manufacturers use specialized microscopes for high-resolution imaging and analysis required to develop and control the manufacturing process.

Companies worldwide use electron microscopes in a variety of industrial applications including aeronautics, automotive manufacturing, clothing and apparel, machining, pharmacology, and many more. Forensic science which is the application of science to law, is one application made popular by the television show ‘CSI’ and others. Microscopic analysis of gunshot residue, blood samples, or clothing fibers to help solve crime is common on TV, and in real life.

In life sciences, electron microscopes are being used to explore the molecular mechanisms of disease, to visualize the 3D architecture of tissues and cells, to unambiguously determine the conformation of flexible protein structures and complexes, and to observe individual viruses and macromolecular complexes in their natural biological context.

In natural resources, the ability to characterize and analyse organic materials is critical for mining companies to analyse millions of micro-scale features in an automated, objective, quantitative and rapid manner. In oil and gas exploration, similar analyses provide quantitative litho type and porosity characteristics of reservoir, seal, and source rocks.

Researchers worldwide are using electron microscopes in the pursuit of a deeper understanding of the structure property function relationships in a wide range of materials and processes such as next-generation fuel cell and solar cell technologies, catalyst activity and chemical selectivity, energy-efficient solid-state lighting, and lighter, stronger and safer materials.

Check Your Progress

7. What is dark field microscopy?
8. How phase contrast microscopes work?
9. Explain the working of fluorescence microscope.
10. What are electron microscopes?
4.8  **CONFOCAL MICROSCOPY: PRINCIPLES AND THEIR APPLICATION**

Confocal microscopy has following benefits in comparison to conventional widefield optical microscopy.

- Elimination or reduction of background information away from the focal plane, which causes image degradation.
- Ability to control depth of field.
- Capability to collect serial optical sections from thick samples.

The fundamental of confocal approach is the usage of spatial filtering techniques for elimination of the out-of-focus light or glare in samples whose thickness exceeds the immediate plane of focus. In recent years, the popularity of confocal microscopy has increased, because of the relative ease with which exceptionally high-quality images can be obtained from samples that are prepared for conventional fluorescence microscopy, and the increase in the number of applications in cell biology, which depends on imaging both fixed and living cells and tissues. Confocal technology has been proved to be the most important advances ever achieved in optical microscopy.

![Fig. 4.9 Confocal and Widefield Fluorescence Microscopy](image)
In a conventional widefield optical epi-fluorescence microscope, secondary fluorescence released by the sample is occurred through the excited volume and obscures resolution of features that are in the objective focal plane. The problem is compounded by thicker samples, which are greater than 2 micrometers, usually exhibit high degree of fluorescence emission in which most of the fine detail is lost. Confocal microscopy gives only a marginal improvement in both axial (z; along the optical axis) and lateral (x and y; in the specimen plane) optical resolution. However, it is able to exclude secondary fluorescence in areas which are removed from the focal plane from resulting images. The resolution can be enhanced with confocal microscopy over conventional widefield techniques, but it is still less than that of the transmission electron microscope. In this aspect, confocal microscopy is considered as a bridge between these two classical approaches.

In Figure 4.9, there are image series that compare certain view fields in traditional widefield and laser scanning confocal fluorescence microscopy. In widefield fluorescence, as seen in Figure 4.9(a), thick section of fluorescently stained human medulla exhibits a large amount of glare from fluorescent structures above and below the focal plane. When imaged with a laser scanning confocal microscope as shown in Figure 4.9(d), the medulla thick section exposes a significant degree of structural detail. Similarly, widefield fluorescence imaging of whole rabbit muscle fibers stained with fluorescein produce blurred images displayed in Figure 4.9(b) lacks detail, while the same sample field in confocal microscopy (Figure 4.9(e) reveals a highly striated topography. In Figure 4.9(c), autofluorescence in a sunflower pollen grain emits an indistinct outline of the basic external morphology, but produces no indication of the internal structure. Comparatively, a thin optical section of the same grain as displayed in Figure 4.9(f), acquired with confocal techniques shows a dramatic difference between the particle core and the surrounding envelope.

4.8.1 Historical Perspective

In the mid-1950, Marvin Minsky developed the concept of confocal microscopy during his studies in Harvard University. Later, in 1957, Minsky got patent for it. Minsky’s reason behind the invention was to image biological events and neural network of brain tissue in the living system. Minsky’s invention was not noticed because of the lack of intense light sources which was necessary for imaging and horsepower of the computer required for handling large data. In 1960, Mojmir Petran and M. David Egger fabricated a multiple-beam confocal microscope that used a spinning (Nipkow) disk for examination of ganglion cells and unstained brain sections. In 1973, Egger
developed the first mechanically scanned confocal laser microscope and issued the first recognizable images of cells. In Late 1970s and 80s, interest in confocal microscopy increases, because of advances in computer and laser technology, and introduction of new algorithms for digital manipulation of images.

After the expiration of Minsky’s patent, many investigators translated practical laser scanning confocal microscope designs into working instruments. In 1979, Dutch physicist G. Fred Brakenhoff developed a scanning confocal microscope and Colin Sheppard also contributed a technique with a theory of image formation simultaneously. In late 1980s, Brad Amos, John White and Tony Wilson developed the utility of confocal imaging in the examination of fluorescent biological specimens. In 1987, instruments for commercial use were demonstrated. In 1990s, optics and electronics afforded high-throughput fiber optics, better thin film dielectric coatings, stable and powerful lasers, detectors with reduced noise characteristics and high-efficiency scanning mirror units. Along with this, fluorochromes started to synthesized. In 1990s, with the advancement of enhanced displays, computer processing speeds and large-volume storage technology, science was ready for a virtual explosion in many applications that could be directed with laser scanning confocal microscopy.

Today’s confocal microscopes is considered as complete integrated electronic systems. In these systems, the optical microscope plays a crucial role in a configuration which, consists a computer, electronic detectors, and several laser systems that can be combined with beam scanning assembly and wavelength selection devices. In many instances, the entire confocal microscope is collectively called as a digital or video imaging system which is capable of creating electronic images. Presently, these microscopes are used for routine investigations on molecules, cells, and living tissues.

4.8.2 Principles of Confocal Microscopy

Figure 4.10 explains the confocal principle in epi-fluorescence laser scanning microscopy diagrammatically. Coherent light is emitted by the laser system (excitation source) passes through a pinhole aperture that is situated in a conjugate plane (confocal) with a scanning point on the sample and a second pinhole aperture positioned in front of the detector, which is a photomultiplier tube. Dichromatic mirror reflects the laser and scanned across the sample in a defined focal plane, secondary fluorescence emitted from points on the sample, which is in the same focal plane pass back through the dichromatic mirror. Then they are focused as a confocal point at the detector pinhole aperture.
The substantial amount of fluorescence emission, which occurs at points above and below the objective focal plane is not confocal with the pinhole. It is termed as Out-of-Focus Light Rays. It forms extended Airy disks in the aperture plane. As only a small fraction of the Out-of-Focus fluorescence emission is transported through the pinhole aperture, most of this extraneous light is not detected by the photomultiplier and does not result to the resulting image. In a wide field epi-fluorescence microscope, the dichromatic mirror, excitation filter, and barrier filter perform similar functions to identical components. When the objective is refocused in a confocal microscope, it shifts the excitation and emission points on a sample to a new plane that becomes confocal with the pinhole apertures of the light source and detector.

In traditional widefield epi-fluorescence microscopy, the entire sample is subjected to intense illumination from an incoherent mercury or xenon arc-discharge lamp. The resulting image of secondary fluorescence emission is viewed directly in the eyepieces or projected onto the surface of an electronic array detector or traditional film plane. Contrary to the simple concept, the mechanism of image formation in a confocal microscope is fundamentally different. The confocal fluorescence microscope consists of multiple laser excitation sources, a scan head with optical and electronic components, electronic detectors (usually photomultipliers), and a computer for acquisition, processing, analysis, and image display.

The scan head is at the heart of the confocal system. It is responsible for rasterizing the excitation scans, and collection of the photon signals.
from the sample that are required to assemble the final image. A typical scan head contains inputs from the external laser sources, a galvanometer-based raster scanning mirror system, fluorescence filter sets and dichromatic mirrors, variable pinhole apertures for generating the confocal image, and photomultiplier tube detectors that are tuned for different fluorescence wavelengths. For a typical commercial unit, the general arrangement of scan head components is presented in Figure 4.11.

In epi-illumination scanning confocal microscopy, the laser light source and photomultiplier detectors are both separated from the sample by the objective that functions as an objective combination and well-corrected condenser. Internal fluorescence filter components, such as the excitation and barrier filters and the dichromatic mirrors and neutral density filters are contained within the scanning unit as shown in Figure 4.11. Interference and neutral density filters are housed in rotating turrets or sliders, which are inserted into the light path by the operator. The excitation laser beam is coupled to the scan unit with a coupler of fiber optic. It is followed by a beam expander, which enables the thin laser beam wrist to completely fill the objective rear aperture. Expanded laser light is passes through the microscope objective that forms an intense diffraction-limited spot. It is scanned by the coupled galvanometer mirrors in a raster pattern across the sample plane (point scanning).

Pinhole aperture is the essential component of the scanning unit. It acts as a spatial filter at the conjugate image plane positioned directly in front of the photomultiplier. Several apertures of varying diameter are usually contained on a rotating turret, which enables the operator to adjust pinhole size and optical section thickness. Secondary fluorescence that are collected
by the objective is descanned by the same galvanometer mirrors that form the raster pattern. Then it passes through a barrier filter before reaching the pinhole aperture. The aperture serves to exclude fluorescence signals from out-of-focus features positioned above and below the focal plane that are instead projected onto the aperture as Airy disks. These disks have a diameter much larger than those image formations. These oversized disks are distributed over a relatively large area so that only a small fraction of light originating in planes away from the focal point can pass through the aperture. The pinhole aperture also serves for elimination of the stray light that passes through the optical system. Coupling of aperture-limited point scanning to a pinhole spatial filter at the conjugate image plane is an important feature of the confocal microscope.

Traditional widefield epi-fluorescence microscope objectives focus a wide cone of illumination over a large volume of the sample that is uniformly and simultaneously illuminated as demonstrated in Figure 4.12. A majority of the fluorescence emission directed back towards the microscope is gathered by the objective, which is depend upon the numerical aperture and projected into the eyepieces or detector. The result is a significant amount of signal due to emitted background light and auto fluorescence originating from areas above and below the focal plane. This helps in reduction of resolution and image contrast.

![Widefield versus Confocal Point Scanning of Specimens](image.png)

**Fig. 4.12** *Widefield Versus Confocal Point Scanning of Specimens*

In Figure 4.12, it is shown that the laser illumination source in confocal microscopy is first expanded to fill the objective rear aperture, and then focused by the lens system to a small spot at the focal plane. The size of the illumination point depends upon the objective numerical aperture, ranges from 0.25 to 0.8 micrometers approximately in diameter and 0.5 to 1.5 micrometers deep at the brightest intensity. Confocal spot size is determined by the microscope design, objective characteristics, scanning unit settings, wavelength of incident laser light, and the sample. Figure 4.12 is a demonstration of the comparison between the typical illumination cones of a widefield (Refer Figure 4.12) and point scanning confocal (Refer Figure 4.12) microscope at the same numerical aperture. The entire depth of the sample over a wide area is illuminated by the widefield microscope. The sample is scanned with a finely focused spot of illumination, which is centered in the focal plane in the confocal microscope.
In laser scanning confocal microscopy, extended sample image is generated by scanning the focused beam across a defined area in a raster pattern, which is controlled by two high-speed oscillating mirrors driven by galvanometer motors. One mirror moves the beam from left to right along the x lateral axis, and the other translates the beam in the y direction. After every single scan along the x axis, the beam is rapidly transported back to the starting point and shifted along the y axis to start a new scan in a process termed as flyback. During the flyback operation, image information is not collected, and the area of interest on the sample in a single focal plane is excited by laser illumination from the scanning unit.

4.8.3 Laser Scanning Confocal Microscope Configuration

Significantly, fluorescent probes, which are employed to add contrast to biological samples and other associated technologies with optical microscopy techniques have been improved. The explosive growth and confocal approach development is a direct result of a renaissance in optical microscopy that are largely fueled by modern optical and electronics technology advances. Among these are stable multi-wavelength laser systems that deliver better coverage of the ultraviolet, visible, and near-infrared spectral regions, sensitive low-noise wide band detectors, improved interference filters (including dichromatic mirrors, barrier, and excitation filters), and powerful computers. Now, the powerful computers are available with relatively low-cost memory arrays, high-resolution video displays, image analysis software packages, and high quality digital image printers. In Figure 4.13, the flow of information is presented diagrammatically through a modern confocal microscope.

Fig. 4.13 Confocal Microscopy Information Flow Schematic Diagram
Many of these technologies are developed independently for a variety of specifically-targeted applications. They have been gradually incorporated into mainstream commercial confocal microscopy systems. In modern microscope systems, classification of designs is based on the technology utilized to scan samples. Scanning can be performed either by translating the stage in the x, y, and z directions while the laser illumination spot is held in a fixed position, or the beam itself can be raster-scanned across the space. As, three-dimensional translation of the stage is cumbersome and prone to vibration, modern instruments employ some type of beam-scanning mechanism.

In modern confocal microscopes, two fundamentally different techniques for beam scanning are developed, single-beam and multiple-beam scanning.

1. **Single-Beam Scanning**: It is one of the popular methods employed in a majority of the commercial laser scanning microscopes. It uses a pair of computer-controlled galvanometer mirrors to scan the sample in a raster pattern at a rate of one frame per second approximately. Faster scanning rates (to near video speed) can be achieved using oscillating mirrors or acousto-optic devices.

2. **Multiple-Beam Scanning**: In multiple-beam scanning, confocal microscopes are equipped with a spinning Nipkow disk that contains an array of pinholes and micro lenses. These instruments use arc-discharge lamps for illumination instead of lasers to decrease sample damage and improves the detection of low fluorescence levels during real time image collection. It also has the ability to readily capture images with an array detector, such as a Charge-Coupled Device (CCD) camera system.

Laser scanning confocal microscope designs are positioned around a conventional upright or inverted research-level optical microscope. However, instead of the mercury arc-discharge or standard tungsten-halogen lamp, one or more laser systems are used as a light source to excite fluorophores in the sample. Image information is collected point by point with a specialized detector such as an avalanche photo-diode or photo-multiplier tube, and then digitized for processing by the host computer that also controls the scanning mirrors and/or other devices to facilitate the collection and exhibition of images. After a series of images (usually serial optical sections) has been developed and stored on digital media, analysis can be conducted by using various image processing software packages, which are available on the host or a secondary computer.
4.8.4 Advantages and Disadvantages of Confocal Microscopy

Advantages of Confocal Microscopy

Laser scanning confocal microscopy has the ability to produce thin (0.5 to 1.5 micrometer) optical sections consecutively through fluorescent specimens. It has a thickness ranging up to 50 micrometers or more. By using a stepper motor, the image series can be collected by coordinating incremental changes in the microscope fine focus mechanism with sequential image acquisition at every step. Image information is constrained to a well-defined plane, instead of signals complications that arise from remote locations in the sample. Because of the reduction in background fluorescence and improved signal-to-noise, definition and contrast are radically improved over widefield techniques. Additionally, optical sectioning eliminates artifacts which occur during physical sectioning and fluorescent staining of tissue samples for traditional forms of microscopy. The non-invasive confocal optical sectioning technique enables the analysis of both living and fixed specimens under various conditions with enhanced clarity.

With confocal microscopy software packages, optical sections can be collected and demonstrated in transverse planes. They are not restricted to the perpendicular lateral (x-y) plane. Vertical sections that are parallel to the microscope optical axis in the x-z and y-z planes, can be created by confocal software programs. Hence, the sample appears to be sectioned in a plane, which is perpendicular to the lateral axis. Practically, vertical sections are achieved by combination of a series of x-y scans and the z axis with the software and projects a view of fluorescence intensity.

![Fig. 4.14 Pollen Grain Serial Optical Sections by Confocal Microscopy](image-url)
In Figure 4.14, auto fluorescence emission wavelengths are illustrated from a stack of optical sections, which is termed a z-series through a sunflower pollen grain that reveals internal variation.

Optical sections are gathered in 0.5-micrometer steps, which is perpendicular to the z-axis, i.e., microscope optical axis) that uses a green helium/neon (543 nanometers; red fluorescence) laser system and dual argon-ion (488 nanometers; green fluorescence).

In many instances, a composite or projection view obtained from a series of optical sections provides important information about a three-dimensional specimen than a multi-dimensional view. For example, a fluorescently labeled neuron that have numerous thin, extended processes in a tissue section is difficult to image using widefield techniques because of out-of-focus blur. Confocal thin sections of the same neuron reveals portion of several extensions, but frequently appear as fragmented streaks and dots and also lack continuity. Composite views that are created by flattening a series of optical sections from the neuron, reveals all of the extended process in sharp focus with well-defined continuity. Functional and structural analysis of other cell and tissue sections also benefits from composite views. It can be opposed to, or combined with, three-dimensional volume rendering techniques.

Because of the advancement in confocal microscopy, multi-dimensional views of living cells and tissues are possible. It includes image information in the x, y, and z dimensions as a function of time. It can also be presented in multiple colors by the use of two or more fluorophores. After volume processing of each image stacks, the resulting data can be demonstrated as three-dimensional multicolor video sequences in real time. Unlike, conventional widefield microscopy, all fluorochromes in multiply labeled sample appear in register by the use of confocal microscope. Temporal data are collected either from time-lapse experiments conducted over extended time-periods or through real time image acquisition in smaller frames for short time period. The potential for use of multi-dimensional confocal microscopy as a powerful tool in cellular biology is continuously growing, as new laser systems are developed to limit cell damage. It also speeds the computer processing and improves storage capacity.

Digitization of the sequential analog image data are collected by the confocal microscope photo-multiplier or any other similar detector helps in facilitating computer image processing algorithms. It is done by transforming the continuous voltage stream into discrete digital increments, which correspond to variations in light intensity. Along with the benefits and speed, which grows from processing digital data, images can be prepared for print output or publication. In cautiously controlled experiments, quantitative measurements of spatial fluorescence intensity are obtained from the digital data either by statically or as a function of time.
Disadvantages of Confocal Microscopy

The limitation of number of excitation wavelengths that are available with common lasers also known as laser lines, that occur over very narrow bands is the main disadvantage of confocal microscopy. They are also quite expensive to produce in the ultraviolet region. Compare to it, conventional widefield microscopes use xenon or mercury based arc-discharge lamps that provides a full range of excitation wavelengths in the visible, ultraviolet and near-infrared spectral regions.

Another disadvantage is the destructive nature of high-intensity laser irradiation to living cells and tissues. This issue is recently addressed by multiphoton and Nipkow disk confocal imaging.

The high cost of purchasing and operating multi-user confocal microscope systems that can range up to an order of magnitude higher than comparable widefield microscopes can limits their implementation in smaller laboratories. This problem can be solved by cost-shared microscope systems that have service in one or more departments in a central facility. Recently, the introduction of personal confocal systems has competitively lowered the price of low-end confocal microscopes and increased the number of individual users.

Check Your Progress

11. Who developed the concept of confocal microscopy?
12. Who developed first mechanically scanned confocal laser microscope?
13. What are the applications of confocal microscopy?
14. What are the two fundamentally different techniques for beam scanning are developed?
15. What is scan head in confocal system?

4.9 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Compound microscopes are either monocular or binocular, depending on whether they have a single eyepiece or two eyepieces.

2. One difference between monocular and binocular microscopes is that in the monocular microscope, the light passes straight from the objective to the eyepiece, while in the binocular microscope it has to be directed into two separate eyepieces.

3. Magnification by a microscope occurs in two stages; first is the magnification by the objective lens and second is the magnification by the eyepiece lenses.
4. The capability of an objective lens to render the outline of the image of an object as clear and distinct is called definition of a compound microscope.

5. The resolving power of a microscope can be defined as its ability to reveal closely adjacent structural details as separate and distinct from each other.

6. Apochromatic’ objectives are made of glass containing the mineral, fluorite.

7. In dark field microscopy, the non-diffracted rays are removed altogether so that the image is composed solely of diffracted wave components. This technique is very sensitive because images based on small amounts of diffracted light from minute phase objects are seen clearly against a black or very dark background.

8. Phase contrast microscopes feature an optical design that transforms differences in the phase of object-diffracted waves to differences in the image, making objects appear as if they have been optically stained.

9. Molecules absorbing the energy of electromagnetic radiation (i.e., photons) will be elevated to a higher energy level, or excited state. These excited molecules will return to the ground state and some molecules will emit radiation on their return to the ground state. This phenomenon is known as fluorescence and fluorescent molecules are known as fluorochromes.

10. Electron microscopes are specifically used to investigate the ultrastructure of a wide range of biological and inorganic specimens including microorganisms, cells, large molecules, biopsy samples, metals, and crystals. Transmission Electron Microscope (TEM) and Scanning Electron Microscope (SEM) are two types of electron microscopes. TEM uses electrons passed through the sample to build a picture of the sample internal structure, whereas SEM uses electrons scattered from the sample surface to build up a picture of the sample surface.

11. Marvin Minsky developed the concept of confocal microscopy.

12. Egger developed the first mechanically scanned confocal laser microscope.

13. Confocal microscopes are used for routine investigations on molecules, cells, and living tissues.

14. The two fundamentally different techniques for beam scanning are single-beam and multiple-beam scanning.

15. The scan head is at the heart of the confocal system. It is responsible for rasterizing the excitation scans, and collection of the photon signals from the sample that are required to assemble the final image.
4.10 SUMMARY

- Microscope is an instrument which is used to view the objects which are too small to be seen by the naked eye.
- A microscope consists of an assembly of lenses which sequentially magnify the image.
- The lenses near the object to be viewed are called objective while those near the eyes are called eyepieces.
- A source of light, concentrating mechanism, a stage for keeping the object and mechanisms to adjust these form the parts of a microscope.
- The magnifying power of a microscope depends on the power of its lenses, however its power to show the details is limited by the wavelength of the light.
- While oil immersion of the objective lens is used to increase its numerical aperture, the optical aberrations are eliminated by using special assembly of lenses.
- Definition depends on the elimination or correction of optical aberrations found in the glass lenses.
- Optical aberrations in microscope are of two types, namely, spherical aberration and chromatic aberration.
- The presence of oil between the object and the lens increases the angle of the cone of rays from the object that enter the objective, thus increasing the numerical aperture of the objective.
- The microscope should be used in the prescribed method and taken good care of to maintain its accuracy and precision.
- Microscopy is used extensively in microelectronics, nanophysics, biotechnology, pharmaceutical research, mineralogy, histopathology and Microbiology.
- Dark field, phase contrast, confocal and fluorescent microscopes are types of optical microscopes whereas transmission electron microscope and scanning electron microscopes are included in electron microscopy. In dark field microscopy the specimen is illuminated from the side and only scattered light enters the objective lens which results in bright objects against a dark background.
- Phase contrast microscopes feature an optical design that transforms differences in the phase of object-diffracted waves to differences in the image, making objects appear as if they have been optically stained.
- Phase contrast images are easiest to interpret when the cells are thin and spread out on the substrate.
Molecules absorbing the energy of electromagnetic radiation (i.e., photons) will be elevated to a higher energy level, or excited state. These excited molecules will return to the ground state and some molecules will emit radiation on their return to the ground state. This phenomenon is known as fluorescence and fluorescent molecules are known as fluorochromes.

The fluorometer is similar to a spectrophotometer, except that the photomultiplier tube to detect the emitted light is located at a right angle from the path of the excitation light.

Electron microscopes are specifically used to investigate the ultrastructure of a wide range of biological and inorganic specimens including microorganisms, cells, large molecules, biopsy samples, metals, and crystals.

Modern electron microscopes produce electron micrographs using specialized digital cameras and frame grabbers to capture the images.

Transmission Electron Microscope (TEM) and Scanning Electron Microscope (SEM) are two types of electron microscopes.

TEM uses electrons passed through the sample to build a picture of the sample internal structure, whereas SEM uses electrons scattered from the sample surface to build up a picture of the sample surface.

The original form of the electron microscope, the Transmission Electron Microscope (TEM), uses a high voltage electron beam to illuminate the specimen and create an image. The electron beam is produced by an electron gun, commonly fitted with a tungsten filament cathode as the electron source.

The Scanning Electron Microscope (SEM) produces images by probing the specimen with a focused electron beam that is scanned across a rectangular area of the specimen (raster scanning).

The fundamental of confocal approach is the usage of spatial filtering techniques for elimination of the out-of-focus light or glare in samples whose thickness exceeds the immediate plane of focus.

Today’s confocal microscopes is considered as complete integrated electronic systems. In many instances, the entire confocal microscope is collectively called as a digital or video imaging system which is capable of creating electronic images. Presently, these microscopes are used for routine investigations on molecules, cells, and living tissues.

4.11 KEY WORDS

- **Magnification**: It is the degree of enlargement or the magnifying power of a microscope.
• **Angle of aperture:** It is the angle formed by the two most divergent rays, starting from the centre of the object, which are focused by the lens on the eye of the observer.

• **Chromatic aberration:** It is a kind of aberration that results from the separation of white light into its component colours while passing through a lens of a microscope.

• **Microscopy:** Technical field that use microscopes to view samples and objects that cannot be seen with the naked eye.

• **Depth:** Depth of field refers to the thickness of the specimen that will be in acceptable focus.

• **Resolution:** The ability to discern fine details. Typically expressed as a linear dimension describing the smallest distance needed between two objects so that both are seen.

• **Fluorescence:** Emission of light by a substance that has absorbed light or other electromagnetic radiation.

### 4.12 SELF ASSESSMENT QUESTIONS AND EXERCISES

**Short Answer Questions**

1. Draw a diagram of a compound microscope and label its various parts.
2. What is magnification? How is the magnifying power of a microscope calculated?
3. What is numerical aperture? How does it affect the resolving power of a microscope?
4. How are the optical aberrations corrected in different types of objectives?
5. Discuss in brief the theory and image interpretation of dark field microscopy.
6. Explain the auto fluorescence emission wavelengths with diagram.
7. Define the various types of electron microscopes.
8. Describe the difference between wide-field and confocal point scanning of samples.
9. What are the components of confocal microscopes?

**Long Answer Questions**

1. Describe the different types of objectives and eyepieces used in a microscope.
2. Describe optical system of a compound microscope.
3. Describe the procedures adopted for the care of microscope.

4. Describe the method of using a light microscope and the precautions and measures that must be kept in mind while using.

5. What are the common difficulties in microscopy? How can they be overcome?


7. Explain the principles of confocal microscopy with illustration also explain its advantages and disadvantages.

### 4.13 Further Readings


UNIT 5  STAINS AND STAINING TECHNIQUES

Structure
5.0 Introduction
5.1 Objectives
5.2 Introduction to Staining Techniques
5.3 Staining and Staining methods
  5.3.1 Staining Methods
  5.3.2 Differential Staining
5.4 Imaging Techniques
5.5 Answers to Check Your Progress Questions
5.6 Summary
5.7 Key Words
5.8 Self Assessment Questions and Exercises
5.9 Further Readings

5.0 INTRODUCTION
Staining techniques are used to observe components that are otherwise not easily visible under an ordinary light microscope. This could be due to the lack of colour contrast between the object being examined and the background or because of the limited resolving power of the light microscope. Bacteria are responsible for many diseases in humans. Therefore, it is essential to study them in order to restrict their growth. Staining of bacteria is of primary importance in the process of studying bacteria. It is needed because the bacteria are nearly transparent under the light microscope. Differential stains impart different colours to bacteria helping to classify them.

To identify the bacterium which is causing a particular disease, it has to be isolated from the specimen. Culture methods are employed to achieve this. The aim is to grow a colony of the bacteria on an artificial medium, so that it can be subjected to further tests for identifying and ascertaining its antibiotic sensitivity. Besides isolation, culture methods are also used to grow the bacteria for production of antigen maintenance of stock cultures, etc.

In this unit, you will study the various techniques of the staining and imaging methods.

5.1 OBJECTIVES
After studying this unit, you should be able to:

• Classify the types of stains
5.2 INTRODUCTION TO STAINING TECHNIQUES

Staining is an auxiliary technique used in microscopy to enhance contrast in the microscopic image. Stains and dyes are frequently used in biology and medicine to highlight structures in biological tissues for viewing, often with the aid of different microscopes. Stains may be used to define and examine bulk tissues (highlighting, for example, muscle fibers or connective tissue), cell populations (classifying different blood cells), or organelles within individual cells.

Biological staining is also used to mark cells in flow cytometry, and to flag proteins or nucleic acids in gel electrophoresis.

Simple staining is staining with only one stain/dye. There are various kinds of multiple staining, many of which are examples of counterstaining, differential staining, or both, including double staining and triple staining.

The bacterium are organisms that can be microscopic, i.e., not visible by the naked eye, so something requires to be done so that they become visible under the microscope. This is where the process of staining comes into picture.

Staining techniques are used to observe components that are otherwise not easily visible under an ordinary light microscope. This could be due to the lack of colour contrast between the object being examined and the background or because of the limited resolving power of the light microscope.

The following are a list of staining techniques commonly used:

(i) **Simple Staining**: Simple staining methods are used to demonstrate the presence and the morphology of bacteria and cells. They employ simple stains.

   - **Methylene Blue Stains**: Neutral methylene blue, Loeffler’s alkaline methylene blue and polychrome methylene blue solutions are useful for the identification of anthrax bacilli by Mcfadyean’s reaction.

(ii) **Differential Staining**: It generally refers to a number of specific processes. It is used to describe staining processes which use more than one chemical stain. Using multiple stains can better differentiate between different microorganisms or structures/cellular components of a single organism.
It also describes medical processes used to detect abnormalities in the proportion of different white blood cells in the blood. The process or results are called a WBC differential. This test is useful because many diseases alter the proportion of certain WBCs. By analysing these differences in combination with a clinical exam and other lab tests, medical professionals can diagnose a disease.

One commonly recognizable use of differential staining is the Gram stain. Gram staining uses two dyes: Crystal Violet and Carbol Fuchsin (which is the counterstain) to differentiate between Gram-Positive (large peptidoglycan layer on outer surface of cell) and Gram-Negative Bacteria.

(iii) **Negative Staining:** It is an established method, often used in diagnostic microscopy, for contrasting a thin specimen with an optically opaque fluid. In this technique, the background is stained, leaving the actual specimen untouched, and thus visible. This contrasts with ‘positive staining’, in which the actual specimen is stained.

For bright field microscopy, negative staining is typically performed using a black ink fluid such as nigrosin. The specimen, such as a wet bacterial culture spread on a glass slide, is mixed with the negative stain and allowed to dry. When viewed under the microscope the bacterial cells, and perhaps their spores, appear light against the dark surrounding background.

(iv) **Special Staining Techniques:** Some of the specialized techniques used for staining have been discussed as follows:

(a) **The Spore Staining:** Technique helps observe the spores of spore-forming bacteria. The structures are protected by a spore coat which helps them to show resistance against various chemical and physical agents. This technique uses rather vigorous heat treatment to force a primary stain into the spores. This is the reason that the presence of spores can be observed and differentiated from vegetative cells very easily.

(b) **Flagella Stain:** The purpose of the protocol is to stain bacterial flagella, thus to reveal the presence or absence of flagella as well as their arrangement on the perimeter of the cell. These traits can be used to characterize bacteria phenotypically, as not all bacteria are flagellated and those that are will possess these structures in various locations extending from the cell membrane. Solid or liquid media cultures can be used for flagella staining. Cultures should be incubated between 16 and 20 hours before staining, as older...
cultures tend to lose flagella. For example, newer cultures are particularly important for *Bacillus* species. that undergo spore formation and lose flagella during this developmental process.

(c) **Capsule stain:** Capsules are the structures positioned outside the cell wall of an organism and that is why these are in direct contact with the environment. Many Bacteria produce capsules under the right conditions. Bacterial capsules are most often composed of long polymers of sugar or sugar derivatives, which are known as polysaccharides. Some of the capsules are composed of polyalcohols or amino acid polymers.

(d) **Spirochaetes:** Spirochaetes fall into the category of a phylum of distinctive double-membrane bacteria, most of which have long spiral-shaped cells. These spirochaetes are differentiated from other bacterial phyla by their position of their flagella. These flagella are sometimes called axial filaments. They run lengthwise between the bacterial inner membrane and outer membrane in periplasmic space. These cause a twisting motion which allows the spirochaete to move about. A spirochaete will undergo asexual transverse binary fission during reproduction.

<table>
<thead>
<tr>
<th>Check Your Progress</th>
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<tbody>
<tr>
<td>1. Which dyes are used in gram staining?</td>
</tr>
<tr>
<td>2. What are methylene blue stains used for?</td>
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### 5.3 STAINING AND STAINING METHODS

Both films as well as tissue sections may need to be stained. The procedure used for staining them, have been discussed ahead.

**Films**

The process to stain films is as follows:

- The stain is poured over the film, covering its entire area.
- The amount of stain should be sufficient to ensure that it doesn’t evaporate during staining.
- The area bearing the identification mark should not be covered by the stain.
• When the staining is done over the slide, it is washed with water and allowed to dry in a vertical position.
• It is then observed under an oil-immersion objective.

Tissue Sections

Stains can be prepared for tissue sections by the following procedure:
• The sections are embedded in paraffin, which is impervious to water. It has to be removed to allow the watery stains to penetrate it.
• Paraffin is removed by keeping the slide bearing the section, in a jar of xylol for some minutes.
• The section is then treated with a few drops of absolute (100 per cent) ethyl alcohol. The section should turn opaque.
• Then a few drops of 50 per cent alcohol are poured on the slide.
• It is then washed in water.
• The section is then stained with the stain required.
• After staining, the section is washed with water and the excess water is removed by pressing the slide between two sheets of blotting paper.
• A few drops of 95 per cent alcohol are poured over the section, immediately.
• This is followed by pouring a few drops of absolute alcohol.
• The slide is then immersed in a jar of xylol.
• When the section becomes clear in appearance it is removed from the xylol.
• The excess of xylol is wiped away and the section is mounted with a number 1 (1 mm thick) cover slip using a drop of Canada balsam.
• It is then observed under an oil-immersion objective.
• The section should not be allowed to dry through this entire procedure.

5.3.1 Staining Methods

Let us look at the various types and characteristics of stains, in detail.

Simple Stains

These stains are used to demonstrate the presence of organisms in the clinical sample. The various simple stains have been given as follows:

1. Loeffler’s Methylene Blue: It is the most commonly used simple stain and is prepared by the use of the following items:
   • Saturated solution of methylene blue is added to absolute alcohol – 300 ml.
Stains and Staining Techniques

NOTES

- KOH 0.01 per cent in water – 1000 ml is added to the above and used.

The procedure is to pour the stain over the film and keep it for three minutes. Then, wash it with tap water.

2. **Polychrome Methylene Blue:** It is slowly ‘ripened’ Loeffler’s methylene blue. The ‘ripening’ is achieved by keeping the Loeffler’s methylene blue in bottles, which are half-filled. These bottles are shaken periodically, so as to aerate the stain. The oxidation of methylene blue leads to the formation of a violet compound which gives the stain its polychrome property. It may take up to a year for the stain to ripen. The ripening process can be quickened by adding 1 per cent potassium carbonate to it. The procedure for its use is similar to Loeffler’s Methylene blue.

3. **Dilute Carbol Fuschin:** It is rarely used because over-staining can occur easily with it.

It is prepared by diluting the strong carbol fuschin (used in Ziehl-Neelsen’s stain), ten to fifteen times, using distilled water. Then, pour the stain over the film and keep it for ten to twenty-five seconds. Finally, wash it with tap water.

**Negative Staining**

It is called negative staining because the background here is stained, while the bacteria are not. India ink preparation is mixed on a glass slide with the culture suspension or the material containing bacteria. A thin film of this mixture is prepared using a loop, which is allowed to dry before being examined. The capsulated bacteria or spirochaetes are seen as clear transparent objects on a dark-brown background.

**5.3.2 Differential Staining**

In microbiology, differential staining techniques are used more frequently than simple stains in order to obtain significant information about Bacteria. Differential staining methods, typically require more than one stain and includes several steps because they document the differentiation of cell types or cell structures. The most important stain is the Gram stain. Other differential staining methods include the endospore stain (to identify endospore-forming Bacteria), the acid fast stain (to discriminate Mycobacterium species from other Bacteria), a metachromatic stain to identify phosphate storage granules, and the capsule stain (to identify encapsulated Bacteria).

These methods are used to differentiate between different types of Bacteria based on the colours they take during these staining procedures.

1. **Gram’s Stain:** This is the most commonly performed staining procedure in any microbiology laboratory. It differentiates the Bacteria...
into two groups, the Gram-Positive and the Gram-Negative Bacteria. This differentiation is clinically very important because the two groups are sensitive to different types of antibiotics.

According to the principle of Gram’s stain, when a basic para-rosaline dye, such as crystal violet (or methyl violet) is used to stain certain type of Bacteria, it can be fixed inside the Bacteria by adding iodine after the para-rosaline dye. In these Bacteria, the subsequent treatment with a decolourizing agent, such as acetone (or alcohol) does not remove the dye.

In the other type of Bacteria, no such ‘fixing’ is observed and the addition of a decolourizing agent removes both the para-rosaline dye and the iodine, rendering such Bacteria colourless. These Bacteria are then counter-stained by a contrasting dye (usually red) to demonstrate them easily.

The group of Bacteria which retain the para-rosaline dye even after application of decolorizing agent (that is, appear violet) is called ‘Gram-Positive Bacteria’, while the group that loses the stain (appears red) is called ‘Gram-Negative Bacteria.’

The methodology of Gram’s stain has been modified by several scientists. Two methods of Gram’s stain are generally used, which are:

(a) **Kopeloff and Beerman’s Method:** This method uses acetone as a decolourizer.

The items need to prepare this reagent are as follows:

- Dissolve 10 gm of methyl violet 6B in 1 ltr of distilled water.
- Dissolve 20 gm of iodine in 100 ml of 4 per cent NaOH. Once it is dissolved completely, add 900 ml of distilled water to it, to make upto 1 ltr.
- Use 100 per cent acetone.
- Dissolve 0.5 gm of basic fuchsin in 1 ltr of distilled water.

The procedure to prepare it is as follows:

- Make a smear of the material to be stained on a clean glass slide.
- Fix the smear by holding it, film upwards, above the Bunsen flame for a few seconds. The fixation is complete when the slide is just too hot to touch on the back of the hand.
- Cover the whole slide with methyl violet and keep it for five minutes.
- Tip off the methyl violet.
- Wash the slide with iodine while holding it at a slope. Remove all the crystalline deposit.
• Cover the whole slide with iodine and keep for two minutes.
• Decolourise with acetone. Tip off all the iodine, then hold the slide at a slope. Keep the tap water running. Pour acetone from top to bottom for three seconds. Immediately wash in running tap water. Over-decolourization may render Gram-Positive bacteria to appear as Gram-Negative, hence the timing of contact with acetone is very important.
• Cover the slide with basic fuchsin and keep it for thirty seconds.
• Wash thoroughly in tap water and let the smear air dry.
• Examine it under an oil immersion objective.

(b) Jensen’s Modification of Gram’s Stain: This method uses alcohol as a decolourizer which is slower in action. It is also less time-consuming. It can be recommended for routine bacteriological work.

The items need to prepare this reagent are as follows:
• Dissolve 5 gm of methyl violet 6B in 1 ltr of distilled water.
• Dissolve 20 gm of potassium iodide in 250 ml of distilled water.
• Then add 10 gm of iodine. When dissolved make upto 1 ltr with distilled water.
• Use 100 per cent ethyl alcohol.
• Add 1 gm of neutral red and 2ml of 1 per cent acetic acid to 1 ltr of distilled water.

The procedure to prepare it is as follows:
• Make the films, dry and fix as stated before.
• Cover the whole slide with methyl violet and keep for thirty seconds.
• Tip off the stain. Wash the slide with iodine while holding it at a slope.
• Cover the slide with fresh iodine and keep for thirty seconds.
• Wash off the iodine with absolute alcohol.
• Cover the slide with alcohol and keep tilting it from one side to another for ten to fifteen seconds (the optimum timing for decolourizing may vary from one sample to another).
• Wash with water.
• Cover the whole slide with neutral red and keep it for one minute.
• Wash thoroughly with water and let the smear air dry.
• Examine under an oil-immersion objective.
• The Gram-Positive Bacteria would appear violet while the Gram-Negative would appear red in colour. To ensure that staining has been proper, a film of pure culture of *Staphylococcus aureus* may be made on one side of the smear to be stained. It should stain violet in colour for the procedure to be called satisfactory.

2. **Acid Fast Bacilli Staining:** Acid-fast bacilli are those which once treated with aniline dyes, resist decolourization even in the presence of a strong acid. The genus mycobacterium which contains the causative organisms of tuberculosis and leprosy is the prime example of Acid Fast Bacteria.

Mycolic acid which is found in the cell wall of mycobacteria is supposed to impart the acid fast property to them. The staining methods used to demonstrate acid fast bacilli are:

(a) **Ziel-Neelsen Staining:** It was originally described by Ehrlich and was modified by Ziehl and Neelsen.

To prepare this reagent, dissolve 5 gm of basic fuchsin in 25 gm of crystalline phenol by keeping them in a 1 ltr flask over a boiling water bath, for five minutes, shaking the flask from time to time. When there is complete solution, add 50 ml of absolute alcohol and mix it thoroughly. Then add 500 ml of distilled water to it. Filter it before use. Take 800 ml of distilled water in a large flask. Add 200 ml of concentrated sulphuric acid to it by slowly pouring it down the side of the flask into the water. About 50 ml of acid should be poured (slowly) at a time. The mixture will become hot. It should be mixed gently and the remainder of the acid poured similarly.

However, care must be taken, never to pour water into acid as the heat of the reaction would cause the water to boil and it may lead to serious burns.

(b) **Loeffler’s Methylene Blue:** The process for preparing Loeffler’s methylene blue has been described earlier.

Acid fast bacilli would appear bright red, while the surrounding tissue cells as well as other bacteria would appear blue. If the tissue cells appear red, then the smear was not decolourized properly. A fresh smear should be prepared and stained in such a case. The slides should be discarded after use and not recycled.

Instead of Loeffler’s methylene blue, malachite green too can be used (the background would appear green instead of blue). Some
people use 95 per cent alcohol as a secondary decolourizer. It is added as an extra step after decolourizing with sulphuric acid. Instead of 20 per cent sulphuric acid one can use ‘Acid-Alcohol’ as a decolourizer. It consists of 3 ml of concentrated hydrochloric acid in 97 ml of 95 per cent alcohol.

**Modifications of Ziehl-Neelsen’s Method**

The given method is used for demonstration of *Mycobacterium tuberculosis*.

*Mycobacterium leprae* is also acid fast but to a lesser degree than tubercle bacillus. Thus, for demonstrating *lepra bacilli* 5 per cent sulphuric acid is used.

Similarly ‘clubs’ caused by actinomycetes and nocardia are acid fast when treated with 1 per cent sulphuric acid. Cultures of some nocardia species are acid fast when decolourized with 0.5 per cent sulphuric acid. Most bacterial spores are also acid fast.

3. **Fluorochrome Staining:** Here the fluorescent dye Auramine O is used instead of Carbol fuchsin. While no heating is involved this procedure requires a fluorescent microscope. It is a costlier but much easier and rapid method.

4. **Albert’s Stain:** Volutin (polyphosphate) granules are found in the cytoplasm of diphtheria bacillus. They appear to be refractile round bodies in bacterial cytoplasm in wet preparations. With toludine blue or methylene blue they stain metachromatically, appearing reddish-purple with the rest of the cytoplasm of the bacteria taking the colour of the counter-stain (green).

The items need to prepare this reagent are as follows:

- Dissolve 1.5 gm of toludine blue and 2 gm of malachite green in 20 ml of 95 per cent ethyl alcohol. Add this mixture to 1 ltr of distilled water containing 10 ml of glacial acetic acid.
- Dissolve 6 gm of iodine and 9 gm of potassium iodide in 900 ml of distilled water.

The procedure to prepare it is as follows:

- Prepare, dry and fix the smear.
- Cover the slide with Albert’s staining solution and keep it for five minutes.
- Wash in tap water.
- Cover the slide in Albert’s iodine and keep for one minute
- Wash in tap water, allow to air dry.
• Examine it under an oil-immersion objective. The granules stain bluish-black, the rest of the protoplasm appears green, while other cells and organisms appear light green.

5. **Cytological Staining:** Capsules of Bacteria present in animal tissues, blood, pus, fluids, etc., are clearly demonstrated by the commonly-used stains. However, when artificial cultures of Bacteria (grown in the laboratory on media) are examined, special methods must be employed for their demonstration. Negative staining using India ink is a preferred method. Wet films are generally preferred to the dry India ink method described earlier. Care should be taken to press the cover slip on the ink drop while it is covered in a blotting paper, so that the film becomes very thin and pale in colour.

The bacterial cell appears as a refractile body with a clear zone around it, abutted by the ink. In non-capsulated Bacteria, no such zone is seen. Positive capsular stains are not reliable and not recommended.

**Staining of Flagella:** Due to their being extremely thin, they are best seen with an electron microscope. To demonstrate them under light microscope, their thickness has to be increased at least ten-fold by superficial deposition of stain. The stain used is basic fuchsin with tannic acid. It is deposited on the flagella and the bacteria from an evaporating alcoholic solution.

**Staining of Intracellular Lipid:** Sudan black is used to stain the intracellular lipids. The lipid inclusions are stained blue-black, while the bacterial cytoplasm takes the colour of the counter-stain (light pink, if dilute carbol fuchsin is used).

**Staining for Bacterial Nuclei:** Bacterial nucleus can be differentiated from the cytoplasm by using Robinow’s method. The cells are first treated with 1mol/litre of HCl at 60°C and then stained with Giemsa’s stain. The nuclei appear dark purple while the cytoplasm is very lightly stained.

**Staining for Bacterial Spores:** Bacterial spores are not stained by ordinary stains like Gram’s stain. In spore-bearing organisms, they appear as a clear area in the cytoplasm of the bacterium. As described earlier, the modified Ziehl-Neelsen method can be used to stain them.

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**Check Your Progress**

3. What is polychrome methylene blue?
4. What does negative staining have an affect on?
5. What is the use of acetone in Gram’s stain?
6. What is responsible for the acid fastness of the Mycobacterium species?
5.4 IMAGING TECHNIQUES

Computer image analysis can be used as a means of objective and quantitative characterizing optical images of the macroscopic, such as microbial colonies, and the microscopic, such as single cell, objects in the microbiological analysis. The fragment of the electromagnetic spectrum that is sensed by our eyes is termed as ‘optical’ and hence such visible objects are termed as the ‘optical images’. The first instrument which helped in discovering the world of microorganisms was the ‘Human visual analyzer’. Antonie van Leeuwenhoek observed single microbial cells by improving image visualization through simple but ingenious device with a lens, called the ‘the Leeuwenhoek microscope’. With the application of photography that studied the visual observations through microbiological optical images, the mysteries of the microbial world were significantly simplified.

The technique Computer Digital Image Analysis (CDIA). CDIP deals with the photograph images that are obtained in or converted into the digital form. A digitized image is a set of small elements in 2D space termed as pixels and in 3D space they are termed as voxels. Each pixel (voxel) contains the digitally coded information on its X – Y – (Z) location in the Cartesian coordinate system. The optical information depends on the type of imaging system used.

For monochrome digital cameras, the information obtained consists of the data in gray scale units, generally 8 bit 256 gray levels. The digital images from conventional three-channel colour cameras and other filter-based imaging systems (colour imaging) can be a set of pixels with the optical information encoded in one of the four colour spaces RGB, HSV, CIE-Lab, and YCrCb. Of these RGB is the most popular as 24 bit combinations of Red, Green and Blue values with 8-bits for each colour. Multispectral imaging systems is capable of capturing the images with information on tens spectral regions or bands in each pixel. The hyperspectral imaging systems provide image information on space distribution of many contiguous spectral band that are close to the continuous spectrum. Both the multispectral and hyperspectral imaging can visualize the visible light as well as near-infrared to infrared. The obtained digital data of the images are considered as the raw data and are preserved and analysed using the computer technology which includes variables of a mathematical model constructed of numerous significant algorithms.

Microscopy imaging techniques are also employed to view the microscopic world. Advances in microscopy enable visualization of a broad range of biological processes and features in the cell structure. Following are some of the advanced techniques used in the study of the microbial structures.
Bright Field Microscopy: It is the most elementary form of imaging a specimen and is generally used with compound microscopes. The technique takes the specimen which is dark and contrasts it by the surrounding bright viewing field. Compound light microscopes are often simply referred to as bright field microscopes.

Oil Immersion Microscopy: Oil immersion microscopy is an essential tool in examining specimens under a compound microscope. Similar refractive indexes allow for large bright images, especially useful to the study of inanimate objects, striated tissue and Bacteria. A mixture of synthetic oils can create the most suitable viscosity to achieve high resolute quality images.

Köhler Illumination: It is a microscopy imaging technique first developed in 1893 through the optimization of a microscope’s optical sequence so as to allow for homogeneously bright light without artefacts and glare.

Dark Field Microscope: It is the optimum microscopy technique for making objects appearing bright against a dark background otherwise their refractive values are similar to the background and they will not be properly imaged. Dark field is achieved by modifying the microscope.

Differential Interference Contrast Microscopy: It is a microscopy imaging technique which benefits from differences in the light refraction by different sections of living cells and transparent specimens and allows for better visibility during microscopic evaluation.

Phase Contrast Microscope: It is mostly used to view the ‘phase objects’ which are transparent, colourless and/or unstained specimens. This requires a microscopy imaging technique and is mostly used in molecular and cellular biology, microbiology and medical research.

Fluorescence Microscope: It employs high-powered light waves to provide unique image viewing options that are unavailable with conventional light microscopes. This imaging technique also uses stains so as to better view more components and details of the inner structures of cells.

Polarizing Microscope: Polarization method is used to enhance contrast and colour to images which provide information about absorption, structure and composition of specimens. The study of rocks and minerals in geology or petrography fields, medicine, biology and metallurgy specifically use this imaging technique.

Confocal Microscope: The confocal microscope technique is used which separate light waves with lasers technology so that the images can be viewed without blurred edges and in higher resolutions. Many images can be taken quickly with a small section of the sample viewed at a time.
Check Your Progress

7. Define the terms ‘optical’ and ‘optical images’.
8. How the technique Computer Digital Image Analysis (CDIA) work?
9. Why the microscopy imaging techniques are used?
10. What is bright field microscopy?

5.5 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Crystal blue and Fuchsin dyes are used in Gram staining.
2. Methylene blue stains are useful for the identification of anthrax bacilli by Mcfadyean’s reaction.
3. Polychrome methylene blue is ripened Loffeler’s methylene blue.
4. Negative staining affects only the background and not the Bacteria.
5. Acetone is used as a decolourizing agent in Gram’s stain.
6. Mycolic acid is responsible for acid fastness of the Mycobacterium species.
7. The fragment of the electromagnetic spectrum that is sensed by our eyes is termed as ‘optical’ and hence such visible objects are termed as the ‘optical images’.
8. The technique Computer Digital Image Analysis (CDIA). CDIP deals with the photograph images that are obtained in or converted into the digital form. A digitized image is a set of small elements in 2D space termed as pixels and in 3D space they are termed as voxels. Each pixel (voxel) contains the digitally coded information on its X – Y – (Z) location in the Cartesian coordinate system. The optical information depends on the type of imaging system used.
9. Microscopy imaging techniques are used to view the microscopic world. Advances in microscopy enable visualization of a broad range of biological processes and features in the cell structure.
10. Bright field microscopy is the most elementary form of imaging a specimen and is generally used with compound microscopes. The technique takes the specimen which is dark and contrasts it by the surrounding bright viewing field. Compound light microscopes are often simply referred to as bright field microscopes.
5.6 SUMMARY

- Staining is an auxiliary technique used in microscopy to enhance contrast in the microscopic image.
- Stains and dyes are frequently used in biology and medicine to highlight structures in biological tissues for viewing, often with the aid of different microscopes.
- Stains may be used to define and examine bulk tissues (highlighting, for example, muscle fibers or connective tissue), cell populations (classifying different blood cells), or organelles within individual cells.
- Biological staining is also used to mark cells in flow cytometry, and to flag proteins or nucleic acids in gel electrophoresis.
- Staining techniques are used to observe components that are otherwise not easily visible under an ordinary light microscope. This could be due to the lack of colour contrast between the object being examined and the background or because of the limited resolving power of the light microscope.
- Simple staining methods are used to demonstrate the presence and the morphology of bacteria and cells. They employ simple stains.
- The neutral methylene blue stain, Loeffler’s alkaline methylene blue and polychrome methylene blue solutions are useful for the identification of anthrax bacilli by Mcfadyean’s reaction.
- Differential staining generally refers to a number of specific processes. It is used to describe staining processes which use more than one chemical stain. Using multiple stains can better differentiate between different microorganisms or structures/cellular components of a single organism.
- Capsules are the structures positioned outside the cell wall of an organism and that is why these are in direct contact with the environment. Many bacteria produce capsules under the right conditions.
- In microbiology, differential staining techniques are used more frequently than simple stains in order to obtain significant information about Bacteria.
- Differential staining methods, typically require more than one stain and includes several steps because they document the differentiation of cell types or cell structures. The most important stain is the Gram stain.
- Gram’s stain differentiates the bacteria into two groups, the Gram-Positive and the Gram-Negative Bacteria. This differentiation is
clinically very important because the two groups are sensitive to different types of antibiotics.

- Acid fast bacilli are those which once treated with aniline dyes, resist decolourization even in the presence of a strong acid. The genus Mycobacterium which contains the causative organisms of tuberculosis and leprosy is the prime example of acid fast bacteria.

- Ziel-Neelsen staining was originally described by Ehrlich and was modified by Ziehl and Neelsen.

- Computer image analysis can be used as a means of objective and quantitative characterizing optical images of the macroscopic, such as microbial colonies, and the microscopic, such as single cell, objects in the microbiological analysis.

- The fragment of the electromagnetic spectrum that is sensed by our eyes is termed as ‘optical’ and hence such visible objects are termed as the ‘optical images’.

- The technique Computer Digital Image Analysis (CDIA). CDIP deals with the photograph images that are obtained in or converted into the digital form.

- A digitized image is a set of small elements in 2D space termed as pixels and in 3D space they are termed as voxels. Each pixel (voxel) contains the digitally coded information on its X – Y – (Z) location in the Cartesian coordinate system. The optical information depends on the type of imaging system used.

- Microscopy imaging techniques are also employed to view the microscopic world. Advances in microscopy enable visualization of a broad range of biological processes and features in the cell structure.

- Bright field microscopy is the most elementary form of imaging a specimen and is generally used with compound microscopes. The technique takes the specimen which is dark and contrasts it by the surrounding bright viewing field. Compound light microscopes are often simply referred to as bright field microscopes.

- In polarizing microscope, the polarization method is used to enhance contrast and colour to images which provide information about absorption, structure and composition of specimens. The study of rocks and minerals in geology or petrography fields, medicine, biology and metallurgy specifically use this imaging technique.

### 5.7 KEY WORDS

- **Staining:** It is the process of depositing a colour on a category of Bacteria in order to differentiate them from other categories.
- **Capsule stain**: Capsules are the structures positioned outside the cell wall of an organism and that is why these are in direct contact with the environment.

- **Negative staining**: It is an established method, often used in diagnostic microscopy, for contrasting a thin specimen with an optically opaque fluid.

- **Computer Digital Image Analysis (CDIA)**: The CDIA technique deals with the photograph images that are obtained in or converted into the digital form.

- **Bright field microscopy**: It is the most elementary form of imaging a specimen and is generally used with compound microscopes to study the specimen which is dark and contrasts it by the surrounding bright viewing field.

- **Kohler illumination**: It is a microscopy imaging technique first developed in 1893 through the optimization of a microscope’s optical sequence so as to allow for homogeneously bright light without artefacts and glare.

### 5.8 SELF ASSESSMENT QUESTIONS AND EXERCISES

**Short Answer Questions**

1. Write short notes on -
   (i) Simple Staining
   (ii) Methylene Blue Stains
   (iii) Differential Staining
2. Describe briefly simple special staining techniques.
3. Explain the process to stain films.
4. What is cytological staining?
5. Why Gram stain is used?
7. List the different types of microscopy imaging techniques.

**Long Answer Questions**

1. Elaborate upon the various staining techniques used.
2. Elaborate staining and staining methods giving example of each type.
3. Explain dilute carbol fuschin in detail.
4. Describe some of the specialized techniques used for staining.
5. Briefly discuss about the differential staining techniques used in microbiology.

6. Explain the Gram staining method with the help of appropriate examples. Also discuss its significance.

7. Explain the procedure of acid fast bacilli staining with the help of appropriate examples.

8. Discuss what modifications are made to the Ziehl-Neelsen’s method.

9. Briefly explain the significance of imaging techniques used in microbiology.

10. Discuss in detail the various types of imaging techniques used in microbiology.

**5.9 FURTHER READINGS**


UNIT 6  CULTURE PRESERVATION TECHNIQUES

Structure

6.0 Introduction
6.1 Objectives
6.2 Preservation Methods of Microbes for Storage and Microscopic Studies
   6.2.1 Microbial Culture Collection
6.3 Sterilization and Disinfection
   6.3.1 Classification of Sterilization Methods
6.4 Answers to Check Your Progress Questions
6.5 Summary
6.6 Key Words
6.7 Self Assessment Questions and Exercises
6.8 Further Readings

6.0 INTRODUCTION

The preservation and maintenance of microbial cultures require special and careful attention, reliable preservation and appropriate quality control to ensure that recovered cultures perform in the same way as the original cultures. This requires a high degree of expertise in the maintenance and management of microbial cultures at ultralow temperatures, or as freeze-dried material, to secure their long-term integrity and relevance for future research, development, and conservation. This chapter outlines some of the important procedures and protocols involved in the conservation, preservation, and maintenance of microbial cultures.

Once a microorganism has been isolated and grown in pure culture, it becomes necessary to maintain the viability and purity of the microorganism by keeping the pure culture free from contamination. Normally in laboratories, the pure cultures are transferred periodically onto or into a fresh medium (subculturing) to allow continuous growth and viability of microorganisms. The transfer is always subject to aseptic conditions to avoid contamination. Since repeated subculturing is time consuming, it becomes difficult to maintain a large number of pure cultures successfully for a long time. In addition, there is a risk of genetic changes as well as contamination. Therefore, it is now being replaced by some modern methods that do not need frequent subculturing. These methods include refrigeration, paraffin method, cryopreservation, and lyophilisation (freeze drying).

Disinfection and sterilization are essential for ensuring that medical and surgical instruments do not transmit infectious pathogens to patients.
Because sterilization of all patient-care items is not necessary, health-care policies must identify, primarily on the basis of the items’ intended use, whether cleaning, disinfection, or sterilization is indicated.

In this unit, you will study about various preservation methods of microbes for storage and microscopic studies, sterilisation and disinfection techniques.

6.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand various preservation methods used for microbes storage
- Explain the importance of microscopic studies
- Explain sterilisation and disinfection techniques

6.2 PRESERVATION METHODS OF MICROBES FOR STORAGE AND MICROSCOPIC STUDIES

The biosphere is dominated by microbes in terms of metabolic impact and numbers. The prokaryotes are the most pervasive life forms with greater metabolic diversity and are contaminated and mixed with several other forms of life. To know more about them they have to be separated from mixed forms and need to be cultured under artificial conditions.

Preservation is to maintain pure culture for extended periods in a viable condition without any genetic change. The most important factor during preservation is to prevent microbial growth by lowering the growth rate:

- To restrict genetic change or mutations.
- To avoid contamination.
- To maintain isolated pure cultures for extended periods.

Production of antibiotics, vitamins, amino acids and other solvents and recombinant proteins is accomplished by large scale cultivation of microbial cells, such as Bacteria, yeast and fungus on industrial scale. In all these industrial applications the metabolic activities or the biochemical pathways are used for the production of specific chemicals with the consumption of substrates or a carbon source. Here the microbial culture acts as a factory, where the substrate is the raw material. It is converted into the product and secreted into the media.

Once a microorganism has been isolated and grown in pure culture it becomes essential to maintain the viability and purity of the microorganism by keeping the pure culture free from contamination. The pure cultures can be transferred periodically onto a fresh medium called subculturing to allow
continuous growth and viability of microorganisms. This transfer is always subject to aseptic conditions to avoid contamination.

Strains can be maintained by periodically preparing a fresh culture from the previous stock. The temperature and the type of medium chosen should support a slow rather than a rapid rate of growth so that the time interval between transfers can be as long as possible.

It is possible to calculate the rate of product formation by a single cell under specific metabolic condition, if we know the quantity of product formed over a period of time and the number of cells in the culture. If we want to produce a specific quantity of the product over a period of time is possible to calculate the number of bacterial or microbial cells required to operate the bioprocess on an industrial scale.

The stock culture collection is used for screening of new effective chemotherapeutic agents as assay tools for vitamins and amino acids, as agents of production of vaccine. The objective is to have ultimate aim of maintain strains alive and uncontaminated to prevent change in their characteristics.

**Methods of Maintenance and Preservation**

Preservation methods involve some or the other limitation restricting the rate of metabolism in the organism. Chances of slow rate of mutation exists during the growth of microorganisms. Lowering of metabolic rate of organisms will reduce the possibilities of such variations.

The principle involved in preserving microorganisms are:

- Reduction in temperature of growth.
- Dehydration of the medium used for growth.
- Limitation of nutrients available to the organism.

**Periodic Transfer to Fresh Media**

Preparation of a fresh stock culture is periodically maintained from the previous stalk culture. It is essential to ascertain culture medium, the storage temperature, and time interval at which the transfers are made for various species. Time interval at which the transfers are made varies with the origin and condition of growth. Normally the time interval is stretched longer between the intervals and ideally the culture should be in slow growth phase. The disadvantage lies in failing to prevent changes in the characteristics of a strain due to the development of variants and mutants.

The disadvantage is of failing to prevent changes in the characteristics of a strain due to the development of variants and mutants. Risk of contamination is more. It may be possible to change in genetic and biochemical characteristics.
**Preservation by Overlaying Cultures with Mineral Oil**

**Agar Slant Cultures**

The agar is inoculated and incubated until good growth appears. By covering the slant tip with sterile mineral oil many bacterial species can be preserved. Oil must cover the slants completely. This is the most economical method of preserving Bacteria and Fungi where they remain viable for several years at room temperature. The paraffin layer prevents the layer of dehydration of the medium and help the sample remain in a dormant state by ensuring an aerobic condition. But the changes in the characteristics can still occur.

**TSI Agar Slant Tubes**: The agar triple-sugar iron is one of the culture media used for the differentiation of most enter Bacteria (Refer Figure 6.1).

![Fig. 6.1 TSI Agar Slant Tubes](image)

High concentration of NaCl is frequently used as an inhibitor of bacterial growth. Bacteria are suspended in 1% salt solution and stored at room temperature. The transfer can be made on an agar slant whenever needed. The organisms are dried over calcium chloride and stored in refrigerator. It is preserved by drying in vacuum.

**Preservation by Lyophilisation**

Lyophilisation (Freeze Drying) is a process where water and other solvents are removed from a frozen product via sublimation. Freeze-dried products are hygroscopic that dehydrates the microbial cells to stop their metabolic activates. Lyophilized pure cultures stored in refrigerators and the microbes retain viability for years in its dormant state. The species of Bacteria can remain unchanged in their characteristics for many years.

**Advantages**

- It is recommended using slow rates of cooling as this will result in the formation of vertical ice crystal structures, allowing more efficient water sublimation from the frozen product.
• The culture are rapidly frozen in liquid nitrogen at -196°C in the presence of stabilizing agents, such as Glycerol or Dimethyl sulfoxide that prevents cell damage.

• The cultures can be revived by transferring the rehydrated culture to a suitable growth medium.

**Cryopreservation**: Freezing in liquid nitrogen at -196 degree C or the gas phase helps survival of pure cultures for long storage times. This liquid nitrogen method has been successful with many species that cannot be preserved by lyophilisation and most species can remain viable under these conditions for 10 to 30 years.

The cells are prepared as a dense suspension in a medium containing a cryoprotective agent such as a glycerol or dimethyl sulfoxide, which prevents cell damage due to crystal formation. The vials with suspension are frozen in liquid nitrogen, this is primarily used with many species that cannot be preserved by lyophilisation without undergoing change in their characteristics. The liquid nitrogen must be replaced for the loss due to evaporation. Figure 6.2 shows the Bacteria in a liquid medium.

![Bacteria in Liquid Medium](image)

Fig. 6.2  *Bacteria in Liquid Medium*

The above Figure 6.2 illustrates Bacteria in liquid medium, Bacteria culture preserved in glycerol stocks can be grown overnight in liquid media to promote propagation.

**Preservation Using Silica Gel**

Desiccant activated silica gel are oven sterilized. After the tubes have cooled a skim milk suspension of conidia or mycelium is dispersed into each tube. The
tubes are quickly cooled to reduce heat generated as the liquid is absorbed and then vortexed to break up clumps. After being dried at 25 degree C they are stored in closed containers with desiccants. Neurospora has been successfully preserved through this method.

**Preservation on Paper**

Pieces of agar containing fruiting bodies are placed on sterile filter paper in a Petri dish. Dried in desiccators under vacuum and stored at room temperatures. The vegetative cells are transferred from the growth medium to the small pieces of sterile filter paper on water agar and incubating fruiting bodies develop. These mature in 8 days. The filter paper are then placed into sterile containers and dried over silica gel in a desiccators.

**Preservation on Beads**

Porcelain beads are autoclaved after cell suspension are prepared from 24-8 hours of culture slants with a 20% sucrose solution. The sterile beads are transferred to a sterile petri dish and inoculated with cell suspension. The beads are returned to the vial dried in vacuum desiccator. Then the beads are broken apart and stored.

### 6.2.1 Microbial Culture Collection

To maintain isolated pure cultures for extended periods in a viable conditions free from contamination the pure cultures are transferred periodically onto a fresh medium known as subculturing. The transfer is always subjected to aseptic conditions to avoid contamination.

Since repeated subculturing is a time consuming process, it poses a difficulty in maintain a large number of pure culture for a long time. In addition it also involves a risk of genetic changes also. Hence it is now replaced by modern techniques avoiding frequent subculturing for cryopreservation and lyophilisation (freeze drying).

Microorganisms are cultured in different ways to get the desired product depending on the different type of microbial system, for example, one can get entirely two different products from the same organism by changing the nutritional or the culturing system.

**Batch Culture**

Batch culture is a closed system. At time = 0, the sterilised nutrient solution in the fermented is inoculated with microorganism and incubation is allowed to proceed a suitable temperature and gaseous environment for a suitable period of time. After the inoculation of sterile nutrient and cultivation different phases of growth are observed. The production of metabolites in the growing medium keeps on changing due to the gradual consumption.
**Fig. 6.3 Growth Characteristics in a Batch Culture of a Microorganism**

The above Figure 6.3 illustrates growth characteristics in a batch culture of a microorganism, in which (1) Lag Phase; (2) Transient Acceleration; (3) Exponential Phase; (4) Deceleration Phase; (5) Stationary Phase and (6) Death Phase.

**Advantages**

Advantages of batch culture are as follows:

- Higher raw material conversion level
- Reduced risk of cell mutation as the growth period is short

**Disadvantages**

Disadvantages of batch culture are as follows:

- Lower productivity level due to time for heating, sterilisation, cooling
- Increased focus on instrumentation due to frequent sterilisation.

**Check Your Progress**

1. What is subculturing?
2. What principles are involved in preserving microorganisms?
3. Give any two advantages of batch culture.
4. Give any two disadvantages of batch culture.
6.3 STERILIZATION AND DISINFECTION

The processes of sterilization and disinfection are defined in various ways. Some of the important definitions of these processes are as follows:

**Sterilization** means complete destruction or elimination of all viable organisms in or on a substance being sterilized. Sterilization procedures involve the use of heat, radiation or chemicals, or physical removal of cells.

**Sterilization** is defined as the process by which an article, surface or medium is freed of all living microorganisms either in the vegetative or spore state. When sterilization is done by a chemical agent, the chemical is called **sterilant**.

Sterilization is absolute and it means that all of the microorganisms have either been removed or killed.

**Disinfection**: It is the killing, inhibition or removal of microorganisms that may cause disease. Disinfection simply means that you reduce the microbial load on an object.

Since this is usually done to render the object less likely to be involved in the transmission of infection, a good disinfection procedure is aimed at specifically reducing the numbers of potentially pathogenic organisms in the context of the use of the object being disinfected.

Microorganisms are ubiquitous. Their control means to inhibit or prevent growth of microorganisms. Control of growth usually involves chemical or physical agents which either kills or prevents the growth of microorganisms to destroy pathogens and prevent their transmission and to eliminate microorganism responsible for contamination. Sometimes only partial destruction may be required.

Agents which kill are called cidal agents. Agents which inhibit the growth of cells are referred to as static agents. The term bactericidal refers to killing Bacteria and bacteriostatic refers to inhibiting the growth of bacterial cells. A bactericide kills Bacteria and a fungicide kills Fungi.

Control of organism can be achieved by a variety of chemical and physical methods. Sterilisation can be achieved by using physical means such as heat, radiation and filtration. Agents which destroy Bacteria are said to be bactericidal. Agents which inhibit the growth and reproduction of Bacteria without destroying them are bacteriostatic. Figure 6.4 shows various sterilization methods.
Fig. 6.4 Various Sterilization Methods

6.3.1 Classification of Sterilization Methods

A number of precautionary measures are taken to prevent contamination during sterilization and creating a germ free or aseptic condition. There are following three methods of sterilization.

- Physical Methods
- Chemical Methods

Physical Methods of Sterilization

The most reliable and authentic physical methods of sterilization are as follows:

**Sunlight**: It has bactericidal activity. It spontaneously sterilizes under natural conditions primarily due to its content of ultraviolet rays.

**Heat**: It is the most reliable and universally applicable method of sterilization. Dry or moist heat may be applied and the materials that may be damaged by heat can be sterilized at lower temperature, for longer periods or by repeated cycles.

**Filtration**: Sterilizing a solution by filtering is one of the most popular methods of sterilization. The solution can be sterilized through this method without heating it. A filter works by passing the solution through a filter with a pore diameter which is very small and so microbes cannot pass through it.

The various types of filters include:

- Earthware Filters
- Asbestos Filters
- Sintered Glass Filters
- Membrane Filters
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Radiation: Another physical method using which you can sterilize is radiation. This is performed by using electron beams, X-rays, gamma rays, or subatomic particles.

The process of non-ionizing radiation sterilization uses ultraviolet light or UV light. The process of irradiation is useful only for sterilization of surfaces and some transparent objects. Ultraviolet irradiation is regularly done to sterilize the interiors of biological safety boxes. The other useful process of sterilization is gamma radiation which involves beam radiation. These are different types of ionizing radiation mostly used in the healthcare industry. The gamma rays are very useful in getting rid of microorganisms.

Very similar to gamma radiation is e-beam irradiation. These e-beams are concentration of various chemical and molecular bonds and are highly charged streams of electrons. Acceleration and conversion of electricity generate these e-beams. These e-beams and gamma radiation sterilize a wide range of devices, including syringes and cardiothoracic devices.

Moist Heat: You need temperature below 100°C to kill vegetative Bacteria but not spores, like pasteurization of milk employed for disinfection at 63°C for 30 minutes or 72°C for 15–20 seconds followed by rapid cooling to 13°C. All non-sporing pathogens such as mycobacteria, brucellae and salmonellae are destroyed by these processes. Vaccines are inactivated in a water bath at 60°C for 1 hour; serum containing congealable proteins can be sterilized by heating for 1 hour at 56°C. Lowenstein–Jensen and Loeffler’s serum are remade sterile by heating at 80–85°C for half an hour on three successive days.

Steam under pressure provides greater lethal action of moist heat; is quicker in heating up articles to be sterilized and can penetrate easily porous material such as, cotton wool stopper.

The various types of steam sterilizers include displacement laboratory autoclave, bench top autoclave, multipurpose laboratory autoclave, high security autoclave, porous load sterilizer and low-temperature steam. An autoclave for use in a laboratory or hospital setting is a large pressure cooker; it operates by using steam under pressure as the sterilizing agent. High pressures enable steam to reach high temperatures, thus increasing its heat content and killing power. Heating power of steam comes from its latent heat of vaporization. This amount of heat content is too large. It takes 80 calories to make 1 litter of boiled water, but 540 calories to convert that boiling water to steam. Thus steam at 100°C has almost seven times more heat than boiling water.
**Uses:** This is used for sterilizing culture media, laboratory supplies, aqueous solutions, rubber material, dressing materials, gowns, dressing, linen, gloves, instruments and pharmaceutical products.

Sterilization controls include a biological control made of an envelope containing a filter paper strip impregnated with 106 spores of *Bacillus stearothermophilus*. After sterilization the strip is inoculated into broth and incubated at 56°C for 5 days. No growth of *B. stearothermophilus* indicates proper sterilization; a Browne’s tube containing red solution changes to green when exposed to temperature of 121°C for 15 minutes in autoclave. It indicates proper sterilization.

**Dry Heat Sterilization:** The lethal effect of dry heat is due to protein denaturation, oxidative damage and toxic effects of elevated levels of electrolytes. Moist heat actually kills microorganisms by coagulation and denaturation of their enzymes and various structural proteins. This is the most important and widely used method. The type of heat, and most importantly, the time of application and temperature are important to ensure destruction of all microorganisms.

Dry heat sterilization is used for needles, inoculating wires, glassware, etc. and objects not destroyed in the incineration process. The rules of relating time and temperature apply, but dry heat is not as effective as moist heat (i.e., higher temperatures are needed for longer periods of time). 160o/2 hours or 170o/1 hour is necessary for sterilization. Hot air sterilizer is the most widely used method of sterilization by dry heat, and it is used for processing materials which can withstand high temperatures for length of time needed for sterilization.

Hot air oven is electrically heated, with heating and it should be fitted with a fan to provide forced air circulation throughout the oven chamber, a temperature indicator, a control thermostat and timer, open mesh shelving and adequate wall insulation. The sterilization hold time is set to 160°C for 2 hour. Oils, glycerol and dusting powder for 1 hour are kept at 150°C. For oils, glycerol, cooling may take up to several hours. Glassware is liable to crack if cold air is admitted suddenly while it is still very hot.

**II. Chemical Methods of Sterilization**

Apart from the physical methods, in some cases, you need to also use chemicals for sterilizing and disinfecting. In this section you will get to know about the chemical methods.

Chemicals used for sterilization include gases ethylene oxide and formaldehyde, and liquids such as glutaraldehyde, ozone and hydrogen peroxide. Several groups of chemical substances such as alcohols, aldehydes, phenols, halogens and so on are used for chemical sterilization.
Types of Antimicrobial Agents

The following are some of the microbial agents which are used frequently.

Disinfectants: Disinfectants are the agents that kill microorganisms, but not necessarily their spores, and so are not safe for application to living tissues. They are used on inanimate objects such as tables, floors, utensils, etc. Examples include, hypochlorite, chlorine compounds, lye, copper sulphate, quaternary ammonium compounds, formaldehyde and phenolic compounds. Table 6.1 shows the various disinfectants with their uses, advantages and disadvantages.

A disinfectant may have a narrow spectrum of activity against microorganisms. However some Gram Negative Bacteria are more resistant, for example, Pseudomonas species. Their action on viruses or spores may be uncertain or absent.

- **Phenols**: Lister introduced antiseptic during surgery. The lethal effects of phenols is due to their capacity to cause cell membrane damage, release cell contents and cause lysis. Low concentration precipitates protein and membrane bound oxidases and dehydrogenases, for example, phenol powerful microbicide

- **Lysol and Cresol**: Active against wide range of microorganisms. Hypochlorite: it is corrosive to meal and textiles and have poor wetting properties. They are readily inactivated by organic materials and detergents. Hypochlorites are corrosive to metal and textiles and against mycobacterium

- **Glutaraldehyde**: It is less corrosive than hypochlorite. It is bactericidal and virucidal disinfectant and effective against mycobacterium and to lesser extent to spores. The decontamination of centrifuges and automated equipments that can resist the damage by glutaraldehyde.

- **Ethyl Alcohol**: Ethanol at 70% in water is highly active against Gram-Positive and Gram-Negative Bacteria and acid fast bacilli. It can be active against spores. It is susceptible in activation by organic matter. It should not be used by soil surface has poor power of penetration. It is used only on perfectly clean surfaces and inoculation chambers for the removal of transient Bacteria.

- **Formaldehyde Disinfection**: For 1000 cubic feet of space, place 500 ml of formaldehyde-40% solution and 100ml of water in an electric boiler and switch on the boiler. Leave the room filled with the disinfectant and seal for 24hrs. After 24 hours open the door and windows to allow the vapours to dispense and neutralise any residual formaldehyde with ammonia exposing 250 ml ammonia per litre of formalin used.
### Table 6.1 Various Disinfectants with their Uses, Advantages and Disadvantages

<table>
<thead>
<tr>
<th>Disinfectant</th>
<th>Uses</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alcohols:</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Isopropyl 60–70%</td>
<td>Intermediate-level disinfectant:</td>
<td>Fast acting</td>
<td>Volatile</td>
<td>Isopropyl alcohol slightly more effective than ethyl alcohol.</td>
</tr>
<tr>
<td>Ethanol 70–90%</td>
<td>disinfect thermometers, external surfaces of some equipment (e.g.</td>
<td>No residue</td>
<td>Evaporation may diminish concentration</td>
<td>70% alcohol more effective than 90%.</td>
</tr>
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<td></td>
<td>stethoscopes).</td>
<td>Non-staining</td>
<td>Inactivated by organic material</td>
<td></td>
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<td></td>
<td>Equipment used for home health care.</td>
<td></td>
<td>May harden rubber or cause deterioration of glues</td>
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<td></td>
<td>Used as a skin antiseptic.</td>
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<td>Use in the operating theatre.</td>
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<tr>
<td><strong>Chlorines</strong></td>
<td></td>
<td>Low cost</td>
<td>Corrosive to metals</td>
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<td></td>
<td>disinfect hydrotherapy tanks, dialysis equipment, cardiopulmonary</td>
<td>Readily available in non-hospital settings and easy to use</td>
<td>Irritant to skin and mucous membranes.</td>
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<td></td>
<td>training manikins, environmental surfaces.</td>
<td></td>
<td>Unaffected by water hardness.</td>
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<td></td>
<td>Effective disinfectant following blood spills, aqueous solutions (5,000</td>
<td>Effective deodorizer and disinfectant.</td>
<td>Unstable when diluted to usable state (1:10 dilution).</td>
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<td></td>
<td>parts per million) used to decontaminate area after blood has been</td>
<td>Does not leave toxic residues.</td>
<td>Use in well-ventilated areas.</td>
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<tr>
<td></td>
<td>removed, sodium dichloroisocyanurate powder sprinkled directly on</td>
<td>Bactericidal activity increases with temperature.</td>
<td>Shelf life shorter when diluted.</td>
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<td></td>
<td>blood spills for decontamination and subsequent cleanup.</td>
<td></td>
<td>Discolouring or bleaching of fabrics can occur</td>
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<td></td>
<td>Equipment used for home health care.</td>
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<td>Requires precleaning of surface prior to disinfection.</td>
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<td></td>
<td></td>
<td></td>
<td>Highly toxic when mixed with ammonia.</td>
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<tr>
<td><strong>Ethylene oxide</strong></td>
<td>Used as gas for the sterilization of heat sensitive medical devices.</td>
<td>Sterilant for heat or pressure sensitive equipment.</td>
<td>Slow acting and requires several hours of aeration to remove residue.</td>
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</tr>
</tbody>
</table>

**NOTES**

- Suitable for low and high-level decontamination of surfaces only.
- For mycobacteria use at high concentrations 1% (10,000 ppm).
- Use with extreme care if used for instrument disinfection because of corrosive activity.
- Wide range of in-use dilutions recommended for different situations therefore ensures dilution is correct for particular use and that it is made up correctly.
<table>
<thead>
<tr>
<th>Chemical</th>
<th>Properties</th>
<th>Uses</th>
</tr>
</thead>
</table>
| Formaldehyde | Very limited use as chemical oxidant.  
|             | Sometimes used to reprocess haemodialyzers.  
|             | Gaseous form used to decontaminate laboratory safety cabinets.               | Active in presence of organic materials.  
|             | Carcinogenic  
|             | Toxic  
|             | Strong irritant  
|             | Pungent odour.  
|             | Limited use because of toxicity.  
|             | Use only under strict supervision of senior staff.                  |                                                      |
| Glutaraldehyde | 2% formulations high-level disinfection for heat sensitive equipment.  
|             | Most commonly used for endoscopes, respiratory therapy equipment and anaesthesia equipment  
|             | Effective against viruses, fungi and bacteria including Mycobacterium tuberculosis. | Non-corrosive to metal.  
|             | Active in presence of organic material.  
|             | Compatible with leased instruments.  
|             | Sterilization may be accomplished in 6-10 hours.  
|             | Extremely irritating to skin and mucous membranes.  
|             | Shelf life shortened when dried (effective for 14-30 days depending on formulation).  
|             | High cost.  
|             | Monitor concentration in reusable solutions.  
|             | Fixative.  
|             | Acts as a fixative, so prior cleaning is essential.  
|             | Toxic, therefore use under conditions that minimize exposure.      |
| Hydrogen peroxide | 3% - low level disinfectant:  
|             | Equipment used for home health care  
|             | Cleans floors, walls and furnishings  
|             | 6% - high level disinfectant:  
|             | Effective for high level disinfection of flexible endoscopes  
|             | Foot care equipment  
|             | Disinfection of soft contact lenses  
|             | High concentrations used as chemosterilants in specially designed machines for decontamination of heat sensitive medical devices. | Strong oxidant  
|             | Fast acting  
|             | Breaks down into water and oxygen.  
|             | Can be corrosive to aluminium, copper, brass and zinc.  
|             |                                                      |
• **Antiseptics**: Antiseptics are applied to the skin and mucous membrane as they are absolutely harmless microbicide agents. However, their internal use is prohibited. These antiseptics and disinfectants are frequently used in hospitals and other treatment centres for a number of topical and hard-surface applications. The antiseptics are used as deterrents for the growth of microorganisms. The Table 6.2 below shows various antiseptics with their uses, advantages and disadvantages.

**Table 6.2 Various Antiseptics with their Uses, Advantages and Disadvantages**

<table>
<thead>
<tr>
<th>Antiseptic</th>
<th>Usage</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodophors (e.g. Betadine)</td>
<td>Surgical scrub, patient preparation, use in genital area, vagina, cervix.</td>
<td>Less irritating to the skin than iodine. Can be used on mucous membranes.</td>
<td>Effectiveness is moderately reduced by blood or other organic material. Effective 1–2 minutes after application.</td>
<td>Effective against a broad range of microorganisms.</td>
</tr>
<tr>
<td>Povidone-iodine solution</td>
<td>Surgical scrub and skin preparation.</td>
<td>Good persistent effect. Remains effective for at least 6 hours after application. Effectiveness not reduced by blood or other organic material.</td>
<td>May cause irritation.</td>
<td>Effective against a broad range of microorganisms, but has a minimal effect on tuberculosis and fungi. May irritate the genital area, vagina, cervix.</td>
</tr>
<tr>
<td>Chlorhexidine gluconate 2% or 4% scrub (e.g. Hibiscrub, Hibiscrub, Hibiscrub) or 0.3% tincture</td>
<td>Surgical scrub and skin preparation.</td>
<td>Good persistent effect. Remains effective for at least 6 hours after application. Effectiveness not reduced by blood or other organic material.</td>
<td>May cause irritation.</td>
<td>Effective against a broad range of microorganisms.</td>
</tr>
<tr>
<td>Iodine 1% Tincture of iodine 2%</td>
<td>Used for skin preparation, but must be allowed to dry and then removed from the skin with alcohol.</td>
<td>Fast acting.</td>
<td>Can cause skin irritation.</td>
<td>Effective against a broad range of microorganisms.</td>
</tr>
<tr>
<td>Alcohol 70% – 90% (isopropyl)</td>
<td>Cannot be used on dry skin. Wash area before applying.</td>
<td>Rapid kill. Effectiveness moderately reduced by blood or other organic material. Drying effect on skin. Cannot be used on mucous membranes.</td>
<td>Effective against a broad range of microorganisms. Alcohol containers should be stored in areas approved for flammable materials.</td>
<td></td>
</tr>
<tr>
<td>Para-chloro-meta-xylenol (PCHX)</td>
<td>Not recommended for routine use. PCMX is available in both antiseptic and disinfectant preparations.</td>
<td>Persistent effect over several hours. Activity only minimally reduced by blood or other organic material. Less effective than chlorhexidine.</td>
<td>Good activity against gram-positive organisms. Its speed is intermediate.</td>
<td></td>
</tr>
</tbody>
</table>
Check Your Progress

5. Define sterilization and sterilant.
6. What is disinfection?
7. What are the chemicals used for sterilization?
8. Where are disinfectants used?
9. When are antiseptics applied?

6.4 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. The pure cultures can be transferred periodically onto a fresh medium called subculturing to allow continuous growth and viability of microorganisms.

2. The principle involved in preserving microorganisms are:
   - Reduction in temperature of growth
   - Dehydration of the medium used for growth
   - Limitation of nutrients available to the organism

3. Advantages of batch culture are as follows:
   - Higher raw material conversion level
   - Reduced risk of cell mutation as the growth period is short

4. Disadvantages of batch culture are as follows:
   - Lower productivity level due to time for heating, sterilisation, cooling
   - Increased focus on instrumentation due to frequent sterilisation

5. Sterilization is defined as the process by which an article, surface or medium is freed of all living microorganisms either in the vegetative or spore state. When sterilization is done by a chemical agent, the chemical is called sterilant.

6. Disinfection is the killing, inhibition or removal of microorganisms that may cause disease. Disinfection simply means that you reduce the microbial load on an object.

7. Chemicals used for sterilization include gases ethylene oxide and formaldehyde, and liquids such as glutaraldehyde, ozone and hydrogen peroxide. Several groups of chemical substances such as alcohols, aldehydes, phenols, halogens and so on are used for chemical sterilization.
8. Disinfectants are used on inanimate objects such as tables, floors, utensils, etc. Examples include, hypochlorite, chlorine compounds, lye, copper sulphate, quaternary ammonium compounds, formaldehyde and phenolic compounds.

9. Antiseptics are applied to the skin and mucous membrane as they are absolutely harmless microbicide agents. However, their internal use is prohibited.

6.5 SUMMARY

- The biosphere is dominated by microbes in terms of metabolic impact and numbers.
- The prokaryotes are the most pervasive life forms with greater metabolic diversity and are contaminated and mixed with several other forms of life.
- Preservation is to maintain pure culture for extended periods in a viable condition without any genetic change.
- Production of antibiotics, vitamins, amino acids and other solvents and recombinant proteins is accomplished by large scale cultivation of microbial cells, such as Bacteria, yeast and fungus on industrial scale.
- In all these industrial applications the metabolic activities or the biochemical pathways are used for the production of specific chemicals with the consumption of substrates or a carbon source.
- Once a microorganism has been isolated and grown in pure culture it becomes essential to maintain the viability and purity of the microorganism by keeping the pure culture free from contamination.
- The pure cultures can be transferred periodically onto a fresh medium called subculturing to allow continuous growth and viability of microorganisms.
- Strains can be maintained by periodically preparing a fresh culture from the previous stock.
- The temperature and the type of medium chosen should support a slow rather than a rapid rate of growth so that the time interval between transfers can be as long as possible.
- It is possible to calculate the rate of product formation by a single cell under specific metabolic condition, if we know the quantity of product formed over a period of time and the number of cells in the culture.
- The stock culture collection is used for screening of new effective chemotherapeutic agents as assay tools for vitamins and amino acids, as agents of production of vaccine.
- Preservation methods involve some or the other limitation restricting the rate of metabolism in the organism.
- Chances of slow rate of mutation exists during the growth of microorganisms. Lowering of metabolic rate of organisms will reduce the possibilities of such variations.
- Preparation of a fresh stock culture is periodically maintained from the previous stalk culture.
- It is essential to ascertain culture medium, the storage temperature, and time interval at which the transfers are made for various species.
- Time interval at which the transfers are made varies with the origin and condition of growth.
- The agar is inoculated and incubated until good growth appears. By covering the slant tip with sterile mineral oil many bacterial species can be preserved.
- The paraffin layer prevents the layer of dehydration of the medium and help the sample remain in a dormant state by ensuring an aerobic condition. But the changes in the characteristics can still occur.
- High concentration of NaCl is frequently used as an inhibitor of bacterial growth. Bacteria are suspended in 1% salt solution and stored at room temperature. The transfer can be made on an agar slant whenever needed.
- Lyophilisation (Freeze-Drying) is a process where water and other solvents are removed from a frozen product via sublimation.
- Freeze-dried products are hygroscopic that dehydrates the microbial cells to stop their metabolic activates.
- Lyophilized pure cultures stored in refrigerators and the microbes retain viability for years in its dormant state.
- The species of Bacteria can remain unchanged in their characteristics for many years.
- Freezing in liquid nitrogen at -196 degree C or the gas phase helps survival of pure cultures for long storage times.
- The cells are prepared as a dense suspension in a medium containing a cryoprotective agent, such as a glycerol or dimethyl sulfoxide, which prevents cell damage due to crystal formation.
- The vials with suspension are frozen in liquid nitrogen, this is primarily used with many species that cannot be preserved by lyophilisation without undergoing change in their characteristics.
- Pieces of agar containing fruiting bodies are placed on sterile filter paper in a Petri dish.
• Dried in desiccators under vacuum and stored at room temperatures. The vegetative cells are transferred from the growth medium to the small pieces of sterile filter paper on water agar and incubating fruiting bodies develop.

• Microorganisms are cultured in different ways to get the desired product depending on the different type of microbial system, for example, one can get entirely two different products from the same organism by changing the nutritional or the culturing system.

• Batch culture is a closed system. At time $= 0$, the sterilised nutrient solution in the fermented is inoculated with microorganism and incubation is allowed to proceed a suitable temperature and gaseous environment for a suitable period of time.

• After the inoculation of sterile nutrient and cultivation different phases of growth are observed.

• The production of metabolites in the growing medium keeps on changing due to the gradual consumption

• Heat is the most reliable and universally applicable method of sterilization.

• Dry or moist heat may be applied and the materials that may be damaged by heat can be sterilized at lower temperature, for longer periods or by repeated cycles.

• Sterilizing a solution by filtering is one of the most popular methods of sterilization. The solution can be sterilized through this method without heating it.

• Radiation is a physical method using which you can sterilize is radiation. This is performed by using electron beams, X-rays, gamma rays, or subatomic particles.

• The process of non-ionizing radiation sterilization uses ultraviolet light. The process of irradiation is useful only for sterilization of surfaces and some transparent objects.

• Ultraviolet irradiation is regularly done to sterilize the interiors of biological safety boxes. The other useful process of sterilization is gamma radiation which involves beam radiation.

• Vaccines are inactivated in a water bath at 60°C for 1 hour; serum containing congealable proteins can be sterilized by heating for 1 hour at 56°C. Lowenstein–Jensen and Loeffler’s serum are remade sterile by heating at 80–85°C for half an hour on three successive days.

• Steam under pressure provides greater lethal action of moist heat; is quicker in heating up articles to be sterilized and can penetrate easily porous material, such as cotton wool stopper.
The various types of steam sterilizers include displacement laboratory autoclave, bench top autoclave, multipurpose laboratory autoclave, high security autoclave, porous load sterilizer and low-temperature steam.

Dry heat sterilization is lethal effect of dry heat is due to protein denaturation, oxidative damage and toxic effects of elevated levels of electrolytes.

The type of heat, and most importantly, the time of application and temperature are important to ensure destruction of all microorganisms.

Dry heat sterilization is used for needles, inoculating wires, glassware, etc. and objects not destroyed in the incineration process.

Hot air oven is electrically heated, with heating and it should be fitted with a fan to provide forced air circulation throughout the oven chamber, a temperature indicator, a control thermostat and timer, open mesh shelving and adequate wall insulation.

The sterilization hold time is set to 160°C for 2 hour. Oils, glycerol and dusting powder for 1 hour are kept at 150°C. For oils, glycerol, cooling may take up to several hours.

Chemicals used for sterilization include gases ethylene oxide and formaldehyde, and liquids such as glutaraldehyde, ozone and hydrogen peroxide.

Several groups of chemical substances such as alcohols, aldehydes, phenols, halogens and so on are used for chemical sterilization.

Disinfectants are the agents that kill microorganisms, but not necessarily their spores, and so are not safe for application to living tissues. They are used on inanimate objects such as tables, floors, utensils, etc.

A disinfectant may have a narrow spectrum of activity against microorganisms. However some Gram Negative Bacteria are more resistant, for example, Pseudomonas species. Their action on viruses or spores may be uncertain or absent.

Lister introduced antiseptic during surgery. The lethal effects of phenols is due to their capacity to cause cell membrane damage, release cell contents and cause lysis.

Low concentration precipitates protein and membrane bound oxidases and dehydrogenases, for example, phenol powerful microbicide.

Ethanol at 70% in water is highly active against Gram Positive and Gram Negative Bacteria and Acid Fast Bacilli. It can be active against spores. It is susceptible in activation by organic matter.

Antiseptics are applied to the skin and mucous membrane as they are absolutely harmless microbicide agents. However, their internal use is prohibited.
6.6 KEY WORDS

- **Sterilization**: Sterilization is defined as the process by which an article, surface or medium is freed of all living microorganisms either in the vegetative or spore state.
- **Sterilant**: When sterilization is done by a chemical agent, the chemical is called sterilant.
- **Disinfection**: It is the killing, inhibition or removal of microorganisms that may cause disease.

6.8 SELF ASSESSMENT QUESTIONS AND EXERCISES

**Short Answer Questions**

1. What is subculturing?
2. What are the principle involved in preserving microorganisms?
3. What is agar slant cultures?
4. What is cryopreservation?
5. How is preservation on paper done?
6. What are the advantages and disadvantages of batch culture?
7. What is sterilisation?

**Long Answer Questions**

1. What are the various methods of preservation of microbes for storage and microscopic studies?
2. How is periodic transfer to fresh media done? Discuss with the help of examples.
3. Explain preservation of lyophilisation.
4. Explain batch collection in detail. Draw a well-labelled diagram of growth characteristics in batch culture of a microorganism.
5. Write a note on sterilisation and its different types.
6. Draw a well labelled diagram to show different sterilization methods.
7. Briefly discuss about disinfectants.
6.9 FURTHER READINGS


UNIT 7 MICROBIAL CULTURE METHODS

Structure
7.0 Introduction
7.1 Objectives
7.2 Axenic and Synchronous Culture
   7.2.1 Axenic Culture
   7.2.2 Synchronous Culture
7.3 Aerobic and Anaerobic Culture
   7.3.1 Culture Methods and Nutritional Types
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7.0 INTRODUCTION

In normal culture, cells are present in all stages of the cell cycle because of several factors influencing the cell cycle. For biological research, culture with a unified cell cycle stage is suitable. As it help in anticipating a particular stage in the cell cycle. Growing of Bacteria in an artificial medium, under suitable conditions in a laboratory is called Bacterial Culture. The growth on a solid medium leads to the formation of aggregates of Bacteria. Each of these aggregates arises from a single bacterium and is called a colony. Since all the Bacteria in a colony are the progeny of a single bacterium, they are supposed to be identical in their biochemical, serological and antibiotic sensitivity characters. Depending on the ability of the Bacteria to survive in the presence of oxygen, they can be divided into Aerobic and Anaerobic Bacteria. Those Bacteria that can survive in the presence of oxygen are called Aerobic Bacteria whereas those that require an absence of oxygen to survive are called Anaerobic Bacteria. Most of the Aerobic Bacteria that grow in the presence of oxygen are also facultative anaerobic, that is, though they...
Microbial Culture Methods

generally grow in the presence of oxygen, they can also grow in its absence. Obligate anaerobes on the other hand, cannot live in the presence of oxygen and are called Anaerobic Bacteria.

In this unit, you will study about axenic and synchronous culture, aerobic and anaerobic culture media and nutritional types, growth curve, generation time and growth kinetics, factors influencing microbial growth in detail.

7.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand axenic and synchronous culture
- Discuss about aerobic and anaerobic culture media and nutritional types
- Explain growth curve, generation time and growth kinetics
- Analyse the factors that influence the microbial growth

7.2 AXENIC AND SYNCHRONOUS CULTURE

In this section, the methodology and significance of axenic and synchronous culture medium essential for the bacterial growth is discussed.

7.2.1 Axenic Culture

Axenic is a culture medium or biological culture that contains only a single species or organism strain and is uncontaminated from other microorganism. Earlier, only Bacteria or unicellular Eukaryotes were cultured. However, multicellular organism can also be cultured in axenic. Axenic culture is a significant tool that helps in studying parasitic and symbiotic organism in a controlled environment. The growth and maintenance of a single species in isolation, free from foreign or contaminating species is axenic culture. Isolation in axenic culture is achieved usually by growing the species in an environment that was sterilised previously from contaminating organisms.

Significance: The contaminants usually encountered are microorganisms, axenic cultures, whether of vertebrates or invertebrates are often referred to as germ free conditions. In addition, the term gnotobiotic is often used interchangeably with axenic.
In Practice it refers to Germ Free Conditions

Axenic culture used as a diagnostic tool that helps in studying symbiotic and parasitic organisms in a controlled environment.

If biological contaminants appear in a culture, it is isolated into a single cell from the culture with a micropipette, and try to establish a new clonal culture. Alternatively, a culture can be streaked on an agar plate in the hope of attaining a colony free of contaminants.

Often to achieve an axenic culture antibiotics must be used. Best results appear when an actively growing culture of Algae is exposed to a mixture of Penicillin and Streptomycin. This drastically reduces the growth of Bacteria increasing the chances of obtaining an axenic cell when using micro-pipetting or agar streaking isolation.

Another approach is to add a range of antibiotic concentrations as growth inhibitors to a number of subcultures of previously available mixed culture and then select the culture that has surviving algal cells but no surviving Bacteria or other contaminant. You can make a dilution series until you expect no cells anymore and plate all those dilution, i.e., do a dilution to extinction. On one of the last plates colonies might be separated well enough to pick a single colony to start a clean culture.

These subcultures are grown until their identity are confirmed. Subculture selection is done by sampling of the target organism from an uncontaminated growth in a mixed culture allowing it to be used as a source of inoculums for the subculture.

A standard approach is that the sample of culture is spread onto an agar plate and incubate the culture for a definite time period. The agar should have enriched medium containing micro and macro nutrients that support organism’s growth. The explants will proliferate into a mass of undifferentiated tissue, callus on such medium which may be exploited for different purposes.

However, some microalgal cultures may die when made axenic, probably due to the termination of obligate symbiotic relationships with Bacteria. Sterility of culture should be checked by adding a small amount of sterile bacterial culture medium and observing regularly for Bacterial Growth. Absence of bacterial culture does not however ensure that the culture is axenic since majority of Bacteria do not respond to standard enrichment medium.

Figure 7.1 shows the procedure for establishing axenic culture of freshwater diatoms.
Applications

Following are the applications of axenic culture:

- Axenic culture are beneficial as organisms are derived from single gene pool except for variations caused due to mutations.
- Tissue completely free from biological contaminants are however essential, as nutrient media will generally support the growth of such contaminants which may then overgrow in the culture or produce metabolites which may influence growth and metabolic responses of cultured material and modify applied treatments.

Demerits

Following are the demerits of axenic culture:

- Axenic cultures are free of any foreign organisms, such as Bacteria and require sterilization of all glassware, culture media and vessels to
avoid contamination. The latter makes it impractical for commercial operations.

- Experimental procedures using axenic culture systems are poorly documented as the plant environment is not optimal and axenic conditions are not rigorously verified. However, in this culture plants were responsive to increasing photo-synthetically competent.
- They exhibit the ability to grow in a medium with increased viscosity and osmotrophic nutrition in pathogenic species. They become complicated because they generally thrive within host tissues.

7.2.2 Synchronous Culture

A synchronous or synchronized culture is a microbiological culture or a cell culture that contains cells that are all in the same growth stage. As numerous factors influence the cell cycle normal cultures have cells in all stages of the cell cycle. The study of the progressive changes is difficult when the cells are randomly distributed with respect to age. Synchronized culture is a culture of Bacterial or animal cells in which all cells are in the same phase of cell division. A synchronous population can be selected from a random population by separation of cells that are at the same stage of development. To resolve the problem methods are devised in which the cells in a culture are brought to the same stage of the cell cycle. So that the entire population can be as though it were a single cell. It is widely used for addressing issues of cell growth and cycle and the effect of various factors on cell growth. Following are some significant features:

- The degree of synchrony that exists in a cell culture can be assessed by cultures mitotic index. This is a measure of the fraction of cells undergoing division at any instant and is given by:
  \[ IM = \frac{NM}{N} \]
  Where IM is the mitotic index, NM is the total number of cells visible in mitosis, N total number of cells present.
- During synchronous growth IM changes from minimum value to maximum value during short interval of time.

Synchronous cultures can be obtained in following methods:

External conditions can be changed, so as to arrest growth of all cells in the culture, and then changed again to resume growth. The newly growing cells are now all starting to grow at the same stage, and they are synchronized. For example, for photosynthetic cells, light can be eliminated for several hours and then re-introduced.

Another method is to eliminate an essential nutrient from the growth medium and later re-introduce it.
Cell growth can also be arrested using chemical growth inhibitors. After growth has completely stopped for all cells, the inhibitor can be removed from the culture and the cells then begin to grow synchronously.

Cells in different growth stages have different physical properties. Cells in a culture can thus be physically separated based on their density or size, for instance. This can be achieved using centrifugation (for density) or filtration (for size).

In the Helmstetter-Cummings technique, a bacterial culture is filtered through a membrane. Most Bacteria pass through, but some remain bound to the membrane. Fresh medium is then applied to the membrane and the bound Bacteria start to grow. New-born Bacteria that detach from the membrane are now all at the same stage of growth; they are collected in a flask that now harbors a synchronous culture.

Following are the two common methods to induce synchrony.

**Synchrony by Induction**

The most frequently employed methods for inducing synchrony involve temperature cycles (temperature shocks), light cycles, and chemical manipulations. When temperature is used to induce synchrony, the cells are subjected to alternating cold and warm periods. Following this sequence, the cell population enters synchronous division.

**The Double Thymidine Block:** A popular procedure for inducing synchrony in cells is to cause the random population to accumulate at the beginning of the S phase by preventing the synthesis of DNA. One way to achieve this is through the addition of high concentrations of the nucleoside thymidine to the culture medium. The addition of the thymidine acts through a feedback mechanism within the cells to prevent the production of other nucleosides that are needed for DNA synthesis. When the thymidine is first added to the culture, any cells that are in the S phase stop DNA synthesis immediately, thereby producing a culture in which some cells are halted at various stages of the S phase while the remaining cells are blocked at the entry into the S phase. Henceforth, the block is lifted for an interval of time greater than the length of the S phase. This allows all cells to complete the S phase regardless of the point in the cell cycle where they were halted after the first application of the thymidine. Therefore, as a result of the ‘double thymidine block’ all the cells in the culture are arrested at the start of the S phase.

**Synchrony by Selection**

Selection techniques involve the mechanical isolation of cells of similar age from a random culture; these cells are then inoculated into fresh medium, where they grow and divide synchronously for some time. Among the methods frequently employed for mechanically isolating cells of similar age
is filtration. This involves the mechanical isolation of cells of similar age from the random culture, these cells are then inoculated into fresh medium, where they grow and divide. In this procedure, a cell culture is passed through a filter that separates the larger cells from the smaller ones. The isolated smaller cells (which are also the younger cells of the population) are then inoculated into fresh medium to start a new culture.

Cells of similar age may also be isolated from a random culture by sedimentation. Because young cells are generally smaller than older cells, they sediment more slowly and may be isolated for subculture. This technique involves filtering an unsynchronised culture of Bacteria through filter. The Helmstetter–Cummings technique based on the fact that Bacteria stick to cellulose nitrate filter.

Through filter, then allowing a flow of fresh medium through it. The only culture of Bacteria in the effluent stream are those which arise through division. Cells in the effluent are newly formed and at the same stage of cell cycle.

Differences between Axenic Culture and Synchronous Culture

- A pure or axenic culture is a population of cells or multicellular organisms growing in the absence of other species or types.
- A pure culture may originate from a single cell or single organism, in which case the cells are genetic clones of one another.
- The most common form of microbial cultures are liquid or solid (agar).
- Synchronous culture is composed of cells which are at the same stage of cell cycle. It is accomplished either by repetitive shifts of temperature or by furnishing fresh nutrients to cultures that have just entered the stationary phase of cells that are at the same stage of develop,

Check Your Progress

1. What is axenic culture?
2. What is the significance of axenic culture?
3. What is synchronized culture?

7.3 AEROBIC AND ANAEROBIC CULTURE

Most of the Aerobic Bacteria are also called facultative anaerobes are members of normal flora. Obligate anaerobes on the other hand cannot live in the presence of oxygen. Aero-tolerant anaerobes can grow poorly in the presence of air. Most can survive in 3%-5% O2. Some are sensitive to O2 concentration as low as 5% O2. Lack the capacity to utilize O2 as a terminal
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...hydrogen acceptor. Anaerobes generate energy by fermentation. Those that can survive the presence of oxygen are called Aerobic Bacteria. Anaerobiosis can be achieved by a number of methods, such as:

- Production of a Vacuum
- Displacement of Oxygen by Other Gases
- Absorption of Oxygen by Chemical or Biological Methods
- Displacement and Combustion of Oxygen
- By Reducing Agents
- Other Anaerobic Culture Systems

So to culture an anaerobic bacterium, the absence of oxygen is required to be produced. In the following section all these methods are discussed.

I. Production of a Vacuum: This is done by incubating cultures in a vacuum desiccator. This is not a satisfactory process as some oxygen is always left behind. In the vacuumed produced fluid culture may boil over and the media may get detached from the plates. Displacement of oxygen with gases, such as hydrogen, nitrogen is sometimes employed.

Drawbacks

- Repeated evacuation and refilling.
- Oxygen can never be removed completely so rarely produces complete anaerobiasis.

II. Displacement of Oxygen by Other Gases: Candle jar is a popular but ineffective method. Inoculated plates are placed inside large air tight container and a lighted candle kept in it. The burning candle will though use all the oxygen in the jar before it extinguishes but some oxygen is always left behind. These candle jar provides carbon dioxide which stimulates the growth of most Bacteria.

III. Absorption of Oxygen by Chemical or Biological Methods

Chemical Methods: Pyrogallic acid method has been used with various modifications. In a large Buchner’s tube containing NaOH solution, pyrogallic acid is added. This tube is then placed inside an air tight jar and provides anaerobiosis but a small amount of carbon monoxide too may be inhibitory to Bacteria.

Gas-Pak Method: Is commercially available in the form of a disposable packet of aluminium foil containing pellets of sodium borohydride and cobalt chloride and of citric acid and sodium bicarbonate.

After the inoculated plates are kept in the jar, water is added to the disposable aluminium foil packets. Reactions between hydrogen and oxygen tales place in the presence of a catalyst, alumina pellets coated with palladium,
to produce anaerobic environment. The gas-pak is simple and effective eliminating the need for drawing a vacuum and adding hydrogen. It yields a more assured volume of carbon dioxide.

An indicator methylene blue is used to check the absence of oxygen which turns blue on exposure to oxygen but remains colourless in anaerobic conditions.

**Biological Methods:** Absorption of oxygen from small closed system has been attempted by incubation along with Aerobic Bacteria, germinating seeds or chopped vegetables.

Two blood agar plates are inoculated with Aerobic Bacteria and the other with anaerobic specimen. Then these two plates are placed one over the other and are incubated. Slow and ineffective process.

**IV. Displacement and Combustion of Oxygen**

**Anaerobic Jars:** For anaerobic atmosphere for obtaining surface growths of anaerobes.

**McIntosh and Fildes Anaerobic Jars:** It is an instrument used for the generation of anaerobic conditions to culture obligate anaerobe, such as *Clostridium* species. Availability of gas is a major drawback of the system (Refer Figure 7.2).

Consists of a jar of stout glass or metal with a gasket to make it air tight. The lid is furnished with two tubes with valves, one as the gas inlet and the other as the outlet. The lid also has two terminals which can be connected to an electric supply. On the under surface it carries a gauze sachet carrying alumina pellets coated with palladium. It acts as a room temperature catalyst for the conversion of hydrogen and oxygen into water.

**Principle:** It works on the principle of evacuation and replacement, where the air inside the chamber is evacuated and replaced with a mixture of gases. The residual oxygen left behind is the converted to water using Spongy palladium. The catalysing agent is a slow combination of hydrogen and oxygen to form water.

**Procedure:** Inoculated culture plates are placed inside the jar with the medium uppermost and downward lid clamped tight. The outlet tube is connected to a vacuum pump and the air inside is evacuated. The outlet tap is closed and inlet tube is connected to the hydrogen supply. Hydrogen is drawn in rapidly. As the gas ceases through the inlet tap there is a decrease of pressure of at least 20mm Hg after the hydrogen is admitted in while the catalyst is active. Incubate the jar at 37 °C for 48 hours.
Disadvantage: When the plates are removed from the jar, it exposes the colonies to oxygen, which is hazardous to the anaerobes during their first 48 hours of growth. A suitable holding system should be used in conjunction with anaerobic jars, placed in oxygen free holding system, removed for examination of the colonies, and then quickly returned to the holding system.

V. By Reducing Agents

Liquid media soon becomes aerobic unless a reducing agent is added. This is done using,

- Thioglycollate Broth.
- Cooked Meat Broth (CMB): Originally known as Robertson’s cooked meat medium has a nutrient broth with fat free cooked minced meat.

Principle

- Unsaturated fatty acids present in meat utilize oxygen for auto-oxidation catalysed by hematin in the meat.
- Certain reducing substance in meat also utilize oxygen.

It permits the growth of strict anaerobes and indicates their proteolytic activities. It is used for the preservation of stock cultures of aerobic organisms.

VI. Other Anaerobic Culture System

Following are some of the anaerobic culture system:

- **Hungate Procedure**: Surface colonies are grown in roll tubes in which a thin layer of agar coats the inside of the tube. The medium must be
transparent for surface colonies to be visible, and this precludes the use of blood agar.

- **Anaerobic Cabinets:** Anaerobic cabinets are commercially produced for the processing of specimens and the subsequent incubation of cultures and subcultures in an oxygen free atmosphere enriched with 5 to 10% CO2.

The advantage is that all of the processing, including periodic examination of plates and preparation of subcultures, can be done without exposure to oxygen.

### 7.3.1 Culture Methods and Nutritional Types

Usually a bacterial species occurs as one component of a large population containing many other species. To explore the characteristics of one species, it must be isolated as pure culture. This can be further maintained or preserved in a culture collection.

**Purpose of Culturing**

- Isolation of Bacteria from a mixed culture or pathological specimen.
- Demonstration of Bacterial properties.
- Production of antigens.
- Typing of Bacteriophages and Bacteriocins Susceptibility.
- To test for antibiotic sensitivity.
- Estimate viable counts.
- Maintain stock culture.

I. **Serial Dilution Technique:** The dilution of 1:100 is achieved by combining one part of a 1:10 dilution with nine parts of diluent. This technique is used as preliminary step to isolate the microbial population from soil, water and air for its industrial use. With the use of sterile distilled water heavy counts of microorganisms are reduced or diluted and sample of each dilution is plated. This would facilitate the isolation of desirable microorganisms from others by plating the various dilutions on their corresponding media. Basal medium are used for the enrichment of the growth of microorganism isolated from soil. If only a few samples of dilution exhibited growth, it is presumed that some of the colonies started from single cells. This method can only be used to isolate the predominant type of organism in a mixed population.

The bacterial colonies formed on the medium can be calculated:

\[
\text{Colony Forming Units} = \frac{\text{Number of Colonies}}{\text{Vol. of Sample Plated} \times \text{Dilution Factor}}
\]

II. **Streak Plate:** Several streaking patterns can be employed for separation of individual Bacterial cells on agar medium. In the streak plate technique
a loophole of bacterial cells is streaked across the Agar. As the streaking continues, fewer and fewer cells are left on the loop and finally loop may deposit single cells on the agar. The plates are kept for incubation under favourable medium to permit the growth of microorganisms. Well isolated colony is then removed, re-suspended in water and again streaked on an agar. The key to this method is by streaking diluents gradient is established across the medium so that while confluent growth occurs on part of the plates where the bacterial cells are not separated, isolated colonies do develop in another region of the plate. The isolated colonies can be picked using sterile loops and re-streaked on a fresh medium to enhance purity. Leaving growth from a colony or slant if a suspension is not streaked, this method is just as reliable as and faster than the pour plate method (Refer Figure 7.3).

**Fig. 7.3 Streak Plate Technique**

The above Figure 7.3 shows Streak Plate Technique, in which (A) Typical Streaking Pattern; (B) An Example of a Streak Plate.

III. **Spread Plate or Lawn Culture**: A small volume of dilute microbial suspensions placed on the centre of an agar plate and spread evenly over the surface by a sterile glass rod. This helps in separating individual microorganisms from the other organisms in suspension and deposited at a discrete location. The dispersed cells develop into isolated colonies. To enable the number of colonies to be equal to the number of viable organisms in a sample, spread plates can be used to count the microbial population.
Number of Bacteria/ml = Number of Colonies on Plate x Dilution of Sample Reciprocal.

Figure 7.4 shows the viable cell count using serial dilution.

IV. **Pour Plate:** In the pour plate method, a suspension of cell is mixed with melted agar and poured into sterile petri dishes using aseptic technique. When the agar solidifies, the cells are immobilised trapping the Bacteria into discrete positions and grow into colonies, even though the medium holds bacteria in place, it allows growth of Bacteria and formation of discrete isolated colonies.

It is often necessary to dilute the suspension before application to the Agar Plate. In order to prevent overcrowding and the formation of confluent growth being derived from a single cell rather than the desired development of isolated colonies.

Tubes containing 15 ml of agar medium are melted and left to cool in a water bath at 45-50 degree C. Dilutions of the inoculums are added in 1ml. Volume to the molten agar. Contents are then poured in sterile petri dishes and allowed to set. After incubation colonies appear that can be enumerated.
using colony counters. This method is recommended to give an estimate of the viable bacterial count in a suspension and is used for quantitative estimation of urine culture.

V. Stab Culture: This is prepared either to demonstrate biochemical property of the bacterium or for producing stock cultures. It is prepared by puncturing with a long straight charged wire in a suitable medium like nutrient gelatine or glucose agar. Medium is allowed to set with the tube in the upright position, providing flat surface at the top of the medium. The tubes are then stored as stock culture. It is primarily used for demonstration of gelatine liquefaction and oxygen requirement of maintenance of the bacterium under study like the triple sugar iron test. After the maintenance of stock culture the medium is incubated further to infer the result.

Uses

Following are the uses of stab culture:
- To study motility of Bacteria in semisolid agar.
- Maintenance of stock culture.
- Demonstration of gelatine liquefaction.
- To demonstrate oxygen requirement of bacterial study.

VI. Shake Culture: It is made up of melting nutrient agar and then cooling to 45 degree C and inoculating it while in molten form depending on the desired size of the inoculums. Withdraw the pipette or wire and flame the mouth of the tube. Mix the contents of the tube by rotation before the agar solidifies. Incubate it at 37 degree C for 24 hours and look for the growth of organisms.

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**Check Your Progress**

4. What is McIntosh and Fildes anaerobic jars?
5. What is Hungate procedure?
6. What are anaerobic cabinets?

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7.4 PROCESS OF GROWTH OF BACTERIA

In this section, we will discuss about the process of growth of Bacteria.

**Generation Time of Bacteria:** The time required by the bacterium to double under optimum conditions is known as the generation time. It is the time taken by the Bacteria between two cell divisions. This is also referred to as population doubling time. Most Bacteria have a generation time of around 20 minutes. However, there are few slow growing Bacteria, which have longer generation time. For example, Mycobacterium tuberculosis has a generation time of around 20 hours. If the bacterial population doubles every
20 minutes then the population reaches an exponential growth of 10^21 cells in 24 hours provided the nutrients are not exhausted and toxic waste products are removed. In practical situation, Bacteria start dying due to the depletion of nutrients and accumulation of waste or toxic products.

**Bacterial Growth Curve:** Growth of Bacteria is regulated by the nutrition present in the environment. The bacterial growth curve is plotted on the basis of studies in a liquid culture medium or broth culture. It classically has four different phases: Lag Phase, Exponential or Log Phase, Stationary Phase, and Decline Phase.

**I. Lag Phase:** Lag phase is the initial phase characterised by cellular activity but not growth. A small group of cells is placed in the nutrient rich medium immediately after the inoculation of liquid medium.

During the initial **lag phase** there is no apparent growth but biochemical analysis shows metabolic turnover. The cells are in the adapting phase and no new growth will eventually begin. In the bacterial growth cycle this phase marks the synthesis of RNA, Enzymes and other molecules to replicate as they are depleted in older cells. These cells increase in size but no cell division takes place in this phase. The phase last depending on the bacterial species, size of inoculums and conditions of growth medium. This period may last longer if the inoculum is old or the medium very different from the original culture to synthesize new enzymes.

The length of lag phase varying considerably. Actively growing cells transferred from one type of media into the same type of media, with the same environmental conditions, will have the shortest lag period.

After this there is a transient acceleration phase as the inoculums begins to grow followed by an exponential phase.

**II. Exponential Phase/Log Phase:** In the exponential phase, microbial growth proceeds at the maximum possible rate for the organism with nutrients in excess and with growth inhibitors absent as the ideal environment parameters. This period is characterised by cell manipulation and predictable doubling of the population. This growth cannot continue indefinitely. The number of new bacteria appearing per unit time is proportional to the present population. Metabolic activity is high as DNA, RNA, cell wall components are generated for division.

Conditions that are optimal for the cells will result in very rapid growth and a steeper slope on the growth curve, while less than ideal conditions will result in slower growth. Cells in exponential growth are the healthiest and most uniform chemically and physiologically, best utilized cells from this phase. Though at high nutrient levels the transport systems are saturated, the growth rate does not rise further with increasing nutrient concentration. Figure 7.5 shows a graphical overview of exponential growth curve and logarithmic growth curve.
III. Stationary Phase: The population growth experienced in the log phase begins to decline as the available nutrients become depleted and waste products start to accumulate. Bacterial cells reaches a plateau where the number of dividing cells equals the number of dying cells.

Physiologically the cells become different at this stage as they try to adapt to the new condition of running out of essential nutrients or its growth is inhibited by its own waste products. The new cells produced are smaller in size with bacilli becoming almost spherical in shape equal to the number of cells dying off resulting in a flattening out of growth on the curve.

Their changes in plasma membrane becoming more permeable and the nucleoid are designed to allow the cells to survive for a longer period of time in adverse conditions. They are in a state waiting for the optimal conditions to return, such as infusion of nutrients.

Cells are also prone to produce secondary metabolites, such as antibiotics. Cells capable of making endospores will activate the necessary genes initiating the sporulation process.

- Closed system population growth eventually ceases, total number of viable cells remains constant.
- Active cells stop reproducing or reproductive rate is balanced by death rate.

![Graphical Overview of Exponential Growth Curve and Logarithmic Growth Curve](image)
Cell division begins to slow down and overall number of cells does not increase.

**Possible Reason for Stationary Phase**

- Nutrient Limitation
- Limited Oxygen Availability
- Toxic Waste Accumulation
- Critical Population Density Reached

**IV. Death Phase:** During death phase, i.e., the last phase population size begins to decrease as number of cells dying more than number of cells produced and the cells lose their ability to divide.

In the last phase the number of viable cells decrease in a predictable fashion. The steepness of the curve corresponds to how fast the viability of the cells is lost. The conditions of the culture medium is deteriorated where the cells are irreparably harmed. The cells fail to show growth when transferred to fresh medium. Presence of autolytic enzymes, accumulation of toxic wastes and depletion of nutrients are all responsible for this decline phase. The bacteria develops involution forms and the number of dying cells continue to rise. Although most of the microbial population dies in the algorithm fashion, the death rate may decrease after the population has drastically reduced. This is due to extended survival of resistant cells. For a cell population complete death of cells is unlikely, as the cell mutates to adapt to their harsh environmental conditions. The small population of the cells cannot be killed and may benefit from the death of their fellow cells. They provide nutrients to the environment as they lyse and release their cellular contents. This is demonstrated by pathogens, where they enter a state of very low metabolism and lack of cellular division, only to resume growth later when conditions improve.

**7.4.1 Measurement of Growth**

An orderly increase in the quantity of cellular constituents is growth. As shown above, it depends upon the ability of the cell to form new protoplasm from nutrients available in the environment. For unicellular organisms, such as the bacteria, growth can be measured in terms of two different parameters. It can be done by recording the changes in cell mass and measuring the changes in cell numbers.

**Measurement of Cell Mass:** This can be done directly by measuring the dry weight, wet weight or volume of cells or measuring the chemical content. Indirect measurement is done by measuring the chemical activity. These methods are as follows:
• Direct physical measurement of dry weight, wet weight, or volume of cells. This is done after centrifugation of the culture so as to concentrate the bacterial cells.

• Direct chemical measurement of some chemical component of the cells, such as total protein or total DNA content.

• Indirect measurement of chemical activity can be done to assess the growth. Activity, such as rate of ATP or oxygen production or consumption, carbon dioxide production or consumption, etc., can be measured.

• Growth of Bacteria results in the turbidity of the medium. The turbidity or optical density of a suspension of cells is directly related to cell mass or cell number, after the construction and calibration of a standard curve. Turbidity measurement can be done by a variety of instruments to determine the amount of light scattered by a suspension of cells. Particulate objects, such as Bacteria scatter light in proportion to their numbers. The method is simple and non-destructive, but the sensitivity is limited to about 10⁷ cells per ml for most Bacteria.

Measurement of Cell Count: Various methods of measuring cell count involve direct counts and indirect viable cell counts. This can be done visually or by using instruments, such as cell counters. These methods are as follows:

• Direct microscopic counts are possible using special slides known as counting chambers. It is an easy method requiring no special equipment. However, it has certain disadvantages. It counts both dead and living cells, as the dead cells cannot be distinguished from living ones. Also, only dense suspensions can be counted (>10⁷ cells per ml). The samples can be concentrated by centrifugation or filtration to increase sensitivity.

• Electronic counting chambers count numbers and measure the size distribution of cells. In order to use such counting devices, the suspending medium must be very clean. Such electronic devices are more often used to count eukaryotic cells, such as blood cells. Flow cytometry is also used to count the cells.

• Indirect viable cell counts are also known as plate counts. It involves plating out or spreading a known quantity of a culture on a nutrient agar surface. The sample or cell suspension can be diluted in nontoxic diluents, such as water or saline before plating. If plated on a suitable medium, each viable unit grows and forms a colony. Each colony that can be counted is called a Colony Forming Unit (CFU) and the number of CFUs is related to the viable number of bacteria in the sample. This method is very advantageous as it is highly sensitive and allows for inspection and positive identification of the organism counted. The disadvantages are that only living cells can develop colonies so only these are counted. Also, as colonies develop only from those
organisms for which the cultural conditions are suitable for growth, the non-growing bacterial cells may be missed. This makes the technique virtually useless to characterize or count the total number of bacteria in complex microbial ecosystems, such as soil or gastrointestinal tract. This method is useful to count the number of bacterial cells in clinical samples, such as urine. Another disadvantage is that bacteria take time to form colonies. Most of the bacteria produce visible colonies on overnight incubation at optimum temperature. Some bacteria may take longer time to grow. This can delay the count.

**Growth Phases:** Bacteria are Prokaryotic organisms that replicate by the asexual process of binary fission. These microbes reproduce rapidly at an exponential rate under favourable conditions. When grown in culture, a predictable pattern of growth in bacterial population occurs. This pattern can be depicted as the number of living cells in a population over time as bacterial growth curve.

Bacterial growth curve represents the number of live cells in bacterial population over a period of time in liquid culture medium or broth culture. Bacteria require certain conditions for growth, such as oxygen, pH, and temperature and light influence microbial growth. The species that populate an environment change over time. Nutrition in the environment regulates the growth of bacteria. A bacterial populations generation time varies between species and depends on how well growth requirements are met.

**Bacterial Growth Rates**

However, in batch cultivations exponential growth is of limited duration as nutrient conditions change, growth rate decreases entering declaration phase, to be followed by stationary phase, when overall growth can no longer be obtained owing to nutrient exhaustion.

Due to the predictability of growth in this phase, the events can be used to calculate the time it takes for the bacterial population to double in number, known as generation time \((g)\). If one knows the cell concentration at the start of the exponential phase of growth and the cell concentration after some period of time of exponential growth, the number of generations can be calculated.

The fixed relationship between \(N_0\) initial number of cells and the start of the exponential phase and \(N\) final cell concentration after a certain period of time, and \(n\) is the number of generations that occurred between the specified periods of time. Generation time \((g)\) can be represented by \(t/n\) where \(t\) is the specified time. The slow rate of cell death is compensated by the formation of new cells by growth. The total count continues to increase while the viable count starts falling. During this phase bacteria stains irregularly, accumulating toxic metabolites represented by constant straight line on growth curve. This
reflects the change in rate of bacterial growth is equal to the change in rate of bacterial death. Figure 7.6 shows the bacterial growth in batch culture that can be modelled with four different phases.

Unicellular growth occurs in a series of following different phases:

A. **Lag Phase**
   - It marks the period of adaptation to the new surroundings.
   - During this phase there is no increase in bacterial cells.
   - However this is phase of intense metabolic activity in which individual organism grows in size.
   - Cell synthesising new components to replenish spent materials.

B. **Exponential Phase:** Exponential growth cannot continue indefinitely. It slows down due to accumulation of metabolic toxic products and the exhaustion of supply of nutrients.
   - When the Bacteria have acclimatised to their new environment and synthesised the enzymes needed to utilise the available substrates.
   - The population of cells double in constant length of time known as generation time so both the number of cells and rate of population increase doubles with consecutive time period.
   - It is a period of rapid growth where Rate of growth and division is maximum and constant and natural logarithm of cell number plotted against time produces a straight line.
   - Cells begin to divide and generation time reaches a constant minimum number of cells produce is more than the number of cells dying.
   - Cells are more susceptible to adverse environmental factors like radiations and antibiotics.
Growth Kinetics

When microorganisms are exposed to any treatment they do not die instantaneously. Only a certain proportion will die during each given period.

A plot of the number of surviving Bacteria cell against the time of exposure gives a graph as shown in Figure 7.7 ((a) and (b)). The curve is exponential theoretically, there will never be zero survivors but will have less than one cell after a while, thus in a given unit volume there will be one in 10 chance of there being a cell present. The longer it takes to eliminate the more no; of cells present. The steepness of the slope shows effectiveness of heat sterilisation. D value applies to a particular temperature and is the time needed to reduce the population by a factor of ten in a heat treatment. Rate of killing advance at higher temperature so D value is reduced. Critical factor is the death rate of most resistant species. Sterilisation protocols should therefore be based on the rate of destruction of endospores.

*Fig. 7.7 Plot of the Number of Surviving Bacteria Cell against the Time*
The batch culture growth model shows change in the growing environment because of decreasing metabolism and cell lysis. The gradual consumption of medium and production of metabolites influences the growing cells. Thus in real situation these four phases are not well defined. The number of living cells decrease by the same proportion giving an exponential curve often never a constant rate. This is seen as a straight line, slope of which is a reflection of slow rate of decaying. In Figure 7.7, the total period under point SA reflects the pressure on the cells to go dormant and the probability to survive. The time period between A and A' is the reduction time D.

7.4.2 Microbial Culture Collection

Microorganisms are cultured in different ways to get the desired product depending on the different type of microbial system, for example, one can get entirely two different products from the same organism by changing the nutritional or the culturing system.

**Batch Culture:** It is a closed system. After the inoculation of sterile nutrient and cultivation different phases of growth are observed. The production of metabolites in the growing medium keeps on changing due to the gradual consumption.

**Advantages**

Following are the advantages of batch culture:
- Higher raw material conversion level.
- Reduced risk of cell mutation as the growth period is short.

**Disadvantages**

Following are the disadvantages of batch culture:
- Lower productivity level due to time for heating, sterilisation, cooling.
- Increased focus on instrumentation due to frequent sterilisation.

**The Continuous Culture of Microorganisms**

Growth in an open system:
- Continual provision of nutrients.
- Continual removal of wastes.
- Maintains cells in log phase at a constant biomass concentration for extended periods.
- Achieved using a continuous culture system.

**Importance:** Constant supply of cells in exponential phase at a known rate. Study of microbial growth at very low nutrient concentrations, close to those present in natural environment. Study of interactions of microbes under conditions resembling the aquatic environments.
**The Chemostat:** Uses dilution rate and nutrient concentration to control growth and population density. Growth rate and yield by adjusting limiting nutrient can be controlled independently of each other. Compared to batch culture it allows:

- To maintain population in exponential phase at a known growth rate for long periods of time.
- Can study microbial growth at very low nutrient concentrations.

When in a steady state a system is in equilibrium, cell number and nutrient status remain constant. The bacterial cells grow at the same rate at which bacterial cells are removed by the overflow. The rate of addition of the fresh medium determines the rate of growth because the fresh medium contains a limiting amount of essential nutrient.

Thus the chemostat removes the inefficiency of nutrients, the accumulation of toxic substances and the accumulation of excess cells in the culture. These factors initiate the stationary phase of growth cycle. If the chemical environment can be constant in a chemostat continuous culture, the cell density is constant in a turbidostat culture, which is also a continuous culture.

In a chemostat, in a steady state adjusting the concentration of one substrate controls the cell growth. In the turbidostat cell growth is kept steady by using turbidity to monitor the biomass concentration and the rate of feed on nutrient solution is adjusted.

**Fig. 7.8 Bacterial Growth Observation Setup**
7.3.3 Factors Affecting Growth

Following are the few factors that affect the growth of Bacteria:

I. Chemical Factors

- Nutrients are substances used in biosynthesis and energy release and are therefore required for growth.
- Chemical factors are supplied by:
  (i) The culture medium (plural - media) that contains substrates required for growth.
  (ii) Culture conditions, i.e., aerobic vs anaerobic conditions.
- Macro and Major elements include C, O, H, N, S, P, K, Ca, Mg, and Fe required in large amounts by the cell. Almost 95% of cells are composed of macromolecules, sometimes called macronutrients C, O, H, N, S, P are components of macromolecules.
  - Carbon: Two carbon dependent types are noted as autotrophs and heterotrophs.
  - Nitrogen: This is an essential atom for proteins and nucleic acids. Protein serve as structural and functional molecule enzymes responsible for metabolic activity of the cell. Some microbes thrive on atmospheric nitrogen.
  - Phosphorus: Component of phospholipids and nucleic acids, nucleotides, such as ATP, some proteins. Available as organic and inorganic forms in the environment.
  - Sulphur: Structural role in methionine and cysteine as well as a number of vitamins (thiamine, biotin), coenzyme A and some carbohydrates available usually from inorganic sources SO4. H2S and organic sulphur compounds, such as cysteine K, Ca, Mg, and Fe are cations in cells and required for a variety of roles, for example, cofactors (K⁺, Ca²⁺, Mg²⁺, and Fe²⁺ or Fe³⁺).
    - Stabilize membranes and ribosomes (Mg²⁺)
    - Contribute to heat resistance of endospores (Ca²⁺)
    - Components of biomolecules, such as cytochromes (Fe²⁺ and Fe³⁺)
  - Trace Elements or Micronutrients
    - Required in lesser or trace amounts.
    - Critical to cell function.
    - Many are metals. Structural role with many enzymes and cofactors.
    - Often trace elements present in medium components or water provide all that is required for growth, Co, Cu, Mn, Mo, Ni, and Zn are needed by most cells.
- Some cells require Cr, Se, W, and V.

0 **Oxygen**

**Aerobic Organisms:** Growth at full atmospheric O2 tensions (21% O2 in the atmosphere). Facultative organisms (under appropriate nutrient and culture conditions) can grow under either aerobic or anaerobic condition. Obligate aerobes require O2 for growth. O2 is poorly soluble, forced aeration is often used in culture systems to provide.

**Anaerobic Organisms:** Obligate (strict) anaerobes, grow only in the absence of O2; sensitive to O2 and brief exposure. Will kill these organisms; perhaps because these organisms are unable to detoxify some of the products of O2 metabolism. Lack a respiratory system and cannot use oxygen as a terminal electron acceptor. These organisms do use oxygen found in cellular materials.

### II. Temperature Ranges for Microbial Growth

Microorganisms vary widely in their range of temperature over which growth is possible. Growth is slower at lower temperatures because enzymes work less efficiently, and loss of membrane fluidity. The optimum and limiting temperatures for an organisms are a reflection of temperature range of its enzyme system. The optimum temperature being closer to the maximum growth temperature. Figure 7.9 illustrates the effect of temperature on microbial growth rate.

Microbes cannot regulate their internal temperature. Enzymes have optimal temperature at which they function optimally. High temperature may inhibit enzyme functioning, organism exhibit distinct cardinal growth temperatures.

- Minimal
- Maximal
- Optimal

Thus psychrophiles are adapted to cool environments by the virtue of their large fatty acids below 0 degrees in their plasma membrane.

![Fig. 7.9 Effect of Temperature on Microbial Growth Rate](image)

*Fig. 7.9 Effect of Temperature on Microbial Growth Rate*
The above Figure 7.9 shows the effect of temperature on microbial growth rate for five environmental classes of Prokaryotes. The factors governing the minimum, optimum and maximum temperatures for a particular organism are indicated. The curve is asymmetrical, with the optimum temperature being closer to the maximum than the minimum.

These reactions are enzyme catalysed reaching a point of optimum temperature where there is rapid increase in the rate of inactivation of heat sensitive cell components like enzymes, DNA, ribosomes. Above this optimum heat denaturation will occur rapidly with a corresponding drop in the rate of growth.

III. Effect of Oxygen: The microbes which require oxygen are termed as aerobes.

Oxygen Concentration: The microbes which require oxygen as terminal electron acceptor for oxidation, as the only means of energy production, the organism will be strict aerobe.

**Obligate Aerobes:** Require O$_2$ for growth for ETS chain in aerobic respiration.

**Obligate Anaerobes:** O$_2$ is toxic substance, which either kills or inhibits their growth. Obligate anaerobic prokaryotes may live by fermentation, bacterial photosynthesis.

**Facultative Anaerobes** are organisms that can switch between aerobic and anaerobic type of metabolism. Under anaerobic conditions they grow by fermentation or anaerobic respiration, but in the presence of oxygen they switch to aerobic respiration.

The response of an organism to O$_2$ in its environment relies on the distribution of various enzymes and oxygen radical that are generated by the cells in its presence. All cells possess enzymes capable of reacting with oxygen.

All organisms which can live in presence of O$_2$ contain superoxide dismutase which prevents the accumulation of lethal superoxide. Nearly all organism contain the enzyme catalase, which decomposes H$_2$O$_2$. These
enzymes detoxify oxygen radicals generated by living system in the presence of oxygen. The distribution of these enzymes in the cell determine their ability to exist in the presence of oxygen. Figure 7.11 shows the action of superoxide dismutase, catalase and peroxidase.

IV. Water Availability: Availability of water is a critical factor that affects the growth of all cells. The availability of water for a cell depends upon its presence of relative humidity or the presence of solution or substance (water activity). Water activity is affected by presence of solutes, such as sugars that are dissolved in water.

V. Osmotic Effects on Growth: Microbes require water to grow, their cells are 80 – 90% water. Water activity (aw) - amount of water that is free to react = availability of water in a substance. Most Bacteria require a water activity of 0.9 for active metabolism. Most organisms are adversely affected by very low water activity.

Halophiles: Grow optimally in the presence of NaCl or other salts at a concentration above about 0.2M. Extreme halophiles require salt concentrations of potassium, cell wall, proteins and plasma membrane require high salt to maintain stability and activity. Microorganisms that row optimally in the presence of NaCl are called halophiles. Figure 7.12 illustrates a graphical overview of NaCl concentration and growth rate.
The environmental osmotic pressure is estimated by solute concentration in the environment. It is inversely related to water activity. A true halophile survives best at salt concentrations where most Bacteria are inhibited. Increased solute concentration means decreased osmolality.

The organisms that survive in extreme conditions of high sugar are called osmophiles. While the ones that live in dry environments due to lack of water are known as xerophiles. This principle is used to prevent bacterial growth in preservation of food by drying or high concentration of sugars. Figure 7.13 shows the growth rate vs. osmolality for different classes of Prokaryotes.

![Figure 7.13](image)

**Fig. 7.13** Growth Rate vs. Osmolality for Different Classes of Prokaryotes

**VI. Effect of pH:** Microbes grow at a wide pH range. Most grow best at neutrality are called neutrophiles. Bacteria usually slightly on an alkaline side called alkaliphiles, for example, algae and fungi on the acid side. However some can grow at extreme high and low value pH. Microorganisms which grow at an optimum pH below neutrality are called acidophiles.

- **Neutrophiles:** pH optimum between 5.5 and 8. Most Bacteria grow well within the pH range of 6 – 9.
- **Alkaliphiles:** Prefer growth under alkaline conditions (pH 8.0 to 11.5) many produce enzymes that work well at high pH – useful for the detergent industry.
- **Acidophiles:** Restricted to growth at low pH values – between 0 and 5.5
  - Fungi are generally more acid tolerant than Bacteria – many grow at pH 4 to 6.
  - Some Bacteria and Archaea are obligate acidophiles, for example, Bacteria – *Thiobacillus*, Archaea – *Sulfolobus*.

Figure 7.14 shows the growth rate vs. pH for three environmental classes of Prokaryotes.
In the preparation of culture media, one must always consider the optimum pH for growth of a desired organism and incorporate buffers in order to maintain the pH of the medium. This is essential as the bacterial waste products accrue during growth. The pH has an important effect on stability of acidophile plasma membrane.

**Intracellular pH**

- Intracellular pH is usually between pH 6 to 8, but the internal pH as low as 4.6 and as high as 9.5 have been measured.
- Maintained by pumping H+ across the membrane, internal buffering and synthesizing new proteins, for example acid shock proteins and heat shock proteins that function by pumping protons or acting as chaperones.

**7.4.4 Culture of Bacteria in Laboratory**

**Culture Media**

Defined Media also known as Synthetic media, is a minimal media that provides only the exact nutritional requirement and the growth factors needed by the organism for growth. Different organisms can be grown optimally on specific defined media.

Chemically defined media helps in studying the nutritional requirement like glucose, amino acids which will supply the needed carbon and energy factors required for enrichment cultures.

- **Fastidious Media**: It is required for those organisms that need to have large numbers of growth factors within their media, for example, *Neisseria gonorrhoea* and *Streptococcus* species.
- **Differential Media**: A differential media formulation distinguishes between different types of Bacteria based on some observable traits in the pattern of growth on the medium. Changes in the media appear by

![Fig. 7.14 Growth Rate vs. pH for Three Environmental Classes of Prokaryotes](image-url)
the end products released by Bacteria during the time they are growing on the media.

- **Selective Media**: It has a component added to it which will inhibit or prevent the growth of certain types or species of Bacteria and promote the growth of desired species. It can allow gram negatives to grow but inhibits gram positives and vice versa (Refer Figure 7.15).

![Selective Media: Geomyces destructans in Culture from Bat Tissues](Fig. 7.15)

The above Figure 7.15 illustrates selective media: *Geomyces destructans* in Culture from Bat Tissues. (2A) Original Culture Tubes of Sabouraud Agar Supplemented with Nine Antibiotics and incubated at 4°C for Six- or Eight-Weeks; notice the profuse Growth of *Geomyces destructans* Strains. (B) Some Fungal Contamination on Individual Isolates was visible as depicted in the close-up of a Culture Tube. (C) Enrichment and Recovery of Pure Fungal Colonies by treating a culture contaminated with Bacteria with Hydrochloric Acid (HCl).

- **Complex Media**: It is one in which the exact chemical amounts are not known but has a variety of nutrients as well as growth factors. Examples of complex media are TSA and Nutrient Broth.

- **Anaerobic Media**: Obligate anaerobes need a media that will protect them from free oxygen.

- **A Reduced Media**: It is one that contains Thioglycollate, which will bond with free oxygen and remove it from the media. Heat is used to drive the bonded oxygen before inoculating the Bacteria into the media.

- **Low Oxygen Cultures**: They are one that prefers as little as oxygen as possible. They prefer a higher percentage of CO2. These types are
used to mimic the environment of the body tissues or respiratory and intestinal tracts.

**Kinetics of Microbial Growth**

Prokaryotes divides by binary fission with each cell that grows to a full size. Before dividing itself into two identical cells its genetic material replicates itself leading to an exponential increase in cell numbers due to increase in cellular constituents. If we plot number of cells in a population against time we would get an exponential curve in. Figure 7.16 shows the graphical overview of temperature vs. growth curve.

![Graphical Overview of Temperature vs. Growth Curve](image)

**Fig. 7.16  Graphical Overview of Temperature vs. Growth Curve**

**Growth Generation Time of Microorganisms**

The binary fission that occurs during the exponential phase requires time by a cell to divide called the generation time or doubling time. In the laboratory under favourable conditions, a growing bacterial population doubles at regular intervals. Growth is by geometric progression. $1, 2, 4, 8, \text{ etc.},$ or $20, 21, 22, 23\ldots\ldots2n$ (where $n = \text{the number of generations}$). This is called exponential growth or logarithmic.

When growing exponentially by binary fission the increase in bacterial population is by geometric progression. If we start with one cell, when it divides there are 2 cells in the first generation, 4 in the second generation, 8 cells in the third generation, and so on.
Measurement of Microbial Growth

Either population number or mass may be calculated and growth leads to both.

- **Direct Measurement of Cell Numbers:** Bacteria can be counted directly on the plate and also called plate counting. Advantage being that the number of viable cells are counted, since conditions of the media need to be maintained it is time consuming and expensive. Bacteria counted are referred to as colony forming units as a single cell or a clump of bacterial cells giving rise to a colony with many cells. In order to get accuracy one must resort to serial dilution method.

- **Most Probable Number (MPN):** It is a statistical method where a sample is diluted repeatedly and inoculate several broth tubes for each dilution point. Count the number of positive tubes in each set. This determines high possibility that a bacterial population falls within a certain range.

- **Direct Microscopic Count:** A specific vol. of bacterial suspension is placed on a microscope with special grid. Stain is added to visualise Bacteria. Cells are counted and multiplied by a factor to obtain concentration

**Advantages**

No incubation time required.

**Disadvantages**

Cannot always distinguish between live and dead Bacteria

Motile Bacteria is difficult to count

Requires a high concentration of Bacteria

- **Membrane Filtration:** This method is used to study if the quantity of Bacteria is very small for example, in aquatic samples and uses membranes with different pore sizes to trap them. The sample is drawn through special membrane filters and placed on the agar medium. The number of colonies produced can be counted after incubation. Selective media or differential media can be used for specific microorganisms.

- **Microscopic Count:** The disadvantage encountered in this method is that fairly large volume is required and also it is difficult to distinguish between living and dead cells. This method is popular for its accuracy with larger cells and used extensively to count red and white blood cells (Refer Figure 7.17).
Check Your Progress

7. Define lag phase.
8. What is exponential phase?
9. What are some of the possible reason for stationary phase?
10. What is a batch culture?

7.5 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Axenic is a culture medium or biological culture that contains only a single species or organism strain and is uncontaminated from other microorganism.

2. The contaminants usually encountered are microorganisms, axenic cultures, whether of vertebrates or invertebrates are often referred to as germ-free. In addition, the term gnotobiotic is often used interchangeably with axenic.

3. Synchronized culture is a culture of bacterial or animal cells in which all cells are in the same phase of cell division.

4. McIntosh and Fildes anaerobic jars is an instrument used for the generation of anaerobic conditions to culture obligate anaerobe, such as Clostridium species. Availability of gas is a major drawback of the system.

5. Hungate procedure is surface colonies are grown in roll tubes in which a thin layer of agar coats the inside of the tube. The medium must be transparent for surface colonies to be visible, and this precludes the use of blood agar.
6. Anaerobic cabinets are commercially produced for the processing of specimens and the subsequent incubation of cultures and subcultures in an oxygen free atmosphere enriched with 5 to 10% CO₂.

7. Lag phase is the initial phase characterised by cellular activity but not growth. A small group of cells is placed in the nutrient rich medium immediately after the inoculation of liquid medium.

8. In the exponential phase, microbial growth proceeds at the maximum possible rate for the organism with nutrients in excess and with growth inhibitors absent as the ideal environment parameters. This period is characterised by cell manipulation and predictable doubling of the population.

9. Some of the possible reason for stationary phase are as follows:
   - Nutrient Limitation
   - Limited Oxygen Availability
   - Toxic Waste Accumulation
   - Critical Population Density Reached

10. Batch Culture is a closed system. After the inoculation of sterile nutrient and cultivation different phases of growth are observed. The production of metabolites in the growing medium keeps on changing due to the gradual consumption.

### 7.6 SUMMARY

- Axenic is a culture medium or biological culture that contains only a single species or organism strain and is uncontaminated from other microorganism.

- Earlier, only Bacteria or unicellular Eukaryotes were cultured. However, multicellular organism can also be cultured in axenic.

- Axenic culture is a significant tool that helps in studying parasitic and symbiotic organism in a controlled environment.

- The growth and maintenance of a single species in isolation, free from foreign or contaminating species is axenic culture.

- Isolation in axenic culture is achieved usually by growing the species in an environment that was sterilised previously from contaminating organisms.

- Axenic culture used as a diagnostic tool that helps in studying symbiotic and parasitic organisms in a controlled environment.

- The study of the progressive changes is difficult when the cells are randomly distributed with respect to age.
• Synchronized culture is a culture of bacterial or animal cells in which all cells are in the same phase of cell division.
• A synchronous population can be selected from a random population by separation of cells that are at the same stage of development.
• A popular procedure is to cause the random population to accumulate beginning of the S phase by preventing the synthesis of DNA.
• Pyrogallic acid, this method has been used with various modifications. In a large Buchner’s tube containing NaOH solution, pyrogallic acid is added.
• Gas-pak method is commercially available in the form of a disposable packet of aluminium foil containing pellets of sodium borohydride and cobalt chloride and of citric acid and sodium bicarbonate.
• Biological methods is absorption of oxygen from small closed system has been attempted by incubation along with aerobic Bacteria, germinating seeds or chopped vegetables.
• The time required by the bacterium to double under optimum conditions is known as the generation time. It is the time taken by the Bacteria between two cell divisions.
• If the bacterial population doubles every 20 minutes then the population reaches an exponential growth of 1021 cells in 24 hours provided the nutrients are not exhausted and toxic waste products are removed.
• Growth of Bacteria is regulated by the nutrition present in the environment. The bacterial growth curve is plotted on the basis of studies in a liquid culture medium or broth culture.
• Lag phase is the initial phase characterised by cellular activity but not growth. A small group of cells is placed in the nutrient rich medium immediately after the inoculation of liquid medium.
• In the exponential phase, microbial growth proceeds at the maximum possible rate for the organism with nutrients in excess and with growth inhibitors absent as the ideal environment parameters.
• The population growth experienced in the log phase begins to decline as the available nutrients become depleted and waste product start to accumulate.
• Bacterial cells reaches a plateau where the number of dividing cells equals the number of dying cells.
• Direct microscopic counts are possible using special slides known as counting chambers. It is an easy method requiring no special equipment.
• Electronic counting chambers count numbers and measure the size distribution of cells.
Indirect viable cell counts are also known as plate counts. It involves plating out or spreading a known quantity of a culture on a nutrient agar surface.

Bacteria are Prokaryotic organisms that replicate by the asexual process of binary fission. These microbes reproduce rapidly at an exponential rate under favourable conditions.

Due to the predictability of growth in this phase, the events can be used to calculate the time it takes for the bacterial population to double in number, known as generation time (g).

Microorganisms are cultured in different ways to get the desired product depending on the different type of microbial system, for example, one can get entirely two different products from the same organism by changing the nutritional or the culturing system.

The production of metabolites in the growing medium keeps on changing due to the gradual consumption.

Facultative anaerobes are organisms that can switch between aerobic and anaerobic type of metabolism.

Availability of water is a critical factor that affects the growth of all cells. The availability of water for a cell depends upon its presence of relative humidity or the presence of solution or substance (water activity).

Water activity is affected by presence of solutes, such as sugars that are dissolved in water.

The organisms that survive in extreme conditions of high sugar are called osmophiles. While the ones that live in dry environments due to lack of water are known as xerophiles.

Microbes grow at a wide pH range. Most grow best at neutrality are called neutrophiles.

Bacteria usually slightly on an alkaline side called alkaliphiles, for example, algae and fungi on the acid side. However some can grow at extreme high and low value pH.

Microorganisms which grow at an optimum pH below neutrality are called acidophiles.

The binary fission that occurs during the exponential phase requires time by a cell to divide called the generation time or doubling time.

### 7.7 KEY WORDS

- **Axenic**: It is a culture medium or biological culture that contains only a single species or organism strain and is uncontaminated from other microorganism.
• **Synchronous culture**: Synchronized culture is a culture of bacterial or animal cells in which all cells are in the same phase of cell division.

• **Complex media**: It is one in which the exact chemical amounts are not known but has a variety of nutrients as well as growth factors.

• **Generation time**: The time required by the bacterium to double under optimum conditions is known as the generation time.

• **Facultative anaerobes**: Facultative anaerobes are organisms that can switch between aerobic and anaerobic type of metabolism.

• **Osmophiles**: The organisms that survive in extreme conditions of high sugar are called osmophiles.

• **Xerophiles**: The organisms that live in dry environments due to lack of water are known as xerophiles.

• **Acidophiles**: Microorganisms which grow at an optimum pH below neutrality are called acidophiles.

### 7.8 SELF ASSESSMENT QUESTIONS AND EXERCISE

**Short Answer Questions**

1. Describe axenic culture and its applications.
2. What is synchronous culture?
4. Write about culture methods and nutritional types.
5. How can you measure the growth of Bacteria?
6. What is growth phase?

**Long Answer Questions**

1. Explain in detail about axenic and synchronous culture giving appropriate examples.
2. Discuss about aerobic and anaerobic culture giving appropriate examples.
3. Describe culture methods and nutritional types.
4. Explain the process of growth of Bacteria in detail.
5. Discuss about the factors that affect the growth of Bacteria.
6. Discuss in detail the cultivation of Bacteria in the laboratory.
7.9 FURTHER READINGS


8.0 INTRODUCTION

A prokaryote is a unicellular organism that lacks a membrane-bound nucleus, mitochondria, or any other membrane bound organelle. Prokaryotes are divided into two domains, Archaea and Bacteria. Species with nuclei and organelles are placed in the third domain, Eukaryota. Prokaryotes reproduce without fusion of gametes. The first living organisms are thought to have been prokaryotes.

In the prokaryotes, all the intracellular water-soluble components (proteins, DNA and metabolites) are located together in the cytoplasm enclosed by the cell membrane, rather than in separate cellular compartments. Bacteria, however, do possess protein-based bacterial micro-compartments, which are thought to act as primitive organelles enclosed in protein shells. Some prokaryotes, such as cyanobacteria, may form large colonies. Others, such as myxobacteria, have multicellular stages in their life cycles.

Molecular studies have provided insight into the evolution and interrelationships of the three domains of biological species. Eukaryotes are organisms, including humans, whose cells have a well-defined membrane-bound nucleus (containing chromosomal DNA) and organelles. The division between prokaryotes and eukaryotes reflects the existence of two very different levels of cellular organization. Distinctive types of prokaryotes include extremophiles and methanogens; these are common in some extreme environments.

In this unit, you will study about Prokaryotic cell, its structure and organisation in detail.
8.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand what Prokaryotic cell is
- Describe Prokaryotic cell – function, structure and organisation

8.2 PROKARYOTIC CELL STRUCTURE AND FUNCTIONS

Prokaryotic cells are cells that do not have a true nucleus or most other cell organelles. Organisms that have prokaryotic cells are unicellular and called Prokaryotes. Bacteria and archaea are Prokaryotes. Prokaryotic cells can be contrasted with eukaryotic cells, which are more complex. Eukaryotic cells have a nucleus surrounded by a nuclear membrane and also have other organelles that perform specific functions in the cell.

Prokaryotic Cell Structure

Prokaryotic cells do not have a true nucleus that contains their genetic material as Eukaryotic cells do. Instead, Prokaryotic cells have a nucleoid region, which is an irregularly-shaped region that contains the cell’s DNA and is not surrounded by a nuclear envelope. Some other parts of Prokaryotic cells are similar to those in Eukaryotic cells, such as a cell wall surrounding the cell (which is also found in plant cells, although it has a different composition). Like eukaryotic cells, Prokaryotic cells have cytoplasm, a gel-like substance that makes up the ‘filling’ of the cell, and a cytoskeleton that holds components of the cell in place. Both Prokaryotic cells and Eukaryotic cells have ribosomes, which are organelles that produce proteins, and vacuoles, small spaces in cells that store nutrients and help eliminate waste. Some Prokaryotic cells have flagella, which are tail-like structures that enable the organism to move around. They may also have pili, small hair-like structures that help Bacteria adhere to surfaces and can allow DNA to be transferred between two prokaryotic cells in a process known as conjugation. Another part that is found in some Bacteria is the capsule. The capsule is a sticky layer of carbohydrates that helps the bacterium adhere to surfaces in its surroundings.

Morphology

Prokaryotic cells have various shapes; the four basic shapes of Bacteria are:

- Cocci: Spherical
- Bacilli: Rod-Shaped
- Spiriochaete: Spiral-Shaped
- Vibrio: Comma-Shaped
- The Archaea Haloquadratum has Flat Square-Shaped Cells
Reproduction

Bacteria and Archaea reproduce through asexual reproduction, usually by binary fission. Genetic exchange and recombination still occur, but this is a form of horizontal gene transfer and is not a replicative process, simply involving the transference of DNA between two cells, as in Bacterial conjugation.

Classification

In 1977, Carl Woese proposed dividing Prokaryotes into the Bacteria and Archaea (originally Eubacteria and Archaebacteria) because of the major differences in the structure and genetics between the two groups of organisms. Archaea were originally thought to be extremophiles, living only in inhospitable conditions, such as extremes of temperature, pH, and radiation but have since been found in all types of habitats. The resulting arrangement of Eukaryota, also called Eucarya, Bacteria, and Archaea is called the three-domain system, replacing the traditional two-empire system.

Evolution

A widespread current model of the evolution of the first living organisms is that these were some form of Prokaryotes, which may have evolved out of protocells, while the Eukaryotes evolved later in the history of life. Some authors have questioned this conclusion, arguing that the current set of Prokaryotic species may have evolved from more complex Eukaryotic ancestors through a process of simplification. Others have argued that the three domains of life arose simultaneously, from a set of varied cells that formed a single gene pool.

There is no consensus among biologists concerning the position of the Eukaryotes in the overall scheme of cell evolution. Current opinions on the origin and position of Eukaryotes span a broad spectrum including the views that Eukaryotes arose first in evolution and that Prokaryotes descend from them, that Eukaryotes arose contemporaneously with Eubacteria and Archeabacteria and hence represent a primary line of descent of equal age and rank as the Prokaryotes, that Eukaryotes arose through a symbiotic event entailing an endosymbiotic origin of the nucleus, that Eukaryotes arose without endosymbiosis, and that Eukaryotes arose through a symbiotic event entailing a simultaneous endosymbiotic origin of the flagellum and the nucleus, in addition to many other models, which have been reviewed and summarized elsewhere.

The oldest known fossilized Prokaryotes were laid down approximately 3.5 billion years ago, only about 1 billion years after the formation of the Earth’s crust. Eukaryotes only appear in the fossil record later, and may have formed from endosymbiosis of multiple prokaryote ancestors. The oldest known fossil Eukaryotes are about 1.7 billion years old. However, some genetic evidence suggests Eukaryotes appeared as early as 3 billion years ago.
While Earth is the only place in the universe where life is known to exist, some have suggested that there is evidence on Mars of fossil or living Prokaryotes. However, this possibility remains the subject of considerable debate and scepticism.

**Characteristics of Prokaryotic Cells**

All Prokaryotic cells have a nucleoid region, DNA and RNA as their genetic material, ribosomes that make proteins, and cytoplasm that contains a cytoskeleton, which organizes and supports the parts of the cell. Prokaryotic cells are simpler than eukaryotic cells, and an organism that is a prokaryote is unicellular; it is made up of only one prokaryotic cell. Prokaryotic cells are usually between 0.1 to 5 micrometers in length (0.00001 to 0.0005 cm). Eukaryotic cells are generally much larger, between 10 and 100 micrometers. Prokaryotic cells have a higher surface area to volume ratio because they are smaller, which makes them able to obtain a larger amount of nutrients via their plasma membrane.

All Prokaryotic organisms are grouped into the kingdom Prokaryotae. Prokaryotic cells do not have a true membrane bound nucleus and organelles, nor flagella with the typical 9+2 arrangement of microtubules , such as found in the Eukaryotes. Most are free living and contain the genetic information, energy producing and biosynthetic systems necessary for growth and reproduction. Microorganisms are heterogenous groups of several distinct classes of living beings. Whittaker’s system recognizes five kingdoms of living things- Monera (Bacteria), Protista, Fungi, Plantae, and Animalia kingdom. Five of the three domains system:

- **Prokaryotes**: Organisms with prokaryotic organization. This includes:
  - Bacteria (Unicellular forms without a definite nucleus).
  - Higher Bacteria (Filamentous actinomycetes to filterable mycoplasmas).
  - Rickettsiae (Parasitic Bacteria having rod shaped appearance).
  - Cyanobacteria (Photosynthetic forms of prokaryotic organisation).

- **Prokaryotes Vs Eukaryotes**

  - In Eukaryotes the genetic information unlike the Prokaryotes is separated from the rest of the cell in the nucleus.
  - The presence of a true nucleus in the Eukaryotes allows much more DNA material per cell to accumulate among chromosomes.
  - The greater complexity of Eukaryotes facilitates the differentiation of cells into the complex tissues and organs in higher organisms.
  - The cytoskeleton present in the Eukaryotes controls the cell division. Whereas Bacteria have similar infoldings of the cell membrane called mesosomes.
• The most important organelle of Eukaryotes are mitochondria and chloroplasts. Both possesses their own DNA, ribosomes similar to Prokaryotes semiautonomous 70S type.

• According to the endosymbionts theory, mitochondria and chloroplast evolved from symbiotic Bacteria which were trapped inside larger eukaryotic cells and lost their independence. Table 8.1 shows the difference between Prokaryotic and Eukaryotic cells.

Table 8.1 Difference between Prokaryotic and Eukaryotic Cells

<table>
<thead>
<tr>
<th></th>
<th>Prokaryotes</th>
<th>Eukaryotes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic information</td>
<td>DNA is circular, usually free-floating in cytoplasm</td>
<td>DNA is linear, found in nucleus</td>
</tr>
<tr>
<td>Organelles</td>
<td>No nucleus or membrane-bound organelles</td>
<td>Has nucleus and membrane-bound organelles (e.g., mitochondria, chloroplasts, Golgi body, ER)</td>
</tr>
<tr>
<td>Size</td>
<td>Small (1-5 micrometers)</td>
<td>Larger (10-100 micrometers)</td>
</tr>
<tr>
<td>Organisms</td>
<td>Bacteria/archaea</td>
<td>Animals, plants, fungi, protists</td>
</tr>
<tr>
<td>Cell structure</td>
<td>Always unicellular</td>
<td>Can be unicellular or multicellular</td>
</tr>
</tbody>
</table>

Distinguishing Characteristics of a Prokaryote: Since Bacteria contains no internal organelles except ribosomes and the Bacterial chromosomes, the structure of Bacterial cell is largely the structure of its surrounding layer. Figure 8.1 shows the anatomy of a Prokaryotic cell.
- The outer layer or cell envelope that consists of two components
  - Cell Wall.
  - Cytoplasmic or Plasma Membrane: Beneath Cell Wall.

- Cellular Appendages: Apart from these essential components, Bacteria may possess additional structure, such as capsule, fimbriae and flagella.

**Capsule:** Bacterial cell has a chemically complex cell envelope in which three basic layers can be identified followed by a cell wall and a cell membrane and a plasma membrane. An outer protective gelatinous covering layer produced by Bacteria outside the cell wall. It is formed of Glycocalyx- tightly bound. It provides protection against chemicals and desiccation. It also protects the bacterium from the attack by phagocytic cells. It helps soil Bacteria to bind soil particles, for example *Azotobacter*.

**Loose Slime:** Viscid, soluble, colloidal material dispersed as loose slime cell wall. Although each layer of cell envelope performs a distinct function, together they act as a single protective unit.

**Flagella:** It is a rigid hollow appendage protruding from the cell wall, an organ of locomotion.

**Cell Interior:** These are structures, for example mesosomes, ribosomes, vacuoles, etc. bound by plasma membrane along with a single circular DNA.

**Cell Envelope**

Bacterial ‘Envelop’: Outer layer of cell envelop

- **Cell Wall:** It is a rigid, porous layer lying outside the plasma membrane. It is a multi-layered structure made of peptidoglycans (polymers of sugar and amino acids. The layer can be distinguished by the Gram stains.
  - **Gram-Positive:** It is thicker, rigid murein network filled with peptides and polysaccharides, for example *Lactobacillus*.
  - **Gram-Negative:** It is thinner, more complex made of murein coated with lipids, for example *E.coli*.

**Gram-Positive Envelop:** Thick cell wall with multiple peptidoglycan layers. No outer membrane or periplasmic space, they possess long chain teichoic acids interwined among them.

**Gram-Negative Envelop:** Single thin peptidoglycan layer, but no teichoic acids possess an outer membrane containing Lipopolysaccharides and a periplasmic space.
Functions of Cell Wall

- To impart shape and rigidity to the cell.
- Prevents rupture when the turgor pressure inside is greater than the outside the cell.
- Takes part in cell division.
- The cell wall surrounds the underlying fragile plasma membrane and protects the interior of the cell from adverse changes in the outside environment.
- Provides specific proteins and carbohydrates for attachment of some Bacterial viruses.

Structure of Cell wall

Cell wall is the second layer of the cell envelope lying below the glycocalyx. It provides a strong structural support to prevent the bacterium from collapsing in a hypotonic solution. The cell wall is composed of macromolecules network. Mucoprotein (peptidoglycan or murein) scaffolding formed by repeated sugars N–Acetylglucosamine and N-Acetylmuramic acid molecules alternating in chains, which are cross-linked with short chains of amino acids.

Peptidoglycan consists of three parts:
- A backbone-composed of alternating N-Acetylglucosamine and N-Acetylmuramic acid.
- A set of identical tetrapeptide side chains attached to N-Acetylmuramic acid.
- A set of identical pentapeptide cross-bridges.

The basic structure remains the same however the tetrapeptide side chains and pentapeptide cross bridges vary from species to species.

Chemical Structure of Cell Wall

- Outer membrane (outside of cell wall) found in Gram-Negative Bacteria.
- Gram-Negative Bacteria have LPS, i.e., Lipopolysaccharide, Lipoprotein and Phospholipids (Refer Figure 8.2). They can cause some of the symptoms of Gram-Negative Bacterial infections (fever, diarrhoea).
Structural Organisation of Prokaryotic Cells

NOTES

Self-Instructional Material

Fig. 8.2 Gram-Negative Bacteria with LPS

The cell envelope, There are two major forms of cell envelope structure, Gram-Positive and Gram-Negative. Originally these two types were identified on the basis of staining reaction. This difference in staining is due to fundamental differences in envelope structure and chemical composition.

The basic difference in the surface structures of the two types explains the result of gram staining. Both take up the small amount of crystal violet and iodine. The CV-I complex is trapped inside the gram positive cell by reduced porosity of thick cell wall.

In contrast the thin peptidoglycan layer and probabilities of membrane adhesion do not interfere in extraction of the complex from the gram negative cell.

Enzymatic removal with lysosomes of cell wall of gram positive results in complete extraction of the CV-I complex leading to a conversion to a gram negative reaction.

Staining: The technique developed by gram is still used today. It involves heat fixing a smear of Bacteria with crystal violet. All Bacteria will take up this stain. The smear is then decolorized with alcohol and propane. Gram-Positive will retain the violet stain appearing purple but Gram-Negative Bacteria do not. Finally the smear is counter stained with a red stain, such as safranin. Gram-Negative would take up this stain and become red. Gram-Positive will stay purple. The different reaction is due to the difference in the structure of two basic type of cell wall.
- Methylene Blue or Toluidine: Red violet colour in contrast with the blue staining of the Bacterial protoplasm and show metachromatic effect.
- Neisser and Albert Staining: The granules can be demonstrated with greater color contrast.
- Acid Fast Staining: Volutin granules are acid fast resisting decoloration by sulphuric acid.
- Wet Films: More refractile than the protoplasm.
- Electron Microscopy: They appear opaque demarcated body.

**Gram Stain: A Differential Stain Procedure**

Figures 8.3 and 8.4 illustrates the Gram staining techniques using different stains for Gram-Positive and Gram-Negative Bacteria.

**Fig. 8.3 Gram-Positive and Gram-Negative Staining**

**Fig. 8.4 Staining using Different Stains**

The basic difference in surface structure explains that both takes up the same amount of Crystal Violet CV and Iodine. The CV-I complex gets
trapped in gram positive cells due to thick porosity of the cell wall. The thin peptidoglycan layer do not interfere the adhesion sites. The disruption of the cell wall results in complete extraction of CV-I complex. The autolytic wall degrading enzymes that cause cell wall breakage may account for Gram negative or variable reactions in culture of gram positive organisms. Figure 8.5 shows the comparison of thick cell walls of Gram-Positive and thin cell wall of Gram-Negative Bacteria.

Fig. 8.5 Comparison of Thick Cell Walls of Gram-Positive and Thin Cell Wall of Gram-Negative Bacteria

The different reaction to the stain is due to the structure of the two basic types of cell wall. Gram-Positive Bacteria have a plasma membrane surrounded by a rigid cell wall. This rigid layer is made of a peptidoglycan, i.e., a polymer of sugar and amino acids. Muerin, which has a complex three dimensional structure.

Structure of Bacteria: Since Bacteria contains no internal organelles except ribosomes and the Bacterial chromosomes, the structure of Bacterial cell is largely the structure of its surrounding layer.

The Cell Envelope: There are two major forms of cell envelope structure, Gram-Positive and Gram-Negative. Originally these two types were identified on the basis of staining reaction. This difference in staining is due to fundamental differences in envelope structure and chemical composition.
Cell Wall

The Gram stain broadly differentiate Bacteria into Gram-Positive and Gram-Negative groups. They differ drastically in organisation of their structure outside the plasma membrane but below the capsule. In Gram-Negative Bacteria theses constitute the cell envelope and in gram positive they form the cell wall. There are two main type of Bacterial cell wall differentiated by their gram staining characteristics.

In Gram-Positive Bacteria, the cell wall consists mainly of peptidoglycans and teichoic acids, whereas in Gram-Negative Bacteria cell wall is more complex anatomically as well as chemically as they include thinner peptidoglycan and outer membrane.

In Gram-Positive Bacteria, the cell wall consists of many layers of peptidoglycans, forming a thick, rigid structure. Gram Negative cell walls will on the other hand contain only a thin layer of peptidoglycan. The matrix substances in the walls of Gram-Positive Bacteria may be polysaccharides or teichoic acids. A major component being lipoteichoic acid. The lipid element is to be found in the membrane where its adhesive properties assists in its anchoring to the membrane. Figure 8.6 shows the peptidoglycan chain.

![Fig. 8.6 Peptidoglycan Chain](image)

**Gram-Negative Bacteria:** The cell walls of Gram-Negative Bacteria are thin and consist of one or a few layers of peptidoglycan adjacent to the cytoplasmic membrane, a polymer of sugar and amino acid. The outer membrane is made up of LPS (Lipopolysaccharides), lipoproteins and phospholipids. The outer membrane has several functions. The outer membrane provides a barrier to antibiotics, digestive enzymes that is formed of a complex:
Structural Organisation of Prokaryotic Cells

NOTES

The chemical structure of the outer membranes Lipopolysaccharides is unique to specific Bacterial subspecies and is responsible for many of antigenic properties of these strains. Lipopolysaccharide, also called endotoxins are composed of polysaccharides and lipid which are responsible for toxicity of Gram-Negative Bacteria. These are found embedded in the membrane instead of phospholipids.

Plasma Membrane

Structure: The cytoplasmic membrane limits Bacterial protoplast, it is a typical ‘unit membrane’ composed of phospholipids and proteins. Lipid molecules are arranged in a double layer with their hydrophilic polar regions aligned externally in contact with a layer of surface protein. Bacterial cytoplasmic membrane is composed of a phospholipid bilayer and displays the general function of a cell. It acts as a permeability barrier for most molecules and facilitates the transport of molecules into the cells. In addition to these functions, prokaryotic membranes also function in energy conversation as the location about which a proton motive force is generated. Bacterial membranes, with some exceptions, for example Mycoplasma generally do not contain sterols like many Eukaryotes do. However, microbes do contain structurally related compounds called hopanoids which likely fulfil the same function. They can have a wide variety of fatty acids within their membranes that the Eukaryotes do not have. The fluidity of the fatty acids can be maintained with the help of the relative proportions of these fatty acids.

There are open channels, ‘prions’ in the outer membrane facilitate passive transport of ions across the membrane.

- The region between the cytoplasmic and outer membranes is marked by periplasm that contains the peptidoglycan layer and many proteins responsible for substrate binding and reception of extracellular signals. Due to presence of high concentration of proteins and peptidoglycans the periplasm exists in a gel-like state. This makes the transportation of substrate across easy using transport and signalling proteins imbedded there.

Functions of Cytoplasmic Membrane

- Semi-Permeability: Controlling the transport of metabolites through protoplasm.
- Possess enzyme system involved in outer membrane synthesis, in the secretion of extra cytoplasmic and extracellular substances.
- Monitoring physical and chemical changes through chemotaxis and sensory proteins.
- Promotes Cell motility.
- Generation of ATP.
- Facilitates chromosomal activity during replication

**Outer Membrane**: A part of gram negatives only. It forms an outer diffusion barrier made of proteins, lipid, and the Lipopolysaccharide (LPS).

Outer membrane Proteins are of three major classes:

- **Structural Proteins**: The lipoprotein is covalently bound to the peptidoglycans help bind the OM to the rest of the cell wall. OM protein A is a structural protein and is also the receptor for the sex pilus during conjugation.

- **The porins are the major proteins of the outer membrane.** There are two or three different porins which form pores through the OM. They are specialised for phosphate transport under appropriate conditions. The porins, lipoprotein and OM protein A are the Bacterial cell proteins.

- **Large specific receptor proteins are found in the OM for certain nutrients to go through porins.** These are of value as receptors for bacteriophages. Figure 8.7 shows the outer membrane found in Gram-Negative Bacteria.

![Fig. 8.7 Outer Membrane Found in Gram-Negative Bacteria](image)

**Capsule**

Prokaryotic cell sometimes secrete large amounts of extracellular polymer outside the cell wall made up of glyocalyx. This highly organised substance firmly attached polymer forms a condensed, well defined layer closely surrounding the cell, it is called the capsule as in the pneumococcus to the cell wall is called capsule. It usually consists of simple or complex carbohydrates.
Structural Organisation of Prokaryotic Cells

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or rarely proteins. A glycocalyx is a network of polysaccharides extending from the surface of Bacteria another cells. Capsules are protective and keep macrophages at a distance. They also protect cells against desiccation.

**Slime Layers**

If the substance is unorganised and only loosely attached to the cell wall, the glycocalyx is described as a slime layer as in *Leuconostoc*. In case glycol is removed from cell wall then the thin layer present known as S layer.

Slime layers and capsules are usually made up of polysaccharide or of polypeptide in some Bacteria. Capsulated Bacteria: Several groups of Streptococci. Slime has little affinity for basic dye and is not visible in gram stained smears.

**Functions of Capsule**

- **Virulence Factor**: Capsules act by protecting the bacterium from ingestion by phagocytosis,
- **Protection of the Cell Wall**: from attack by various kinds of antibacterial agents, for example, Lysozyme and lytic agents
- **Identification and typing of Bacteria**: with the help of capsular antigens.

Suspending Bacteria in Indian ink is an easy way to demonstrate capsules. Ink particles cannot penetrate the capsular material and encapsulated cells appear to have a halo around them. This is the Quellung reaction.

**Bacterial Appendages**

**Fimbriae or Pili**

These terms are usually interchangeable depending on their function. Many Gram-Negative Bacteria have short, hair like appendages on their surface. Pili are longer and thinner than flagella. They originate in the cytoplasmic membrane and are composed of structural. Protein monomers arranged helically termed pilins. They are found in motile and motile strains.

**Functions**: Based on the functions they perform

- **Ordinary Pili**: Fimbriae functions as an organ of adhesion that allow attachment to other cells. This is helpful to many enteric Bacteria in holding them to nutritionally favourable microenvironment. These adhesive properties help in Bacterial colonisation of epithelial surfaces referred to as colonization factors.
- **Sex Pili**: These are structurally similar to fimbriae but are functionally different. These are longer and fewer in number. These are genetically determined by sex factors or conjugative plasmids found on male gametes. Sex pili formed by male cells are involved in binding male
and female cells for transfer of DNA during mating. They act as specific receptors for these bacteriophages to bind male with female Bacteria during conjugation.

Fimbriae is made up of fimbrin protein and range hundreds in number. They help in motility they are antigenic and helps recognition. They are used for attachment, for example Fimbriae of Bacterial Neisseria gonorrhoeae helps to colonize in mucous membrane and fimbriae of \textit{E.coli} helps to adhere in small intestine (Refer Figure 8.5).

![Image of Fimbriae](image)

\textbf{Fig. 8.8} Pili and Fimbriae

**Flagellum**

Prokaryotic Flagella: They are different from eukaryotic. It consists of single filament made of helically arranged protein subunits. They are found in motile Bacteria except spirochetes, unbranched long filaments called flagella as organs of locomotion.

\textbf{Structure}: They are long, hollow helical filaments originating in the Bacterial protoplasm and emerge from the cell wall. They are powered by the proton motive force very different to eukaryotic flagella that uses ATP. They are made up of globular protein flagellin, similar to myosin- the contractile muscle protein: the flagellar protein called H-antigen. Flagella are highly antigen inducing specific antibodies. Though flagella of different genera of Bacteria have the same chemical composition they are antigentically different. They can be found on both Gram-Positive and Gram-Negative bacilli.

\textbf{Motility}

The thrust that propels the Bacteria forward is provided by counter clockwise rotation of the basal body which causes the helically twisted filament to whirl. The ability of Bacteria to swim by means of propel like action of the flagella provides them with the mechanical means to perform chemotaxis. Response
Structural Organisation of Prokaryotic Cells

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to chemical stimuli involves a sophisticated sensory system of receptors that are located in the cell surface and periplasm. This transfers information to chemotaxis protein that controls the flagellar motor. Genetic studies have revealed the existence of mutants with altered biochemical pathways for flagellar motility and chemotaxis.

Mutations in any of these gene products may result in loss or impairment of motility. Flagellin protein are immunogenic and constitute a group of protein antigen which are characteristic of a given species. The species specificity of the flagellin reflects differences in the primary structure and the proteins. Flagella can be sheared from the Bacterial cell without affecting the viability of the cell. The cell becomes non-motile temporarily. With times it synthesises new flagella and regains motility. Sometimes protein synthesis inhibitor may block the regeneration of flagella. Figure 8.9 shows the parts and attachment of a flagellum of a Gram-Negative and Gram-Positive Bacteria.

![Fig. 8.9 Parts and Attachment of a Flagellum of a Gram-Negative and Gram-Positive Bacteria](image)

**Parts**

- **Filament**: The longest portion which extends from cell surface to the tip.
- **Hook**: the hook is short, curved segment at the end of filament made up of proteins.
- **Basal Body**: made up of proteins, to which the hook is anchored and which imparts motion to the flagellum.

The basal body attaches the flagellum to the cell wall and plasma membrane. It is composed of a central rod inserted into a series of rings. In Gram-Positive Bacteria, only a distal pair of rings is present while in Gram-
Negative Bacteria two pairs of proximal and distal rings are connected by a central rod. The hook of Gram-Positive Bacteria slightly longer than the Gram-Negative Bacteria. The filament is made up of globular protein called flagellin which are arranged in several chains and form a helix around a hollow core. The flagella provides the Bacteria with mechanical means to perform chemotaxis (movement in response to repellent substances in the environment) Response to chemical stimuli involves a sensory system of receptors located in the surface periplasm. The protein synthesis inhibitor can block the flagellar movement of Bacteria. Figure 8.10 illustrates insertion of Bacteria into bacterial cell.

**Fig. 8.10 Insertion of Bacteria into Bacterial Cell**

The number and distribution of flagella on Bacterial cell are useful in classifying and identifying Bacteria. Based on typical arrangement of flagella on Bacterial surface following types can be listed.

- Monotrichous: Bacterial cells carry a single flagellum.
- Lophotrichous: If the Bacteria carries a single tuft of flagella.
- Amphitrichous: When the tuft appears at both the ends.
- Peritrichous: Bacteria covered all over with flagella.

**Fig. 8.11 Flagella**
Mesosome: These are convoluted membranous bodies formed as invaginations of plasma membrane into the cytoplasm. Mesosomes develop from the sites of cross, wall formation more abundant in Gram-Positive Bacteria from the nuclear body.

Functions

- Compartmenting DNA: These involved in compartmenting of DNA at cell division and sporulation.
- Sites of the Respiratory Enzymes: There is increased surface area and principle sites of the respiratory enzymes in Bacteria, chemiosmosis in nitrifying Bacteria and are analogous to mitochondria of Eukaryotes.

The Nucleoid

The Prokaryotic nucleoid equivalent of the Eukaryotic nucleus is structurally simpler than the true eukaryotic nucleus is structurally simpler than true eukaryotic nucleus, which has complex mitotic apparatus and surrounding nuclear membrane.

The Bacterial nucleoid, which contains the DNA fibrils, lacks a limiting membrane. The Bacterial nucleoid is a structure containing a single chromosome. The chromatin does not contain basic histone proteins.

Bacterial Chromosomes in ‘Nucleoid Area’ (Refer Figure 8.12).

- Found inside the Bacterial cytoplasm
- Ribosome

Plasmids

There could be extra nuclear genetic elements in the cytoplasm consisting of DNA called plasmids (Refer Figure 8.13) or episomes. Theses can exist and replicate independently of the chromosomes or may be integrated with it.
• Inside the Bacterial cytoplasm.
• Plasmid – Extrachromosomal DNA, not part of ‘genome’, different genes.
• Can transfer in a process- conjugation across sex pilus, change the genetic character of the recipient or through the bacteriophages.
• plasmids are not essential for host growth and reproduction, but may confer on it certain properties.

![Fig. 8.13  Plasmids](image)

**Mitochondria**

- Site of cellular respiration-redox reactions. Oxygen is reduced. Krebs cycle, then, etc. chain and oxidative Phosphorylation. Which makes ATP.
- Double membrane bound organelle that have a sedimentation rate of 70S being composed of a 30S and 50S subunit.
- Outer membrane - Contains transport protein porin.
- Inner membrane - Very selective permeable
- Cristae - Inner membranes that hold the respiratory assemblies.
- Content - Its own DNA, its own ribosomes and protein synthesising ability enzymes for cellular respiration.

**Ribosomes**

- Ribosomes complex structure made of RNA and proteins.
- Slightly smaller than eukaryotic ribosome that promotes selective toxicity.
- Site of cellular protein synthesis.
- Free in cytoplasm as dimmers individual subunits.
- Attached to mRNA molecules in a polysome.
- Involved in the synthesis of proteins to be transported out of the cell.

The Figure 8.14 below shows inside of a Ribosome.
Structural Organisation of Prokaryotic Cells

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**Fig. 8.14 Inside of a Ribosome**

**Intracytoplasmic Inclusion**

The synthesis of intracellular inclusions in different Prokaryotes is usually dependent upon both nutritional ability and growth factors present. Since these are not essential structures they may be absent under certain growth conditions.

They perform functions of storage, maintain turgidity and consists of volutin, lipid, glycogen, starch or sulphur.

**Metachromatic or Volutin Granules**

Volutin granules function as storage reservoirs for phosphate. Used in the ATP synthesis. Generally found in cells that grow in phosphate rich environment.

They could be found in diphtheria bacillus and plague bacillus, mycobacterium.

**Polysaccharide Granules:** These typically consists of glycogen, and starch. Their presence can be demonstrated when iodine is applied to the cells.

**Lipid Inclusion:** It is a common lipid storage material appearing in many species of Mycobacterium, Bacillus, Azotobacter and other genera. They are revealed by staining cells with fat-soluble dyes, such as Sudan dyes.

**Sulphur Granules:** Certain Bacteria derive energy by oxidizing sulphur and sulphur containing compounds. These Bacteria may deposit sulphur granules in the cell, where they serve as energy reserve.

**Carboxysomes:** They are inclusions that contain the enzyme ribulose 1, 5, biphosphate carboxylase. This enzyme is used by photosynthetic Bacteria to fix carbon dioxide, i.e., cyanobacteria and thiobacillus.

**Gas Vacuoles:** These are hollow cavities that consists of rows of several individual gas vesicles. Found in cyanobacteria they help in maintaining buoyancy in order to receive sufficient amount of light, oxygen and nutrient.
**Magnetosomes**: These are inclusions of iron oxide surrounded by invaginations of plasma membrane. These are formed by several Gram-Negative Bacteria. They act like magnets protecting cells against hydrogen peroxide accumulation.

**Endospores**: they are Bacterial survival structures that are highly resistant to environmental stresses. It permits the survival of some Bacteria in salt crystals and limited to several genera of Gram-Positive Bacteria, such as *Bacillus* and *Clostridium*. Figure 8.15 shows the ultrastructure of a bacterial endospore.

*Fig. 8.15 Ultrastructure of a Bacterial Endospore*

**Significance**: The main ecological role appears to be survival in the dry state in a non-nutrient environment.

**Table 8.2 Comparison between Vegetative Cell and Endospore**

<table>
<thead>
<tr>
<th>Vegetative cell</th>
<th>Endospore</th>
</tr>
</thead>
<tbody>
<tr>
<td>Usually stains Gram positive. Readily stained by ordinary stains.</td>
<td>Not readily stained by ordinary stains.</td>
</tr>
<tr>
<td>Sensitive to various physical and chemical agents (heat radiation, disinfectant and antibiotic).</td>
<td>Resistant to various physical and chemical agents (heat radiation, disinfectant and antibiotic).</td>
</tr>
<tr>
<td>Relatively low sulfur containing amino acid.</td>
<td>High sulfur containing amino acid and calcium.</td>
</tr>
<tr>
<td>Calcium ions and dipicolinic acid (DPA) absent. Metabolically active.</td>
<td>Calcium ions and DPA is present. Metabolically inactive (activities are negligible).</td>
</tr>
</tbody>
</table>

**L-Forms**: L-forms or spheroplasts are synthesised from complete Bacteria by the action of penicillin on them retaining residual cell wall. They are osmotically less sensitive and can easily grow on agar based culture media.
They are unstable bodies discovered by Kleineberger-Nobel upon studying colonies of *Streptobacillus moniliformis* with the appearance of fried eggs and non-pathogenic animals.

Advances in genomic DNA sequencing of microorganisms the classification of three domains has been widely accepted a taxonomic level higher than kingdom

**Domain Archaeabacteria:** This term refers to the ancient origin of the Bacteria, which seem to have diverged from the very early forms of Eubacteria. They are inhabited mostly in extreme environments. They are classified on the base of the extreme environments they live in. Methanogens, extremophiles and non-extreme Archaeabacteria. Figure 8.16 shows the universal phylogenetic tree.

**Domain Bacteria:** The most abundant organism the eubacteria plays critical role like cycling carbon and sulphur. Much of the photosynthesis is carried out by eubacteria

**Domain Eukarya**

It has four kingdoms; first Protista-unicellular organisms, Followed by kingdom Fungi, plants, and animals. Multicellularity and sexuality are two unique characters that differentiate from Prokaryotes and Eukaryotes.

**Check Your Progress**

1. Give the morphology of Prokaryotic cell.
2. Name the organisms with Prokaryotic organization.
3. Write down some of the major differences between Prokaryotes and Eukaryotes.
4. Differentiate between Gram-Negative and Gram-Positive envelope.

**8.3 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS**

1. Prokaryotic cells have various shapes; the four basic shapes of Bacteria are:
   - Cocci: Spherical
   - Bacilli: Rod-shaped
• Spirochaete: Spiral-shaped
• Vibrio: Comma-shaped
• The Archaea Haloquadratum has flat square-shaped cells.

2. Prokaryotes organisms with Prokaryotic organization includes:
• Bacteria (unicellular forms without a definite nucleus).
• Higher Bacteria (filamentous actinomycetes to filterable mycoplasmas).
• Rickettsiae (Parasitic Bacteria having rod shaped appearance.
• Cyanobacteria (photosynthetic forms of prokaryotic organisation).

3. Some of the major differences between Prokaryotes and Eukaryotes are as follows:
• In Eukaryotes the genetic information unlike the Prokaryotes is separated from the rest of the cell in the nucleus.
• The presence of a true nucleus in the Eukaryotes allows much more DNA material per cell to accumulate among chromosomes.
• The greater complexity of Eukaryotes facilitates the differentiation of cells into the complex tissues and organs in higher organisms.
• The cytoskeleton present in the Eukaryotes controls the cell division. Whereas Bacteria have similar infoldings of the cell membrane called mesosomes.

4. Gram-Positive envelope: Thick cell wall with multiple peptidoglycan layers. No outer membrane or periplasmic space, they possess long chain teichoic acids interwined among them. Gram-Negative envelope: Single thin peptidoglycan layer, but no teichoic acids possess an outer membrane containing Lipopolysaccharides and a periplasmic space.

8.4 SUMMARY
• Prokaryotic cells are cells that do not have a true nucleus or most other cell organelles.
• Organisms that have prokaryotic cells are unicellular and called Prokaryotes.
• Prokaryotic cells can be contrasted with eukaryotic cells, which are more complex.
• Eukaryotic cells have a nucleus surrounded by a nuclear membrane and also have other organelles that perform specific functions in the cell.
• Prokaryotic cells do not have a true nucleus that contains their genetic material as eukaryotic cells do.
Instead, prokaryotic cells have a nucleoid region, which is an irregularly-shaped region that contains the cell’s DNA and is not surrounded by a nuclear envelope.

Some other parts of prokaryotic cells are similar to those in Eukaryotic cells, such as a cell wall surrounding the cell (which is also found in plant cells, although it has a different composition).

Like eukaryotic cells, prokaryotic cells have cytoplasm, a gel-like substance that makes up the ‘filling’ of the cell, and a cytoskeleton that holds components of the cell in place.

Both prokaryotic cells and eukaryotic cells have ribosomes, which are organelles that produce proteins, and vacuoles, small spaces in cells that store nutrients and help eliminate waste.

Some prokaryotic cells have flagella, which are tail-like structures that enable the organism to move around. They may also have pili, small hair-like structures that help Bacteria adhere to surfaces and can allow DNA to be transferred between two prokaryotic cells in a process known as conjugation.

The capsule is a sticky layer of carbohydrates that helps the bacterium adhere to surfaces in its surroundings.

Bacteria and archaea reproduce through asexual reproduction, usually by binary fission.

Genetic exchange and recombination still occur, but this is a form of horizontal gene transfer and is not a replicative process, simply involving the transference of DNA between two cells, as in Bacterial conjugation.

A widespread current model of the evolution of the first living organisms is that these were some form of Prokaryotes, which may have evolved out of protocells, while the Eukaryotes evolved later in the history of life.

The oldest known fossilized Prokaryotes were laid down approximately 3.5 billion years ago, only about 1 billion years after the formation of the Earth’s crust.

Eukaryotes only appear in the fossil record later, and may have formed from endosymbiosis of multiple prokaryote ancestors. The oldest known fossil Eukaryotes are about 1.7 billion years old.

All prokaryotic cells have a nucleoid region, DNA and RNA as their genetic material, ribosomes that make proteins, and cytoplasm that contains a cytoskeleton, which organizes and supports the parts of the cell.
• Prokaryotic cells are simpler than eukaryotic cells, and an organism that is a prokaryote is unicellular; it is made up of only one prokaryotic cell. Prokaryotic cells are usually between 0.1 to 5 micrometers in length (0.00001 to 0.0005 cm).

• Eukaryotic cells are generally much larger, between 10 and 100 micrometers.

• Prokaryotic cells have a higher surface area to volume ratio because they are smaller, which makes them able to obtain a larger amount of nutrients via their plasma membrane.

• Bacterial cell has a chemically complex cell envelope in which three basic layers can be identified followed by a cell wall and a cell membrane and a plasma membrane.

• An outer protective gelatinous covering layer produced by Bacteria outside the cell wall. It is formed of Glycocalyx- tightly bound. It provides protection against chemicals and desiccation.

• Gram-Positive envelop is thick cell wall with multiple peptidoglycan layers. No outer membrane or periplasmic space, they possess long chain teichoic acids interwined among them.

• Gram-Negative envelop is single thin peptidoglycan layer, but no teichoic acids possess an outer membrane containing Lipopolysaccharides and a periplasmic space.

• Cell wall is the second layer of the cell envelope lying below the glycocalyx. It provides a strong structural support to prevent the bacterium from collapsing in a hypotonic solution.

• The cell wall is composed of macromolecules network. Mucoprotein (peptidoglycan or murein) scaffolding formed by repeated sugars N-acetyl glucosamine and N-Acetylmuramic acid molecules alternating in chains, which are cross-linked with short chains of amino acids.

• The chemical structure of the outer membranes Lipopolysaccharides is unique to specific Bacterial subspecies and is responsible for many of antigenic properties of these strains.

• Lipopolysaccharide, also called endotoxins are composed of polysaccharides and lipid which are responsible for toxicity of Gram-Negative Bacteria.

• Prokaryotic cell sometimes secrete large amounts of extracellular polymer outside the cell wall made up of glycocalyx.

• If the substance is unorganised and only loosely attached to the cell wall, the glycocalyx is described as a slime layer as in Leuconostoc.

• Sex pili are structurally similar to fimbriae but are functionally different. These are longer and fewer in number.
Structural Organisation of Prokaryotic Cells

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- Motility is the thrust that propels the Bacteria forward is provided by counter clockwise rotation of the basal body which causes the helically twisted filament to whirl.
- Mutations in any of these gene products may result in loss or impairment of motility. Flagellin protein are immunogenic and constitute a group of protein antigen which are characteristic of a given species.
- The species specificity of the flagellin reflects differences in the primary structure and the proteins.
- Mesosome are convoluted membranous bodies formed as invaginations of plasma membrane into the cytoplasm. Mesosomes develop from the sites of cross, wall formation more abundant in Gram-Positive Bacteria from the nuclear body.
- Lipid inclusion is a common lipid storage material appearing in many species of Mycobacterium, Bacillus, Azotobacter and other genera. They are revealed by staining cells with fat-soluble dyes, such as Sudan dyes.
- Certain Bacteria derive energy by oxidizing sulphur and sulphur containing compounds. These Bacteria may deposit sulphur granules in the cell, where they serve as energy reserve.
- Carboxysomes are inclusions that contain the enzyme ribulose 1, 5, biphosphate carboxylase. This enzyme is used by photosynthetic Bacteria to fix carbon dioxide, i.e., cyanobacteria and thiobacillus.
- Gas vacuoles are Hollow cavities that consists of rows of several individual gas vesicles. Found in cyanobacteria they help in maintaining buoyancy in order to receive sufficient amount of light, oxygen and nutrient.
- Magnetosomes are inclusions of iron oxide surrounded by invaginations of plasma membrane. These are formed by several Gram-Negative Bacteria. They act like magnets protecting cells against hydrogen peroxide accumulation.
- Endospores are Bacterial survival structures that are highly resistant to environmental stresses.

8.5 KEY WORDS

- **Capsule**: The capsule is a sticky layer of carbohydrates that helps the bacterium adhere to surfaces in its surroundings.
- **Sex pili**: These are structurally similar to fimbriae but are functionally different. These are longer and fewer in number.
- **Motility**: The thrust that propels the Bacteria forward is provided by counter clockwise rotation of the basal body which causes the helically twisted filament to whirl.

- **Mesosome**: These are convoluted membranous bodies formed as invaginations of plasma membrane into the cytoplasm.

- **Lipid inclusion**: It is a common lipid storage material appearing in many species of Mycobacterium, Bacillus, Azotobacter and other genera.

- **Carboxysomes**: They are inclusions that contain the enzyme ribulose 1, 5, biphosphate carboxylase.

- **Gas vacuoles**: These are hollow cavities that consists of rows of several individual gas vesicles.

- **Magnetosomes**: These are inclusions of iron oxide surrounded by invaginations of plasma membrane.

- **Endospores**: They are bacterial survival structures that are highly resistant to environmental stresses.

## 8.6 SELF ASSESSMENT QUESTIONS AND EXERCISES

### Short Answer Questions

1. Define Prokaryotic cell.
2. Write about the reproduction of Prokaryotic cell.
3. Draw a well-labelled diagram of a Prokaryotic cell.
4. What is staining?
5. Write the functions of cytoplasmic membrane.
6. What is endospore?

### Long Answer Questions

1. Write a note on Prokaryotic cell, its morphology, reproduction and classification.
2. Discuss about the evolution of Prokaryotic cell.
3. Explain with the help of table major differences between Prokaryotic cell and Eukaryotic cell.
4. Write about the structural organisation of Prokaryotic cell.
5. What is motility? Explain with the help of well-labelled diagram.
8.7 FURTHER READINGS


UNIT 9  MICROBIAL DIVERSITY

Structure
  9.0  Introduction
  9.1  Objectives
  9.2  Algae: General Introduction
    9.2.1  Microalgae and Microalgae
  9.3  Characters of Different Classes of Algae Given By F.E.Fritsch
  9.4  Answers to Check Your Progress Questions
  9.5  Summary
  9.6  Key Words
  9.7  Self Assessment Questions and Exercises
  9.8  Further Readings

9.0  INTRODUCTION

Algae, singular alga, members of a group of predominantly aquatic photosynthetic organisms of the kingdom Protista. Algae have many types of life cycles, and they range in size from microscopic Micromonas species to giant kelps that reach 60 metres (200 feet) in length. Their photosynthetic pigments are more varied than those of plants, and their cells have features not found among plants and animals. In addition to their ecological roles as oxygen producers and as the food base for almost all aquatic life, algae are economically important as a source of crude oil and as sources of food and as a number of pharmaceutical and industrial products for humans. The taxonomy of algae is contentious and subject to rapid change as new molecular information is discovered. The study of algae is called phycology, and a person who studies algae is called a phycologist.

Macroalgae are multicellular marine algae which form a real plant. They have a stem and leaves. Depending on water depth they are brown, red or green. Marine algae stick with their root on rock and are completely rinsed from sea water. They are 4000 different marine algae but only a few suitable for human consumption. Algae contain valuable nutrients, among others lots of trace elements including one very important: iodine. Another essential element is algin used in various matters especially in food production. Microalgae are different. They are one cell algae. Spirulina, Chlorella or also blue green algae are very popular in this variety. They are cultivated in fresh water tanks. The ‘algae bloom’ which is driving at certain seasons in coastal regions is also one of the microalgae.
In this unit, you will study about algae and its classification based on Fritsch system, general characteristics of microalgae and macroalgae, and their biological and ecological importance in detail.

### 9.1 OBJECTIVES

After going through this unit, you will be able to:
- Understand algae and its classification
- Outline the characters of different classes of algae proposed by Fritsch
- Discuss the general characteristics of microalgae and macroalgae and their biological and ecological importance

### 9.2 ALGAE: GENERAL INTRODUCTION

Algae is an informal term for a large, diverse group of photosynthetic eukaryotic organisms that are not necessarily closely related, and is thus polyphyletic. Including organisms ranging from unicellular microalgae genera, such as Chlorella and the diatoms, to multicellular forms, such as the giant kelp, a large brown alga which may grow up to 50 m in length. Most are aquatic and autotrophic and lack many of the distinct cell and tissue types, such as stomata, xylem, and phloem, which are found in land plants. The largest and most complex marine algae are called seaweeds, while the most complex freshwater forms are the Charophyta, a division of green algae which includes, for example, *Spirogyra* and the Stoneworts.

Algae constitute a polyphyletic group since they do not include a common ancestor, and although their plastids seem to have a single origin, from cyanobacteria, they were acquired in different ways. Green algae are examples of algae that have primary chloroplasts derived from endosymbiotic cyanobacteria. Diatoms and brown algae are examples of algae with secondary chloroplasts derived from an endosymbiotic red alga. Algae exhibit a wide range of reproductive strategies, from simple asexual cell division to complex forms of sexual reproduction.

Algae lack the various structures that characterize land plants, such as the phyllids (leaf-like structures) of bryophytes, rhizoids in non-vascular plants, and the roots, leaves, and other organs found in tracheophytes (vascular plants) (Refer Figure 9.1). Most are phototrophic, although some are mixotrophic, deriving energy both from photosynthesis and uptake of organic carbon either by osmotrophy, myotrophy, or phagotrophy. Some unicellular species of green algae, many golden algae, euglenids, dinoflagellates, and other algae have become heterotrophs (also called colorless or apochlorotic algae), sometimes parasitic, relying entirely on external energy sources and have
limited or no photosynthetic apparatus. Some other heterotrophic organisms, such as the apicomplexans, are also derived from cells whose ancestors possessed plastids, but are not traditionally considered as algae. Algae have photosynthetic machinery ultimately derived from cyanobacteria that produce oxygen as a by-product of photosynthesis, unlike other photosynthetic Bacteria, such as purple and green sulphur Bacteria. Fossilized filamentous algae from the Vindhya basin have been dated back to 1.6 to 1.7 billion years ago.

**Fig. 9.1 Comparison of Algae and Plant Structure**

**General Characteristics**

- Diverse group of simple eukaryotic photosynthetic autotrophs.
- Unicellular or multicellular plant like organisms that belong to Kingdom Protista.
- They can be Prokaryotes or Eukaryotes. All blue green algae are Prokaryotes.
- Most are found in the ocean or other water bodies in their pursuit for reproduction, support and nutrition with high productivity.
- Absorbs nutrients from water and form the base of the food chain.
- All reproduces asexually and a few sexually lacking sterile jacket of cells around reproductive cell.
- Most are not pathogens and produce harmful toxins.
- They lack vascular (conducting) tissues with no xylem or phloem; no roots, stems or leaves.
• They store food as starch, proteins or lipids.
• Most have a variety of additional ‘accessory’ pigments to increase photosynthetic efficiency.

Distribution and Abundance

• **Epiphytes:** These algae forms grow on other plants. However they do not derive food from the plant they grow on but take support only, for example, *Coleochaete* in association with *Chara* and *Nitella.*

• **Halophytes:** Certain algae propagating in saline water, for example, *Chlamydomonas.*

• **Symbionts:** Large number of algae in connection with dissimilar organism for their advantage, for example, *Nostoc, Anabena* in the coloroid roots of Cyclics. Lichens for some of the best examples of association between algae and fungal cells.

• **Epizoic:** Growing in the sheels of Molluscs, turtles and fins of fishes, for example, *Cladophora* found in snails.

• **Endozoic:** Algae found inside the body of aquatic animals. Zoochlorella occurs in the coelom of hydra.

• **Parasitic Algae:** Few algae depend on other plants for obtaining food. Common intercellular parasitic algae is *Cephaleuros* that grows in tea leaves causing disease to tea plant.

Vegetative Structure of Multicellular Algae

• Thallus body lacks conductive tissue and is nonvascular.
• Holdfasts anchor alga to rock in benthic forms.
• Stipes: Hollow, stem-like structures, do not support weight.
• Blades, leaf like structures.
• Pneumatocysts: Floating gas filled bladder.

Multicellularity of Cell

• Evolved in Alga.
• Better root, gamete protection, grow tall to obtain sunshine.
• Getting water to all cells.
• Plants with evolved vascular tissue, xylem and phloem.

9.2.1 Microalgae and Microalgae

Algae are simple, non-flowering, aquatic organisms, consisting of large assemblage of single-celled forms and seaweeds. **Macroalgae** are multicellular marine algae which form a real plant. They have a stem and
leaves. Depending on water depth they are brawn, red or green Marine algae stick with their root on rock and are completely rinsed from sea water. They are 4000 different marine algae but only a few suitable for human consumption. Algae contain valuable nutrients, among others lots of trace elements including one very important: iodine. Another essential element is algin used in various matters especially in food production. **Microalgae** are different, they are one cell algae. *Spirulina, Chlorella* or also blue green algae are very popular in this variety. They are cultivated in fresh water tanks. The ‘Algae Bloom’ which is driving at certain seasons in coastal regions is also one of the microalgae.

Microalgae do not have roots, stems or leaves. Microalgae that are able to perform photosynthesis are vital to life on earth; they produce around half of the oxygen found in the atmosphere, they utilize the sun’s light and carbon dioxide in their growth. This process is called photosynthesis. They are imperative to the life of our oceans and lakes as they are at the very bottom of the food chain, without microalgae the whole ecosystem would collapse. Microalgae are found in the aquarium, mainly in the form of nuisance slime algae (*Cyanobacteria*) and as the somewhat more useful Calcifying Algae (*Coralline*). This contradicts the initial description of needing a microscope to see them, this is because as a single cell they are invisible to the human eye but when a vast number of these single sells join themselves together they become visible to the human eye.

Macroalgae and microalgae are used in a variety of commercial products with many more in development. Foods, nutraceuticals, and feeds are the major commercial products from algae. Well-known culinary products include Nori, Wakame, Kombu and Dulse, from whole macroalgal biomass. The microalgae *Spirulina* and *Chlorella* have been widely marketed as nutritional supplements for both humans and animals. Several microalgae with a high nutritional value and energy content are grown commercially as aquaculture feed. The major processed products from macroalgae are the hydrocolloids, including Carrageenan, Agars, and Alginates, used as gelling agents in a variety of foods and healthcare products. Pigments extracted from algae include β-carotene, astaxanthin, and phycobiliproteins. These are generally used as food colorants, as additives in animal feed or as nutraceuticals for their antioxidant properties. Polyunsaturated Fatty Acids (PUFAs) are another high value product derived from microalgae. Other potential products include fertilizers, fuels, cosmetics and chemicals. Algae also have application in bioremediation and CO₂ sequestration, as well as producing many interesting bioactive compounds. Algae have great potential to produce a wide range of valuable compounds, beyond their current exploitation.
Similarities between Microalgae and Macroalgae:

- Both algae and microalgae show large diversity
- They are both primary producers of oxygen during photosynthesis.
- Both serve as natural filters of phosphates and nitrogenous wastes.
- They both serve as excellent food sources in aquatic ecosystems.

The main difference between algae and microalgae is that algae are simple, autotrophic organisms, consisting of huge diversity among them whereas microalgae are the microscopic type of algae.

**Cyanobacteria (Blue Green Bacteria)**

These are Prokaryotic microorganisms similar to the true bacteria, they are photosynthetic but not true algae due to absence of membrane bound nuclei. They are ancient life forms as the first living forms to evolve on Earth present as fossil remains.

**Structure:** Typical prokaryotic cell with a naked coil of DNA and no true nucleus. The cell wall is similar in structure and composition to that of Gram Negative Bacteria. They perform photosynthesis with the help of phycocyanin and phycoerythrin and protein is synthesised on 70 S ribosome. Some blue-green algae like *Anabena*, have special thick walled cells called heterocysts that can fix free nitrogen in the air.

**Ultra Structure of the Cell**

The Eukaryotic algal cell is surrounded by a cell wall. A few are enclosed by an outer matrix outside the cell wall. This appears similar to bacterial capsules usually in gelatinous forms. Mitochondrial structure varies greatly in the algae. Algae like Euglenids have discoid cristae or lamellar cristae as in green and red algae while the rest have tubular cristae.

**Organisation of Thallus**

There are two types of structures present in algae:

- Prokaryotic Cell
- Eukaryotic Cell

Alga thalli show a wide range of variation and ranges from unicellular microscopic structures to large sea weeds. It is noteworthy that no single type of thallus conforms a division. There are many similarities found among different division of alga. A typical Eukaryotic cell (Refer Figure 9.2) is characterised by well-defined nucleus and membrane bound organelle.
Fig. 9.2 A Typical Eukaryotic Cell of Algae

- **Nucleus**: They can exhibit Coenocytic condition or multinucleated condition. Each nucleus possess one or more endosomes, dark stained nuclei. Along with a nucleolous a linear association of small nucleoli are found (Refer Figure 9.3 (A)).

- **Golgi Bodies**: These with cell metabolism is associate arranged in stacks called dictyosomes.

- **Eye Spot**: It is a photoreceptor organelle, highly pigmented. It is found in vegetative cells that are motile and reproductive cells of algae (Refer Figure 9.3 (B)).

Fig. 9.3 (A) in Chlamydomonas; and Eye Spot (B) in Volvox
Algae are either unicellular like *Chlamydomonas* or multicellular like *Spirogyra*. Multicellular algae may be in the form of colonies like volvox or in the form of filaments as *Spirogyra*. A few algae are parasites, some grow in special habitats, for example, Parasites, Symbiotic Cryophytes and Thermophytes.

The plastids in algae, contain pigments which are of three types:

- **Chlorophylls**: Five types chlorophyll (a, b, c, d and e) are found in different algae. Chlorophyll a is present in all algae.

- **Carotenoids**: These are the yellow and orange pigments (namely carotenes and xanthophylls) are found in varied quantities in different algae.

- **Biliproteins or Phycobilins**: These pigments include phycocyanin (blue in colour) and phycoerythrin (red in colour) and presence of these pigments is the characteristic feature of certain types of algae.

The chloroplast with pigments in it contains one or more spherical bodies called **pyrenoids** where formation of starch takes place.

**Life Cycle**

Reproduction in algae is of three types, vegetative, asexual and sexual. Vegetative reproduction takes place by fragmentation, fission, budding, etc. Asexual reproduction by production of asexual spores (motile or non-motile). Sexual reproduction takes place during unfavorable conditions by producing gametes.

**Plant Life: Alternation of Generation**

Plants spend part of their life cycle in the haploid (1n) stage, and part in the diploid (2n) stage – both stages are multicellular.

![Life Cycle Diagram](image)

**Life Cycle Animal vs. Plants**

Plants display an alternation of haploid and diploid phases in their life cycle. Animals like humans live in 2n stage. 2n stage is dominant. Single celled gametes are 1n.
Alga are most common to aquatic systems and live at the water interface and termed as neustonic. They also live as endosymbionts within plants. Alga are associated with Fungi to form Lichens.

**Haploidic Type:** The sporophyte representing the plant body and the major portion of the life cycle is diploid. The haploid stage is represented by the gametes, for example, *Spirogyra, Chara*.

**Diplomatic Type:** The sporophyte representing the plant body and the major portion of the life cycle is diploid. The haploid stage is represented by the gametes, for example, *Fucus, Sargassum*.

**Diplohaplontic Life Cycle:** In this type there is alternation of two distinct vegetative individuals - sporophyte and gametophyte having not only different functions but different number of chromosomes, this results in sporogenic meiosis in diphasic life cycle, for example, *Cladophora*.

**Triphasic Life Cycle:** Depicts succession of three generations.

**Haplobiontic**, for example, Rhodophyceae. Here two well-developed haploid phases are present in life cycle. The zygote indicates the diploid phase, for example, plant body of batrachospermum is gametophyte bearing sex organs. Male and female gamete after fusion forms diploid zygote on a haploid plant body. Somatic haploid generation alternates with diploid phase, Batrachospermum.

Diplobiontic, in polysiphonia life cycle, for example, there are two distinct diploid phases that alternate with haploid gametophytic phase. The male and female gametophytes give rise to the gametes that forms a zygote on fusion. The zygote divides mitotically and form small diploid sporophyte attached to the gametophytic plant. Sporangia are formed with diploid carpospores. These on germination forms free living tetrasporophyte. The adult form bears tetrasporangia 2n. After meiosis four haploid tetraspores are formed. This produces a free living gametophytic plant.
9.3 CHARACTERS OF DIFFERENT CLASSES OF ALGAE GIVEN BY F.E. FRITSCH

Algae are heterogenous assemblage of individuals from two kingdom, namely,
- Monera
- Protista

The Prokaryotic Algae

Blue green algae, Cyanobacteria placed under Monera. The algae do not belong to Kingdom of Plantae. They are considered as lower forms of plants due to their photosynthetic characteristics in comparison to other vascular plants.

The Eukaryotic Algae

Possess membrane bound organelles, for example, nuclei, mitochondria and plastids unlike Prokaryotes.

The most comprehensive classification of Algae was given by F.E. Fritsch in his book titled The Structure and Reproduction of Algae based on then following criteria:
- Pigmentation
- Type of Flagella
- Assimilatory Products
- Thallus Structure
- Method of Reproduction

Class Chlorophyceae (Green Algae)

The Chlorophyceae are one of the classes of green algae, distinguished mainly on the basis of ultrastructural morphology, for example, the Chlorophyceae CW clade, and Chlorophyceae DO clade, are defined by the arrangement of their flagella.
- Occurrence: The algae grow in fresh and salt water or within other organisms as parasites or commensals.
Pigment: The photosynthetic pigments are chlorophyll a and b. Along with specific carotenoids.
Reserve Food: Starch.
Structure: They possess cellulosic cell walls. They possess a holdfast to anchor them to hard surfaces.
Reproduction takes place sexually and asexually, isogamous to oogamous type.

Most common genera are Chlorella, Volvox, etc. From Chlymadomonas several distinct lines of evolutionary specialization have evolved in the green alga. There first line is marked by a non-motile unicellular green alga, such as Chlorella. Volvox represent a second major line of evolutionary specialization.

Class Xanthophyceae (Yellow Green Algae)

Yellow-green algae or the Xanthophyceae (xanthophytes) are an important group of heterokont algae. Most live in fresh water, but some are found in marine and soil habitats. They vary from single-celled flagellates to simple colonial and filamentous forms.

- Occurrence: Few are marine but normally fresh water.
- Pigment: Abundant yellow xanthophyll.
- Reserve Food: Oil, Paranoids absent.
- Structure: Unicellular motile to simple filamentous forms. Cell wall rich in pectic compounds. Motile cells with unequal flagella.
- Reproduction: Isogamous rare sexual reproduction.
- Example: Vaucheria.

Class Chrysophyceae (Stoneworts /Brittleworts)

The Chrysophyceae, usually called chrysophytes, chrysomonads, golden-brown algae or golden algae are a large group of algae, found mostly in freshwater. Golden algae is also commonly used to refer to a single species, Prymnesium parvum, which causes fish kills.

- Occurrence: They are largely distributed from fresh to brackish water. They are visible in shallow ponds as a dense covering.
- Pigment: brown or orange chromatophores. Phycocyanin serve as a chief accessory pigments.
- Structure: There is a covering made up of limestone in some species from the precipitates of calcium and magnesium carbonate, for example, Chara and Nitella.
- Reserve Food: Fat and Leucosin.
- Reproduction: Rare isogamous type.
Bacillariophyceae (Diatoms)

Diatoms are a major group of algae, specifically microalgae, found in the oceans, waterways and soils of the world. Living diatoms number in the trillions: they generate about 20 percent of the oxygen produced on the planet each year, take in over 6.7 billion metric tons of silicon each year from the waters in which they live, and contribute nearly half of the organic material found in the oceans. The shells of dead diatoms can reach as much as a half mile deep on the ocean floor, and the entire Amazon basin is fertilized annually by 27 million tons of diatom shell dust transported by East-to-West (easterly) transatlantic winds from the bed of a dried up lake once covering much of the African Sahara.

- Occurrence: Fresh water, sea soil and terrestrial habitats.
- Pigments: Chromatophores, yellow or golden brown.
- Reserve Food: Fat and Volutin.
- Structure: Cell wall of silica and pectic. It consists of two halves and each has two or more pieces. Cell wall is richly ornamented.
- Reproduction: forms are diploid, sexual reproduction occurs by fusion of protoplast, for example, *Pinnularia*.

Class Cryptophyceae

The cryptomonads are a group of algae, most of which have plastids. They are common in freshwater, and also occur in marine and brackish habitats. Each cell is around 10–50 μm in size and flattened in shape, with an anterior groove or pocket. At the edge of the pocket there are typically two slightly unequal flagella.

- Occurrence: Both in marine and freshwater.
- Pigments: Chromatophores usually parietal and diverse forms.
- Reserve Food: Starch or Solid Carbohydrate.
- Structure: Motile cells that are advanced coccoid.
- Reproduction: Isogamous.
- Example: *Chroomonas*.

Class Dinophyceae

Dinoflagellates (Dinophyceae, Dinophyta) comprise both photosynthetic and heterotrophic flagellates, with about 130 genera and 2000 species (van den Hoek *et al.*, 1995). The principal storage polysaccharide is starch, a (1, 4; 1, 6)-α-glucan, which occurs in the form of grains outside the chloroplasts. The cells are surrounded by a complex theca and in some cases a thin additional layer, the pellicle. Although the pellicle is usually referred to as
cellulosic, linear (1, 3; 1, 4)-β-glucans were found in Peridinium westii (Nevo and Sharon, 1969).

- Occurrence: Widely as sea water planktons.
- Reserve Food: Starch and Oil.
- Pigments: Chromatophores are dark yellow, brown.
- Structure: Unicellular motile to branched filaments.
- Reproduction: Rare isogamous type.

**Class Chloromonadineae**

The Raphidophyceae (raphidophytes, formerly referred to as Chloromonadophyceae and Chloromonadineae) are a small group of eukaryotic algae that includes both marine and freshwater species. All raphidophytes are unicellular, with large cells (50 to 100 μm), but no cell walls. Raphidophytes possess a pair of flagella, organized such that both originate from the same invagination (or gullet). One flagellum points forwards, and is covered in hair-like mastigonemes, while the other points backwards across the cell surface, lying within a ventral groove. Raphidophytes contain numerous ellipsoid chloroplasts, which contain chlorophylls a, c₁, and c₂. They also make use of accessory pigments including β-carotene and diadinoxanthin. Unlike other heterokontophytes, raphidophytes do not possess the photoreceptive organelle (or eyespot) typical of this group.

- Occurrence: All are fresh water forms.
- Pigments: bright green chromatophore with excess chlorophyll.
- Reserve Food: Oil.
- Structure: Motile, flagellate with two equal flagella.

**Class Euglenineae**

- Occurrence: They occur in fresh, brackish, marine waters and in moist soils. They often form water blooms in ponds, for example, Euglena are found in water with high organic load.
- Pigment: Like chlorophyta and charophyta euglenoids have chlorophyll a and b.
- Reserve Food: A Polysaccharide composed of >1, 3 Glucose the primary storage product is Paramylon which is very unique to Euglenoids.
- Structure: Motile flagellates, prominent nucleus with complex vascular system.
- Reproduction: Isogamous.
Class Phaeophyceae (Brown algae)

The brown algae (singular: alga), comprising the class Phaeophyceae, are a large group of multicellular algae, including many seaweeds located in colder waters within the Northern Hemisphere. Most brown algae live in marine environments, where they play an important role both as food and as habitat.

- Occurrence: Mostly marine.
- Pigments: Chlorophyll a, c, carotenes, xanthophylls, chlorophyll b absent.
- Reserve Food: Mannitol as well as Laminarin and Fats.
- Structure: Can be bulky parenchymatous forms. Several plants attain giant size.
- Display internal and external differentiation.
- Reproduction: Ranges from isogamous to oogamous. Varied alternation of generation.
- Example: Ectocarpus, Sargassum

Class Rhodophyceae (Red Algae)

Rhodophyceae is a class name, but is effectively synonymous with Rhodophyta the red algae. It does not appear in all taxonomic systems. ITIS (Integrated Taxonomic Information System) records a subdivision, i.e., subphylum of Rhodophyta with the name Rhodophytina and synonym Rhodophyceae.

- Occurrence: Unicellular seaweeds found under this class but most members are filamentous and multicellular.
- Pigments: Chromatophores are red and blue like r-phycoerythrin and blue r-phycocyanin, chlorophyll a, d, and carotenes. The presence of phycobilin proteins enables the members to live at depths of 100m or more.
- Reserve Food: Floridean Starch composed of 1, 4, or 1, 6, Glucose residues is stored as food reserve in them.
- Structure: Simple filamentous form, attaining considerable complexity of structure. Motile structures are not known.
- Reproduction: Sexual reproduction is advanced and oogamous type. The male organ produces non motile gametes and female organ has a long receptive neck. After sexual reproduction special spores are produced.
- Examples: Batrachospermum, Polysiphonia.
Class Myxophyceae (Cyanophyceae or Blue Green Algae)

Cyanophyceae is a primitive group of algae, consists of 150 genera and about 2,500 species. In India, the division is represented by 98 genera and about 833 species. Members of the class Myxophyceae (Cyanophyceae) are commonly known as blue green algae. The name blue green algae is given because of the presence of a dominant pigment c-phycocyanin, the blue green pigment.

In addition, other pigments like chlorophyll a (green), c-phycoerythrin (red), β-carotene and different xanthophylls are also present. The members of this class are the simplest living Autotrophic Prokaryotes.

- Occurrence: Fresh and sea water as well as moist places.
- Pigment: Chlorophyll, carotenes, xanthophylls and c-phycocyanin and c-phycoerythrin. The ratio of last two pigments exhibits color variation, commonly blue green.
- Reserve Food. Sugars and Glycogen.
- Structure: Simple type from cell to filamentous. Some of the filamentous forms show false or true branching, very rudimentary nucleus, no proper chromatophores, the photosynthetic pigment diffused throughout the peripheral position. No motile stages.
- Reproduction: There is no sexual reproduction
- Examples: Oscillatoria, Nostoc.

Algae Pigments

Algae are photoautotrophic, unicellular colony as well as multicellular filamentous. Cell walls are made up of cellulose or pectin, and require high moisture for their growth. Reproduction is through sexual as well as asexual.

Green Algae: They are filamentous unicellular as well as multicellular organisms, contain chlorophyll a and b. They live close to water like pond scum and store starch like plants. They are considered the ancestors of plants, for example, Volvox, Spirogyra.

Phaeophyceae (Brown Algae or Kelp): They are rapidly growing and macroscopic, mostly found at intermediate steps. They produce dark pigment fucoxanthin, products of photosynthetic alcohol, fat and sugar organized into large sea weeds multicellular organisms, for example, Ectocarpus, Fucus and Laminaria. Figure 9.4 shows the life cycle of Laminaria showing alternation of generations.
Zygnematophyceae, a fossil group with two genera, has been suggested by Fritsch. Internal morphology is higher to chlorophyceae while spore tetrads are similar to Rhodophyceae.

**Red Algae:** They are photoautotrophic living at greater ocean depth. Red pigment allows them to absorb blue light penetrating deepest in the ocean. Agar is extracted from many red algae. Spermatia carried by water currents to the trichogyne of the female cell. Some produce lethal toxins, for example, Sea weeds, Polysiphonia Zygnematophyceae, a fossil group with two genera has been suggested by Fritsch. Internal morphology is higher to chlorophyceae while spore tetrads are similar to Rhodophyceae.

**Diatoms:** They are light brown pigment producing unicellular or filamentous organisms. They have complex cell walls made of pectin and silicon oxide. They store energy in the form of oil.

**Dinoflagellates (Plankton):** Chromatophores are dark yellow, brown, etc., and contain a number of special pigments. Flagella display bioluminescent.

**Reproduction**

Sporophyte are multicellular diploid structures that produce cells that undergo meiosis in sporangia to produce 1n spores grow into 1n organisms called gametophytes that can then undergo syngamy fertilization to produce 2n zygote that is the sporophyte. Sporophytes of this seaweed are usually found in water below the line of the lowest tides attached to the rocks by extending holdfast. Sporangia produces zoospores by meiosis. Structure of Zoospores are similar to each other. But a few of them have the ability of developing into a male gametophyte and female gametophyte. Gametophytes look
different in comparison to sporophyte, short, branched filaments that grow on the surface of sub tidal rocks. Male gametophytes release sperms, and female gametophytes produce eggs, which remain attached to gametophyte. Sperm fertilize the eggs. The zygotes grow into new sporophytes, starting life attached to the remains of the old gametophyte.

**Euglena**

Euglena is a genus of single cell flagellate eukaryotics. It is the best known and most widely studied member of the class Euglenoidea, a diverse group containing some 54 genera and at least 800 species. Species of Euglena are found in freshwater and salt water. Figure 9.5 illustrates the structural diagram of Euglena.

- Small group found mostly in freshwater, unicellular, and motile by flagellum.
- Mainly in eutrophic ponds and pools due to lots of organic material and nutrients.
- Lack of Cell wall as the most unusual feature.
- They have a flexible pellicle covering like a Protozoa.
- They have conspicuous Stigma or eyespot and the paraflagellar body near the base of the flagellum which is a photoreceptor attracted to light.
- It has chloroplast but can survive as heterotrophs like a Protozoa.

![Fig. 9.5 Euglena: Structural Diagram](image-url)
**Reproduction**

It is of following types.

**Asexual Reproduction**

- Mitosis

**Sexual Reproduction**

- Meiosis
- Zoospores
- Plus and Minus Gametes
- Zygospore
- Reproduction in Multicellular Algae

**Oedogonium Reproduction**

- Antheridium releases the flagellated sperm that swim to the oogonium.
- Oogonium houses the zygote which is the diploid spore.
- The spores undergoes meiosis and produces four haploid zoospores. One of them forms a root like holdfast and the other grows into a new filament.
- Two distinct multicellular phases.

**Gametophyte Haploid (1n):** Haploid 1n, multicellular, release gametes in alternation of generations.

**Sporophyte Diploid (2n):** Diploid 2n, multicellular, release spores in alternation of generations.

**Vegetative Reproduction**

**Protonema:** The secondary protonema developed either from rhizoidal node of primary protonema or from the basal node of primary rhizoid, for example, *Chara*.

**Asexual Reproduction:** Generally occurs in favourable conditions. In Cyanophyceae the sexual reproduction is absent. It is uniparental where two sex strains are not required and mode of cell division is mitotic.

**On the Basis of Structure of the Spores**

- **Zoospores:** They are motile with naked structures with two, four or many flagella. These are produced in Zoosporangium in both unicellular and filamentous algae, for example, *Ulothrix, Ectocarpus*. The zoospores of Xanthophyceae are called synzoospore which bears number of flagella and is multinucleate structure. Motile spores absent in Cyanophyceae and Rhodophyceae.
• **Aplanospores**: These are non-motile spores, either a single cell produces an aplanospore or the protoplast may divide to form many aplanospores, for example, *Ulothrix, Vaucheria*.

• **Hypnospores**: Aplanospores secrete a thick wall to overcome prolonged period of desiccation. Under unfavourable conditions hypnospores germinate and grow into new individuals. The hypnospores of *Chlamydomonas nivalis* are red in colour due to deposition of pigment, hematochrome, in their cell walls, for example, *Chlamydomonas*.

• **Tetraspores**: Diploid plants of some algae form aplanospore which are four in number.

• **Autospore**: Aplanospores that are similar to the parent cell.

• **Akinete**: In some alga vegetative cells develop into thick walled spores with abundant food reserves. These are resting cells, for example, *Nostoc*. They can survive unfavourable conditions and primarily for perennation rather than multiplication.

• **Carpospores**: In Rhodophyceae, for example, these are formed when zygote divides as in Batrachospermum.

• **Exospores**: These are formed externally by the protoplast.

• **Endospores**: These are non-motile and produced by division of protoplast, for example, in Cyanophyceae.

**Sexual Reproduction**

Usually takes place under adverse condition. The gametes can be from the same parent, i.e., monoecious, or from two different parent, i.e., dioecious. It involves three phases:

• Plasmogamy: Fusion of Cytoplasm.

• Karyogamy: Fusion of Two Different Gamete Nuclei.

• Meiosis: Division of Zygote to produce Haploid Cells.

On the basis of structure and physiological behavior of sex organ and their complexity.

• **Isogamy**: The fusion of two morphological similar gamete that are haploid and naked. In chlamydomonas the mature adult may directly function as gamete (hologamy) (Refer Figure 9.6).

• **Heterogamy**: Fusion occurs between morphologically as well as physiologically different gametes.
  
  o **Anisogamy**: Dissimilar gametes participate where male is more active and smaller in size-microgamete and females less active and bigger-macrogamete (Refer Figure 9.7).
o **Physiological Anisogamy**: Fusing gametes with different strains are morphologically similar but physiologically different.

o **Oogamy**: It is the most advanced stage of sexual reproduction in which male gamete develops within antheridium while female gamete is formed within non-motile oogonium.

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**Fig. 9.6 Isogamy Sexual Reproduction**

**Fig. 9.7 Anisogamy Sexual Reproduction**
Type Study

*Chlamydomonas* reproduces asexually by fragmentation and cell division and sexually. All cells are similar cells including the vegetative cells. Asexual reproduction by cell division occurs most frequently and sexual reproduction is induced by unfavorable condition. Here zygotic meiosis takes place for sexual reproduction except for zygote which is haploid. Figure 9.8 illustrates the diagrammatic life cycle of *Chlamydomonas*.

![Diagram of Chlamydomonas life cycle](image)

*Fig. 9.8 Life Cycle of Chlamydomonas*

Unfavorable conditions, such as nitrogen deprivation, (+) and (–) haploid strains of cells produce through mitosis haploid gametes. Gametes pairing takes place under plasmogamy and karyogamy resulting in diploid zygote, the zygospore. It has thick protective wall that can withstand unfavorable conditions. On return of favorable environment zygotic meiosis takes place forming four haploid cells.

*Volvox* is at the end of evolutionary pinnacle of a colonial green colony alga that is based on Chlamydomonas derived cells. The cells of the colonial green alga are connected by cytoplasmic connections that allow cell to cell communication. On the periphery the cells are biflagellate and the colonies are hollow. The synchronous beating of flagellar movement brings about displacement of colony. There is a photoreceptor eye spot present in each cell.

The cells that are specialised for sexual reproduction are through oogamous zygotic meiosis. Some are specialised for asexual reproduction
and undergo mitosis to form daughter colonies within the parent colony. On maturing daughter colony break from parent colony and becomes free living.

**Spirogyra** During sexual reproduction a conjugation tube forma between two filaments. This may have fertilisation in them or with gametes migrating into the tube of the other filament. The zygote becomes surrounded by thick wall to pass through the unfavorable conditions.

**Ecological Importance of Algae**

- Important part of food chain in aquatic ecosystem and can be used by heterotrophs for fixing carbon dioxide.
- 80% of the earth’s oxygen is believed to be produced by planktonic algae.
- Algal blooms are indicator of water pollution.
- Grow rapidly in water with high concentration of organic material (sewage or industrial waste).
- Petroleum and natural reserves were formed primarily from diatoms and plankton, many unicellular algae are symbionts in animals.
- Many symbiotic in coral animals as zooxanthellae base of food chain to tropical coral reefs since waters are poor in nutrients.

**Alga in Sewage Disposal:** Alga like *Chlorella, Euglena*, are grown in sewage oxidation ponds with suitable bacteria (Algal-Bacterial System). They provide surplus oxygen for aerobic decomposition. The amount of nitrates and phosphates from effluents are picked up for metabolism releasing $\text{O}_2$ in the process. The Aerobic Bacteria then decomposes raw sewage, thus purifying the waste.

**Biological Importance**

- Algae with their increasing versatility are finding applications in many emerging spheres as nutritional supplement, fertilizer, etc.
- Algae as a source of biofuels have been recognized as the rich energy source and have been utilized to produce biofuels, such as bioethanol, biodiesel and many more. These renewable fuels produced are being termed third generation fuels.
- Algae as a bio-fertilizers includes the Blue Green Algae, which act as bio-fertilizers. They have the capacity to accumulate mineral like red and brown algae are rich in potassium. They can be used as organic fertilizers. It contain microorganisms which has the capacity to restore the soils natural nutrient cycle and build soil organic matter.
- Alga in binding of soil particle. It plays an important role as binding agent on the surface of the soil. It can also be used in the reclamation of barren alkaline soil.
• Algae as a medicines include the Green Alga *Chlorella* which produces Antibiotic Chlorellin, very effective against certain Pathogenic Bacteria. Similar antibiotics obtained from a diatom *Nitzschia palea* against *E.coli* due to high iodine contents. Many seaweeds are used in goitre medicine preparation due to high percentage of iodine content in them.

• Alga in industry include the four major products derived from algae, namely Diatomite, Agar, Carrageenin and Alginates.

  **Diatomite** is a porous sedimentary rock formed by the accumulation of amorphous silica remains of dead diatoms in marine sediments. This rightly known as Diatomaceous Earth has many applications. **Kelps** are a rich source of Soda, Iodine, Potash and Aliginic Acid.

  **Agar Agar** is a non-nitrogenous jelly like substance extracted from different species of Red Algae. It used as a culture medium in laboratories because of its ability to afford good range of temperature for culturing.

  **Alginates:** Salts of Alginic Acid present in cell wall of Phaeophyceae is found in sea weeds and isolated from laminaria. They are used as thickeners and emulsifiers in various commercial products like Syrups and Ice Cream.

**Detrimental of Algae**

• Blooms of Freshwater Algae.
• Toxins accumulated in Food Chain.
• Red Tides.
• Fouling of Ships and other surfaces covered with Water.
• Fouling of Shells of Commercial Bivalves.

### Check Your Progress

5. What is a Prokaryotic algae?
6. What does Eukaryotic algae contain?
7. What are diatoms?
8. What are Chrysophyceae?
9. What is green algae?

### 9.4 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Algae lack the various structures that characterize land plants, such as the phyllids (leaf-like structures) of bryophytes, rhizoids
in non-vascular plants, and the roots, leaves, and other organs found in tracheophytes (vascular plants).

2. General characteristics of algae are as follows:
   - Diverse group of simple eukaryotic photosynthetic autotrophs.
   - Unicellular or multicellular plant like organisms that belong to Kingdom Protista.
   - They can be Prokaryotes or Eukaryotes. All blue green algae are Prokaryotes.
   - Most are found in the ocean or other water bodies in their pursuit for reproduction, support and nutrition with high productivity.
   - Absorbs nutrients from water and form the base of the food chain.
   - All reproduce asexually and a few sexually lacking sterile jacket of cells around reproductive cell.

3. Macroalgae are multicellular marine algae which form a real plant. They have a stem and leaves. Depending on water depth they are brown, red or green. Marine algae stick with their root on rock and are completely rinsed from sea water. They are 4000 different marine algae but only a few suitable for human consumption. Algae contain valuable nutrients, among others lots of trace elements including one very important, iodine. Another essential element is alginate used in various matters especially in food production. Microalgae are different, they are one cell algae. *Spirulina, Chlorella* or also blue green algae are very popular in this variety. They are cultivated in fresh water tanks. The ‘*Algae Bloom*’ which is driving at certain seasons in coastal regions is also one of the microalgae.

4. Similarities between microalgae and macroalgae:
   - Both algae and microalgae show large diversity.
   - They are both primary producers of oxygen during photosynthesis.
   - Both serve as natural filters of phosphates and nitrogenous wastes.
   - They both serve as excellent food sources in aquatic ecosystems.

5. Blue green algae, Cyanobacteria: placed under Monera. The algae do not belong to Kingdom of Plantae. They are considered as lower forms of plants due to their photosynthetic characteristics in comparison to other vascular plants.

6. The Eukaryotic algae possess membrane bound organelles, for example, nuclei, mitochondria and plastids unlike Prokaryotes.

7. Diatoms are a major group of algae, specifically microalgae, found in the oceans, waterways and soils of the world. Living diatoms number in the trillions, they generate about 20 percent of the oxygen produced on the planet each year, take in over 6.7 billion metric tons of silicon each
year from the waters in which they live, and contribute nearly half of
the organic material found in the oceans. The shells of dead diatoms
can reach as much as a half mile deep on the ocean floor, and the entire

8. The Chrysophyceae, usually called chrysophytes, chrysomonads,
golden-brown algae or golden algae are a large group of algae, found
mostly in freshwater. Golden algae is also commonly used to refer to
a single species, *Prymnesium parvum*, which causes fish kills.

9. Green algae are filamentous unicellular as well as multicellular organisms,
contain chlorophyll a and b. They live close to water like pond scum
and store starch like plants. They are considered the ancestors of plants,
for example, *Volvox, Spirogyra*.

### 9.5 SUMMARY

- Algae is an informal term for a large, diverse group of photosynthetic
eukaryotic organisms that are not necessarily closely related, and is
thus polyphyletic.
- Most are aquatic and autotrophic and lack many of the distinct cell
and tissue types, such as stomata, xylem, and phloem, which are found
in land plants.
- The largest and most complex marine algae are called seaweeds, while
the most complex freshwater forms are the Charophyta, a division of
green algae which includes, for example, *Spirogyra* and the Stoneworts.
- Algae constitute a polyphyletic group since they do not include a
common ancestor, and although their plastids seem to have a single
origin, from Cyanobacteria, they were acquired in different ways.
- Green algae are examples of algae that have primary chloroplasts derived
from endosymbiotic Cyanobacteria.
- Diatoms and brown algae are examples of algae with secondary
chloroplasts derived from an endosymbiotic red alga.
- Algae exhibit a wide range of reproductive strategies, from simple
asexual cell division to complex forms of sexual reproduction.
- Algae lack the various structures that characterize land plants,
such as the phyllids (leaf-like structures) of bryophytes, rhizoids
in non-vascular plants, and the roots, leaves, and other organs found
in tracheophytes (vascular plants).
- Most are phototrophic, although some are mixotrophic, deriving
energy both from photosynthesis and uptake of organic carbon either
by osmotrophy, myotrophy, or phagotrophy.
• Some unicellular species of green algae, many golden algae, euglenids, dinoflagellates, and other algae have become heterotrophs, also called colorless or apochlorotic algae sometimes parasitic, relying entirely on external energy sources and have limited or no photosynthetic apparatus.

• Some other heterotrophic organisms, such as the apicomplexans, are also derived from cells whose ancestors possessed plastids, but are not traditionally considered as algae.

• Algae have photosynthetic machinery ultimately derived from cyanobacteria that produce oxygen as a by-product of photosynthesis, unlike other Photosynthetic Bacteria, such as purple and green sulphur Bacteria.

• Algae are simple, non-flowering, aquatic organisms, consisting of large assemblage of single-celled forms and seaweeds.

• Macroalgae are multicellular marine algae which form a real plant. They have a stem and leaves. Depending on water depth they are brawn, red or green Marine Algae stick with their root on rock and are completely rinsed from sea water.

• Algae contain valuable nutrients, among others lots of trace elements including one very important: iodine. Another essential element is algin used in various matters especially in food production.

• Microalgae are one cell algae. *Spirulina, Chlorella* or also blue green algae are very popular in this variety. They are cultivated in fresh water tanks. The ‘Algae Bloom’ which is driving at certain seasons in coastal regions is also one of the microalgae.

• Microalgae do not have roots, stems or leaves. Microalgae that are able to perform photosynthesis are vital to life on Earth; they produce around half of the oxygen found in the atmosphere, they utilize the sun’s light and carbon dioxide in their growth.

• Microalgae are found in the aquarium, mainly in the form of nuisance slime algae (cyanobacteria) and as the somewhat more useful calcifying algae (coralline).

• Macro and microalgae are used in a variety of commercial products with many more in development.

• Well-known culinary products include Nori, Wakame, Kombu and Dulse, from whole macroalgal biomass.

• The microalgae *Spirulina* and *Chlorella* have been widely marketed as nutritional supplements for both humans and animals.

• Several microalgae with a high nutritional value and energy content are grown commercially as aquaculture feed.
• Pigments extracted from algae include β-carotene, astaxanthin, and phycobiliproteins.
• Algae have great potential to produce a wide range of valuable compounds, beyond their current exploitation.
• The Eukaryotic algal cell is surrounded by a cell wall. A few are enclosed by an outer matrix outside the cell wall.
• Alga thalli show a wide range of variation and ranges from unicellular microscopic structures to large sea weeds.
• Algae are either unicellular like Chlamydomonas or multicellular like Spirogyra. Multicellular algae may be in the form of colonies like Volvox or in the form of filaments as Spirogyra.
• A few algae are parasites, some grow in special habitats, for example, Parasites, Symbiotic Cryophytes and Thermophytes.
• Reproduction in algae is of three types, vegetative, asexual and sexual. Vegetative reproduction takes place by fragmentation, fission, budding, etc.
• Sexual reproduction in algae takes place during unfavorable conditions by producing gametes.
• The sporophyte representing the plant body and the major portion of the life cycle is diploid. The haploid stage is represented by the gametes, for example, Spirogyra, Chara.
• The Chrysophyceae, usually called chrysophytes, chrysomonads, golden-brown algae or golden algae are a large group of algae, found mostly in freshwater. Golden algae is also commonly used to refer to a single species, Prymnesium parvum, which causes fish kills.
• Diatoms are a major group of algae, specifically microalgae, found in the oceans, waterways and soils of the world.
• Living diatoms number in the trillions, they generate about 20 percent of the oxygen produced on the planet each year, take in over 6.7 billion metric tons of silicon each year from the waters in which they live, and contribute nearly half of the organic material found in the oceans.
• The brown algae (singular: alga), comprising the class Phaeophyceae, are a large group of multicellular algae, including many seaweeds located in colder waters within the Northern Hemisphere.
• Most brown algae live in marine environments, where they play an important role both as food and as habitat.
• Cyanophyceae is a primitive group of algae, consists of 150 genera and about 2,500 species.
In India, the division is represented by 98 genera and about 833 species. Members of the class Myxophyceae (Cyanophyceae) are commonly known as blue green algae.

The name blue green algae is given because of the presence of a dominant pigment c-phycocyanin, the blue green pigment.

Green Algae are filamentous unicellular as well as multicellular organisms, contain chlorophyll a and b. They live close to water like pond scum and store starch like plants.

Zygnematophyceae a fossil group with two genera has been suggested by Fritsch. Internal morphology higher to chlorophycceae while spore tetrads are similar to Rhodophyceae.

Red Algae are photoautotrophic living at greater ocean depth. Red pigment allow them to absorb blue light penetrating deepest in the ocean.

Agar is extracted from many red algae. Spermatia carried by water currents to the trichogyne of the female cell.

Diatoms are light brown pigment producing unicellular or filamentous organisms. They have complex cell walls made of pectin and silicon oxide. They store energy in the form of oil.

Euglena is a genus of single cell flagellate Eukaryotics. It is the best known and most widely studied member of the class Euglenoidea, a diverse group containing some 54 genera and at least 800 species.

Protonema is the secondary protonema developed either from rhizoidal node of primary protonema or from the basal node of primary rhizoid, for example, Chara.

Asexual reproduction: generally occurs in favourable conditions. In Cyanophyceae the sexual reproduction is absent. It is uniparental where two sex strains are not required and mode of cell division is mitotic.

Isogamy is the fusion of two morphological similar gamete that are haploid and naked.

In heterogamy fusion occurs between morphologically as well as physiologically different gametes.

In anisogamy dissimilar gametes participate where male is more active and smaller in size-microgamete and females less active and bigger-macrogamete.

Physiological anisogamy is the fusing gametes with different strains are morphologically similar but physiologically different.
• Oogamy is the most advanced stage of sexual reproduction in which male gamete develops within antheridium while female gamete is formed within non-motile oogonium.

• *Volvox* is at the end of evolutionary pinnacle of a colonial green colony alga that is based on *Chlamydomonas* derived cells.

### 9.6 KEY WORDS

• **Diatoms**: Diatoms are light brown pigment producing unicellular or filamentous organisms.

• **Isogamy**: Isogamy is the fusion of two morphological similar gamete that are haploid and naked.

• **Heterogamy**: In heterogamy fusion occurs between morphologically as well as physiologically different gametes.

• **Anisogamy**: In anisogamy dissimilar gametes participate where male is more active and smaller in size-microgamete and females less active and bigger-macrogamete.

• **Physiological anisogamy**: Physiological anisogamy is the fusing gametes with different strains are morphologically similar but physiologically different.

• **Oogamy**: Oogamy is the most advanced stage of sexual reproduction in which male gamete develops within antheridium while female gamete is formed within non motile oogonium.

### 9.7 SELF ASSESSMENT QUESTIONS AND EXERCISES

**Short Answer Questions**

1. What is algae?
2. Write the general characteristics of algae.
3. Draw a well-labelled diagram to distinguish algae from a land plant.
4. What are the similarities that can be found between microalgae and macroalgae?
5. Write a short note on cyanobacteria.
6. Write a short note on chlorophyceae.
7. How is algae ecologically important?
Long Answer Questions

1. Discuss about algae, its general characteristics, distribution in detail.
2. Elaborate a note on microalgae and macroalgae. Write about it economic importance as well.
3. Draw a well-labelled diagram of a typical Eukaryotic cell of algae.
4. Write a note on the characters of different classes of algae given by Fritsch.
5. With the help of well labelled diagram explain the life cycle of Chlymadomonas.
6. What are the ecological and biological importance of algae?

9.8 FURTHER READINGS

UNIT 10 THE WORLD OF PROTOZOA

Structure
10.0 Introduction
10.1 Objectives
10.2 Protozoa: An Introduction
   10.2.1 Cytoskeleton
10.3 Importance of *Entamoeba histolytica* and *Plasmodium* Species
   10.3.1 Life Cycle of *Plasmodium*
10.4 Answers to Check Your Progress Questions
10.5 Summary
10.6 Key Words
10.7 Self Assessment Questions and Exercises
10.8 Further Readings

10.0 INTRODUCTION

The Protozoa are considered to be a subkingdom of the kingdom Protista, although in the classical system they were placed in the kingdom Animalia. More than 50,000 species have been described, most of which are free living organisms. Protozoa are found in almost every possible habitat. The fossil record in the form of shells in sedimentary rocks shows that Protozoa were present in the Pre-Cambrian era. Virtually all humans have Protozoa living in or on their body at some time, and many persons are infected with one or more species throughout their life. Some species are considered commensals, i.e., normally not harmful, whereas others are pathogens and usually produce disease. Protozoan diseases range from very mild to life-threatening. Individuals whose defenses are able to control but not eliminate a parasitic infection become carriers and constitute a source of infection for others.

Many protozoan infections that are inapparent or mild in normal individuals can be life-threatening in immunosuppressed patients, particularly patients with Acquired Immune Deficiency Syndrome (AIDS). Evidence suggests that many healthy persons harbor low numbers of *Pneumocystis Carinii Pneumonia* (PCP) in their lungs. PCP is a life-threatening lung infection that can affect people with weakened immune systems. *Entamoeba histolytica* is an anaerobic parasitic amoebozoan which infects humans and other primates causing Amoebiasis. *Plasmodium* is a genus of unicellular Eukaryotes that are obligate parasites of vertebrates and insects.

In this unit, you will study about Protozoa and its general characteristics, importance of *Entamoeba histolytica* and *Plasmodium* species in detail.
10.1 OBJECTIVES

After going through this unit, you will be able to:

- Explain the general characteristics of Protozoa
- Discuss about *Plasmodium* species
- Understand what *Entamoeba histolytica* is

10.2 PROTOZOA: AN INTRODUCTION

The term protozoa comes from the Greek words for ‘*Proto*’ meaning ‘First’ and ‘*Zoa*’ meaning ‘Animals’. Protozoa are therefore termed as the ‘First Animal’ with single celled organisms having animal like characteristics. Scientists consider that Protozoa may resemble the first Eukaryotes that evolved from Prokaryotes. Originally a term envisaged by Haeckel, who defined that Protista represents a diverse group of organisms. This was based on the assumption of their Eukaryotic characters and failure to fit satisfactorily in animals, plant or fungal kingdoms. With the help of molecular evidence Protists are divided into plant like Algae and animal like Protozoa. On the basis of molecular and cytological comparisons, the animal like protozoan is closely related to photosynthetic and plant like Euglena.

The domain Eukaryotes is defined by the presence of a unique diagnostic feature, the cell nucleus. All members of the domain possess a nucleus, chromosomes, where DNA is complexed with histones, cell division via mitosis, and a system of structural and contractile proteins called the cytoskeleton.

Protista is a taxon, a kingdom has the following important biological concerns:

- Check for animal, plant and fungal origins.
- Check for Eukaryotic origins.
- Check for Eukaryotic photosynthesis and related metabolic pathways.

Protozoa are a diverse assemblage with mixed affinities, such as:

- They lack a cell wall.
- They have at least one motile stage in the life cycle.
- They ingest their food.

General Characteristics of Protozoan Phyla

- A protozoan is a complete organisms in which all life activities are carried on within the limits of a single plasma membrane.
- Phylogenetic studies show that Protozoa do not form a monophyletic group.
• Although they are unicellular organism but the protozoan cell organelles are highly specialized.
• They are ecologically diverse, but are limited to narrow environmental ranges.
• Some are colonial with multicellular stages but have non-colonial forms.
• Protozoans have only one non reproductive cell type and lack embryonic development. The embryonic development is one of the criteria for metazoan.
• Protozoa are Protists displaying heterotrophic nutrition. Presence of moisture is essential for their survival as they are susceptible to desiccation.
• A Protozoan cell may be more complex than a single animal cell and have the same morphology and physiology of a typical Eukaryotic cell.
• There are certain evolved structures as well like that of the contractile vacuole which is a fluid filled vacuole involved in the osmoregulation of certain Protists.
• This is vital for freshwater protozoans, since they live in hypotonic environment.
• Most protozoans have heterotrophic mode of nutrition and carry out their digestion in phagocytic vacuoles.

Protistan Habitat

They are mostly aquatic, damp soil, leaf litter, and other terrestrial habitats that are sufficiently moist. Many are bottom dwellers that attach themselves to rocks. Thus they are planktons, the microscopic communities of organisms that drift passively near the water interface. These commonly found planktonic Eukaryotic Algae, along with Prokaryotic Cyanobacteria are the bases of most marine and freshwater food webs. Phytoplankton are primary photosynthetic producers of half the organic matter in the world. They help support abundant heterotrophic Protists, Prokaryotes and Animals.

Many are symbionts that inhabit body fluids, tissues or cells of hosts, from mutualism to parasitism. Ciliates are a diverse group most of them being solitary fresh water inhabitants that use cilia to move and to feed. Dinoflagellates are all unicellular and mostly marine of greater ecological evolutionary and morphological interest. Abundant aquatic phytoplanktons suspend near the water surface and provide foundation of most marine and water food webs. Most of them are unicellular and provide heterotrophic species of dinoflagellates. Their population blooms causes red tides in coastal waters. The presence of predominant pigment in the plastids - Xanthophylls give a reddish appearance to it. *Pfiesteria piscicida* is a carnivorous
The world of Protozoa

NOTES

Locomotion in Protists

Amoeboid Movement: Commonly found gliding movement in the Protists though cell molecular mechanisms still not clear in them. Long fingerlike projections called pseudopodia stream forward over a substrate. This involves interaction between proteins called actin and myosin. This is closely related to muscle movement in animals. But the molecular sequence of the movement is yet to be discovered.

Flagellar and Ciliated Movement: Flagella and cilia are whiplike organelle that are identical structures. They are composed of nine sets of doublet microtubules arranged around two central single microtubules. Short cilia occur usually on a cell in abundance. Flagella are usually found singly or in pairs are long. Both function like whiplike organelles that push or pull the cell through its aqueous environment, or they may move over the surface of the cell. They differ in their lengths and their patterns of beating. They are made up of major motor protein dynein. An undulating motion occurs as dynein molecules walk down microtubules.

Both appear different the structures found in bacteria that are made up of a protein called flagellin. Instead of undulating the flagella rotate to produce movement.

10.2.1 Cytoskeleton

The cytoskeleton of Eukaryotes possess a set of long thin fibres called the cytoskeleton. These play the following roles:

- Maintains Cell Shape and Support.
- Facilitates Various Types of Movements.
- Supports Motor Proteins which help the Cell to Move.

The cytoskeleton has three main components: microfilaments, intermediate filaments and microtubules.

Microfilaments can exists as single filaments, in bundles or networks made up of actin, a protein that exists in several forms constituting many functions in the animal species. The polymerisation forming a double helix of actin into microfilaments is reversible and they can disappear from cells when required into free units of free actin.

- Microfilaments help the entire cell to contract.
- They stabilize cell shape.

Actin: In muscles cells, actin fibres are associated with another protein called myosin, and they together bring about contraction of muscles. In other
The World of Protozoa

Somatic cells they are concerned with localized changes of shape in cells cytoplasmic streaming. Microfilaments are also involved in the formation of cellular extensions, called pseudopodia.

**Intermediate Filament:** They are composed of fibrous protein of keratin family, with the same general structure for stabilizing cell structure.

**Microtubules:** These are long and hollow, unbranched cylinders assemblage of tubulin. They,
- Form a rigid internal skeleton especially with cell extensions.
- Framework on which motor protein can move structures in the cell.

Tubulin is made up of two polypeptide monomers, called Alpha Tubulin and Beta Tubulin. Addition of tubulin makes microtubules capacity for rapid elongation.

**Microtubules Power Cilia and Flagella:** There is a basal body found at the base of every Eukaryotic flagellum. The nine microtubules doublets extend into the basal body. The doublet are linked by proteins. The motion of cilia and flagella results from sliding of the microtubules, driven by a motor protein called dynein, which undergo changes in its shape driven by energy from ATP (Adenosine Triphosphate). One microtubule bind to another tubule. Dynein molecules change shape to bring about the movement of the microtubule.

**Characteristic Features of Representative Types**
- Protozoa have intracellular specialization or organization of organelles in cells.
- Cells have distinct function. The colonial Protozoa have separate somatic and reproductive zooids.
- Asexual reproduction occurs by mitotic division.
- True sexual reproduction is with zygote formation.
- Responses to stimuli represent the simplest reflexes and inborn behaviours.
- Shelled Protozoa have the simplest exoskeleton.
- Basic enzyme support all type of nutrition, namely the autotrophic, heterotrophic and saprozoic.
- Many have developed means of locomotion.
- No germ layers present.
- No organ tissue are formed but have specialised organs to serve these functions.
- Most are naked, but some have a simple endoskeleton or exoskeleton.
Economic Importance

Protozoa play a significant role in the economy of nature. They make up a large part of plankton that are important link in aquatic food chains and food webs. They are useful in biochemical and molecular investigations. Some of the diseases caused by them are discussed below:

Pathogenic Protozoa

Amoeboflagellates (Phylum Sarcomastigophora): Use pseudopods or false feet or flagellum for locomotion.

I. Amoebas (Subphylum Sarcodina)

Move around by extending blunt projections called pseudopods. This also helps them in engulfing food and bring about phagocytosis.

Some species cause amoebic dysentery.

- *Entamoeba hystolytica*: Feed on blood cells. Causative agent of dysentery and intestinal cyst.
- *Dientamoeba fragilis*: Causes diarrhea, found as commensals.
- *Keratitis*: Caused by *Acanthamoeba*. Results in blindness with perpetual use of contact lenses.

II. Flagellates (Subphylum Mastigophora)

Flagella used as locomotory organ. These are spindle shaped projecting from anterior end. Outer membrane is tough pellicle with an oral groove or cytostome for digestion. Table 10.1 shows the Pathogenic Protozoa.

Important Pathogens

- *Trichomonas vaginalis*: Causes the genital and urinary infection transmitted sexually by fomites.
- *Giardia lamblia*: Causes a persistent intestinal infection (Giardiasis) with diarrhea, nausea, flatulence, and cramps. In U.S. most common cause of waterborne diarrhea. About 7% of U.S. population are healthy carriers.
- *Trypanosoma cruzi*: Hemoflagellate that causes Chagas disease, a cardiovascular disease common in Texas and Latin America. Transmitted by kissing bug living in mud houses or blood transfusions.
### Table 10.1 Pathogenic Protozoa

<table>
<thead>
<tr>
<th>Protozoan Group</th>
<th>Genus</th>
<th>Host</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoebae</td>
<td>Entamoeba</td>
<td>Mammals</td>
<td>Amebiasis</td>
</tr>
<tr>
<td></td>
<td>Iodamoeba</td>
<td>Pig</td>
<td>Enteritis</td>
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<tr>
<td>Sporozoa</td>
<td>Babesia</td>
<td>Cattle</td>
<td>Sarcocystis</td>
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<tr>
<td></td>
<td>Theileria</td>
<td>Cattle, Sheep, Mammals, Birds</td>
<td>Theileriasis</td>
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<tr>
<td></td>
<td>Sarcocystis</td>
<td>Cats</td>
<td>Sarcosporidiosis</td>
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<tr>
<td></td>
<td>Toxoplasma</td>
<td>Dogs</td>
<td>Toxoplasmosis</td>
</tr>
<tr>
<td></td>
<td>Isospora</td>
<td>Cattle, Cats, Chickens, Pig</td>
<td>Coccidiosis</td>
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<tr>
<td></td>
<td>Eimeria</td>
<td></td>
<td>Coccidiosis</td>
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<tr>
<td></td>
<td>Plasmodium</td>
<td>Many Animals</td>
<td>Malaria</td>
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<tr>
<td></td>
<td>Leucocytozoon</td>
<td>Birds</td>
<td>Leucocytozoosn</td>
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<tr>
<td></td>
<td>Cryptosporidium</td>
<td>Mammals</td>
<td>Cryptosporidiosis</td>
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<tr>
<td>Ciliates</td>
<td>Balantidium</td>
<td>Pig</td>
<td>Balantidiasis</td>
</tr>
<tr>
<td>Flagellates</td>
<td>Leishmania</td>
<td>Dogs, Horses Sheep, Cattle</td>
<td>Leishmaniasis</td>
</tr>
<tr>
<td></td>
<td>Trypanosoma</td>
<td>Most Animals</td>
<td>Trypanosomiasis</td>
</tr>
<tr>
<td></td>
<td>Trichomonas</td>
<td>Horses, Cattle Birds</td>
<td>Trichomoniasis</td>
</tr>
<tr>
<td></td>
<td>Histomonas</td>
<td>Birds</td>
<td>Blackhead Disease</td>
</tr>
<tr>
<td></td>
<td>Giardia</td>
<td>Mammals</td>
<td>Giardiasis</td>
</tr>
</tbody>
</table>

#### Morphological Variations

- Mostly they are unicellular, aerobic and chemoheterotrophic in nature of kingdom Protista.
- Reproduction is asexual by fission; budding or schizogony as well as sexually like Paramecium. They grow well in moisture. Protozoa is usually covered with pellicle.
- Feeding is through special structures and digestion takes place inside the vacuoles. Life cycle has two forms one is trophozoite which is a vegetative and growing stage, and another one is the cyst stage.
- They switch from one host to another in order to resist adverse environmental conditions. Cysts is a protective capsule that help them survive in the unfavourable conditions and turn back to their vegetative stage when favourable conditions returns.

The cytoplasm in some species appears semisolid or gelatinous ectoplasm, giving some rigidity to the cell body. The base of flagella or cilia are embedded in the ectoplasm. Inside the ectoplasm is the more granular endoplasm. Table 10.2 shows a brief outline classification of the Protozoa.
### Table 10.2 Classification of Protozoa

<table>
<thead>
<tr>
<th>Taxonomic Group</th>
<th>Characteristics</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phylum: Sarcomastigophora</strong></td>
<td>Locomotion by flagella, pseudopodia, or both; Sexual reproduction is essentially syngamy</td>
<td>Giardia, Leishmania, Trichomonas, Trichonympha, Trypanosoma</td>
</tr>
<tr>
<td><strong>Subphylum: Mastigophora</strong></td>
<td>One or more flagella; Division by longitudinal binary fission; Sexual reproduction in some groups</td>
<td>Amoeba, Coccodiscus, Elphidium</td>
</tr>
<tr>
<td><strong>Class: Zoomastigophorea</strong></td>
<td>Parasitic without chromatophores; One to more flagella; Sexuality known in some groups</td>
<td>Labyrinthula</td>
</tr>
<tr>
<td><strong>Subphylum: Sarcodina</strong></td>
<td>Locomotion by pseudopodia; Shells present; Flagella restricted to reproductive stages; Asexual reproduction by fission</td>
<td>Cryptosporidium, Eimeria, Plasmodium, Taxoplasma, Nosema, Nosema</td>
</tr>
<tr>
<td><strong>Superclass: Rhizopoda</strong></td>
<td>Locomotion by pseudopodia or by protoplasmic flow; Some contain tests</td>
<td>Pleistophora</td>
</tr>
<tr>
<td><strong>Phylum: Labyrinthomorpha</strong></td>
<td>Spindle-shaped cells capable of producing mucous tracks; Trophic stage as ectoplasmic network; Nonamoeboid cells parasitic on algae and seagrass</td>
<td>Hoplosporidium</td>
</tr>
<tr>
<td><strong>Phylum: Apicomplexa</strong></td>
<td>Parasitic, spore-forming and have an apical complex; Sexuality by syngamy; Cysts often present; Cilia absent</td>
<td>Myxosoma</td>
</tr>
<tr>
<td><strong>Phylum: Myxospora</strong></td>
<td>Unicellular spores with spiroplasm containing polar filaments; Obligate intracellular parasites</td>
<td>Myxosoma</td>
</tr>
<tr>
<td><strong>Phylum: Ascetospora</strong></td>
<td>Spore with one or more spiroplasms; No polar capsules or filaments; Parasitic in invertebrates</td>
<td>Myxosoma</td>
</tr>
<tr>
<td><strong>Phylum: Paramecium</strong></td>
<td>Spores of multicellular origin; One or many polar capsules; All parasitic, especially in fish</td>
<td>Myxosoma</td>
</tr>
<tr>
<td><strong>Phylum: Ciliophora</strong></td>
<td>Simple cilia or compound ciliary organelles in at least one stage in the life cycle; Two types of nuclei; Contractile vacuole present; Binary fission transverse; Sexuality involving conjugation; Most species free living, but many commensal, some parasitic</td>
<td>Balantidium, Didinium, Entodinium, Ichthyophihirius, Nyctotherus, Paramecium</td>
</tr>
</tbody>
</table>

**Source:** Prescott et al. (2005).

- **Phylum Sarcomastigophora:** The phylum Sarcomastigophora belongs to the Protista kingdom and it includes many unicellular or colonial, autotrophic, or heterotrophic organisms. It is characterized by flagellae, pseudopodia, or both, for locomotion. The common genera are Giardia, Leishmania, Trichomonas, Trichonympha, Trypanosoma, Entamoeba, etc. It also includes various types of symbiotic amoebae, of which most require other animals as host to survive, such as Endamoeba and Entamoeba.
- **Phylum Labyrinthomorpha**: This phylum includes Protists which are non-amoeboïd vegetative cells that are spindle shaped or spherical. These move in network of mucous tracks by gliding motion.

- **Phylum Apicomplexa**: Collectively called as sporozoans. Lack locomotory organs so have a spore forming stage in their life cycle. They are inter or intra cellular parasites of animals. They are parasites distinguished by a unique apical complex collection of microtubules, microfibrils located at one end of cell plasmodium.

- **Phylum Micospora**: Obligatory intracellular parasites lacking mitochondria. Many are pathogenic and have an important role in the biological control of certain insects.

- **Phylum Ascetospora**: Parasitic Protists that lack spore lacking polar caps or polar filaments. Ascetosporans found in tissues of molluscs.

**Protozoa**

Mostly they are unicellular, aerobic and chemoheterotrophic in nature of kingdom Protista (Refer Figure 10.1). Reproduction is asexual by fission; budding or schizogony as well as sexually like paramecium. They grow well in moisture. Protozoa is usually covered with pellicle Feeding is through special structures and digestion takes place inside the vacuoles. Life cycle has two forms one is trophozoite which is a vegetative and growing stage, and another one is the cyst stage. They switch from one host to another in order to resist adverse environmental conditions. Cysts is a protective capsule that help them survive unfavourable conditions and turn back to their vegetative stage when favourable conditions returns.

*Fig. 10.1 Some Protozoa*
Plant Life: Alternation of Generation. Life cycles are highly varied in them. Though mitosis occurs in nearly all protists but variations found are not known in other eukaryotes. They undergo sexual processes of meiosis and syngamy, thereby shuffling genes between two individuals followed by asexual reproduction. The haploid stage is the main vegetative feeding and growing stage of most protists with zygote being the only diploid cell.

Meiosis and fertilization is common to all sexually producing organisms.

- The only haploid cells are gametes. They fuse to form diploid zygote. This product divided by mitosis into diploid multicellular individual.
- Zygote under meiosis forming haploid cells that then undergo meiosis to produce haploid multicellular organisms.
- Alternation of generations, includes both a diploid multicellular stage and a haploid multicellular stage.

Protozoa is not a coherent taxonomic group with a common ancestor but a collection of phylogenetically diverse species. Each of the group have a close evolutionary relationship with certain algal groups.

On the basis of gene sequencing and motility it can be arranged as below:

1. Archaea: They are spindle shaped and lack mitochondria. They are having flagella at the front end and common symbionts in animals, for example Giardia. Originally zooflagellates may be symbiotic, or free living or parasitic. The latter has the, for example, Trapanosoma. They possess a unique organelle kinetoplast found in the cells single mitochondria containing its own DNA. The flagellum originates from the undulating membrane that gives trypanosome its characteristic locomotion. The infectious form T. brucei develops in the salivary glands of the intermediate host the Tse-Tse Fly. On entering the human host it enters the CNS by way of blood and lymphatics. Inflammation of the brain and spinal cord result in characteristic lethargy, coma and death.

The choanoflagellate – specialised group of zooflagellates represents closest single celled relatives of animals. They have microvilli surrounding each flagellum. This arrangement is similar to the one found in multicellular animals sponges. This connection is supported by molecular evidence. Thus they both share the flat lamellar cristae in mitochondria.

The Diplomonads is worthy of note under them. They have multiple flagella and two nuclei per cell but lack mitochondria. Giardia is an, for example, causing intestinal disturbance. It does not have a secondary host and survives as a resistant cyst before it is given up in infected water. It occupies a very distant branch in phylogenetic tree.
2. **Apicomplexa (Sporozoans):** Consists of obligate intracellular parasites, non-motile in mature form. Usually transmitted by insects, and have complex life cycle with different stages in different hosts, for example *Plasmodium*. They have complex life cycles involving both haploid and diploid phases and infecting more than one host.

3. **Amoeboida (Sarcodina):** This group contains causative agent of dysentery, and they move with the help of pseudopods, for example, *Entamoeba*.

4. **Ciliophora:** Only one pathogen in group called *Balantium coli*, which is also a causative agent of dysentery. They are motile because the presence of cilia present on the surface of cell, for example, *Paramecium*.

5. **Euglenozoa:** They have asexual mode of reproduction and movement with the help of flagella called zooflagellates. It can be divided into two groups.
   - **Euglenoids:** They are photoautotrophs as well as chemoautotrophs. They possess chlorophyll a and move with the help of flagella.
   - **Hemoflagellates:** They are long slender cells with undulating membrane and flagellum. They are transmitted through insects and live in host blood as the name implies, for example *Trapanosoma*.

**The Ciliates**

This is the most complex and largest group of protozoans. It shows the highest level of internal organisation in any single celled organism. A unique feature they have two distinct types of nuclei. Figure 10.2 shows the *Paramecium* conjugation between opposite mating strains.

- ** Macronuclei:** It encode enzymes and other proteins required for cells metabolic function. They are polyploidy, containing many copies of the genome.
- **Micronuclei:** It involves only in sexual reproduction by conjugation.

**Ciliated Protozoan**

![Fig. 10.2 Paramecium Conjugation between Opposite Mating Strains](image)
The Sporozoans: They have complex life cycles involving both haploid and diploid phases infecting more than one host. They are characterised by a spore like stage called sporozoite involved in transmission of parasite to a new host.

Check Your Progress

1. Give any four characteristic features of Protozoa.
2. What is phylum Sarcomastigophora?
3. What type of cells does phylum Labyrinthomorpha have?
4. What is apicomplexa?

10.3 IMPORTANCE OF ENTAMOEBA HISTOLYTICA AND PLASMODIUM SPECIES

10.3.1 Life Cycle of Plasmodium

Infectious Agent of Malaria has following two hosts.

*Plasmodium vivax* and *Plasmodium falciparum*: Cause malaria in human host (intermediate host). Four different species cause malaria. Transmitted by *Anopheles* (definitive host). DDT (Dichloro-Diphenyl-Trichloroethane) was used extensively in an attempt to eradicate the mosquito vector. Successful vaccine not available yet. Infected mosquito bites and infects a person. Sporozoites enter liver cells. These undergo multiple divisions to become merozoites (specialized spore) that uses apical complex to penetrate RBC. Merozoites reproduce asexually in RBC and lyse cells at 48 or 72 hours intervals. Some merozoite infect new RBCs, some divides to form gametocytes, these infect a biting female mosquito to complete life cycle in her body. Gametocytes form gametes and fertilization occurs in mosquito digestive tract. Zygote is the only diploid stage in the life cycle. Oocytes develop in walls of mosquito gut. Thousands of sporozoites develop in the oocyst and then migrate to mosquito’s salivary gland. They have complex life cycles involving both haploid and diploid phases and infecting more than one host.
Subphylum Sarcodina

Super Class: Rhizopoda, for example, *Amoeba proteus*.

- The cell membrane encloses ectoplasm and endoplasm.
- Amoeba feed on algae, rotifers by phagocytosis.
- Reproduction is by binary fission using mitosis.
- *Entamoeba coli* in the intestine and *E. Gingivalis* in the mouth are not disease agents.
- *Entamoeba histolytica* lives in human large intestine and attacks the intestinal wall with enzymes, causing severe and often fatal diarrhea.
- Some rhizoids have silicious or chitinoid test for protection; pseudopodia project from opening.
- *Foraminiferans* are shelled rhizopods found mainly in oceans, some have complex haploid and diploid cycles.
- Slime moulds in class Eumycetozoa live on forest detritus.
- They stream together to form a pseudoplasmodium with discrete cells or a multinucleate plasmodium producing a fruiting body.
- Radiolarians reproduce by binary fission, budding and sporulation.

10.3.2 *Entamoeba histolytica*

The amoeba goes through three stages of life cycle:

- **Trophozoit Amoeba**: This is the growing or feeding, most active form pathogenic to humans. The nucleus with a single chromatin enclosed by a delicate nuclear membrane. Ingested blood cells appear in the cytoplasm.
- **Encystation:** Trophozoites are discharged in the lumen from the intestinal wall and are transformed into cystic forms. This results in different stains.
  - **Precystic Amoeba:** Motility ceases and ingested particles are extruded from these ovoid blunt pseudopodias.
  - **Cystic Amoeba:** The parasite surrounds itself by a highly refractile cyst wall.

There are a few chromatid bodies present. The uninucleate cyst undergoes double fission so that four nuclei are produced. This stage is considered as the mature cyst (Refer Figure 10.4).

- **Excystation:** The release of the parasite from the cyst is known as excystation. These mature forms are ready for infection. They reach the alimentary canal along with contaminated food and water. The cyst wall is digested by the action of intestinal trypsin as the cyst reaches the caecum through amoeboid movement. The cytoplasm divides to eight metacystic amoebulae. The active young forms invade the intestinal tissue to reach the mucous and sub mucous layers.

**Fig. 10.4** *Entamoeba histolytica*, A. Trophozoite Stage, B. Precystic Stage, C-E Cysts, C. Uninucleate, D. Binucleate, E. Quadrinucleate Stage

**Reproduction in Entamoeba histolytica**

*Entamoeba* reproduces by binary fission, the rate of multiplication being very high. Figure 10.5 shows the life cycle in *Entamoeba histolytica*. 
The nucleus begins to elongate.
- A modified mitosis divides nucleus into two.
- The cytoplasmic mass begins to elongate and the parasite is divided into two equal halves.
- Each half receives one daughter nucleus producing two individuals.

**Modes of Infection**

The life cycle completes in one host - in this case being the man. Transmission from one host to the other is brought about by fecal contamination of drinking water. Eating of uncooked vegetables with infected forms can cause infection. Mature quadrinucleate cysts are infective forms. The viable cysts can pass through the intestine of fly or cockroach and infest new host through contaminated food.

**Protistan Taxonomy**

Traditional classification was based on morphological features, such as possession of flagella, chloroplast and other structures. This suggests that separate groups are closely related at molecular level. The Kinetoplastids and the euglenophytes both flagellate forms were distantly separated because latter contained chloroplasts. They are now known to be closely related in phylogenetic terms. Kinetoplastids previously placed with choanoflagellates and zooflagellates show very few similarities in evolutionary terms confirmed by molecular analysis.
Trypanosoma, a zooflagellate (Refer Figure 10.6) has a kinetoplast within a single large mitochondria. The flagellum is continuous with undulating membrane belonging to kinetoplastid that stains darkly with histochemical techniques. Kinetoplastid has:

- Mitochondrial DNA.
- Located near Base of Flagellum.

Initially believed to function in movement that gives *Trypanosoma* its characteristic locomotion.

**Fig. 10.6 Trypanosoma - A Zooflagellate**

### Check Your Progress

5. Write any three characteristics of subphylum Sarcodina.
6. What is Trophozoit Amoeba?
7. How is trophozoites discharged?

### 10.4 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Characteristic features of Protozoa are as follows:
   - Protozoa have intracellular specialization or organization of organelles in cells.
   - Cells have distinct function: Colonial protozoa have separate somatic and reproductive zooids.
   - Asexual reproduction occurs by mitotic division.
   - True sexual reproduction with zygote formation.
   - Responses to stimuli represent the simplest reflexes and inborn behaviours.

2. The phylum Sarcomastigophora belongs to the Protista kingdom and it includes many unicellular or colonial, autotrophic, or heterotrophic organisms. It is characterized by flagellae, pseudopodia, or both, for locomotion. The common genera are *Giardia, Leishmania, Trichomonas, Trichonympha, Trypanosoma, Entamoeba*, etc. It also
The World of Protozoa

includes various types of symbiotic amoebae, of which most require other animals as host to survive, such as *Endamoeba* and *Entamoeba*.

3. Phylum Labyrinthomorpha has non-amoeboid vegetative cells that are spindle shaped or spherical. These move in network of mucous tracks by gliding motion.

4. Apicomplexa collectively called as sporozoans, they lack locomotory organs so have a spore forming stage in their life cycle. They are inter or intra cellular parasites of animals. They are parasites distinguished by a unique apical complex collection of microtubules, microfibrils located at one end of cell plasmodium.

5. Characteristics of Subphylum Sarcodina are as follows:
   
   Super class: Rhizopoda, for example, *Amoeba proteus*.
   
   - The cell membrane encloses ectoplasm and endoplasm.
   - Amoeba feed on algae, rotifers by phagocytosis.
   - Reproduction is by binary fission using mitosis.

6. Trophozoit Amoeba is the growing or feeding, most active form pathogenic to humans. The nucleus with a single chromatin enclosed by a delicate nuclear membrane. Ingested blood cells appear in the cytoplasm.

7. Trophozoites are discharged in the lumen from the intestinal wall and are transformed into cystic forms. This results in different stains.

10.5 SUMMARY

- The term protozoa comes from the Greek words for ‘Proto’ meaning ‘First’ and ‘Zoa’ meaning ‘Animals’. Protozoa are therefore termed as the ‘First Animal’ with single celled organisms having animal like characteristics. Scientists consider that Protozoa may resemble the first Eukaryotes that evolved from Prokaryotes.

- Originally a term envisaged by Haeckel- Protista represents a diverse group of organisms. This was based on the assumption of their eukaryotic characters and failure to fit satisfactorily in animals, plant or fungal kingdoms.

- With the help of molecular evidence protists are divided into plant like Algae and animal like Protozoa.

- On the basis of molecular and cytological comparisons the animal like protozoan is closely related to photosynthetic and plant like Euglena.

- The domain Eukaryotes is defined by the presence of a unique diagnostic feature, the cell nucleus.
The World of Protozoa

NOTES

- All members of the domain possess a nucleus, chromosomes, where DNA is complexed with histones, cell division via mitosis, and a system of structural and contractile proteins called the cytoskeleton.
- A protozoan is a complete organism in which all life activities are carried on within the limits of a single plasma membrane.
- Phylogenetic studies show that protozoa do not form a monophyletic group.
- Protozoans have only one non reproductive cell type and lack embryonic development, embryonic development is one of the criteria for metazoan.
- Protozoa are protists displaying heterotrophic nutrition. Presence of moisture is essential for their survival as they are susceptible to desiccation.
- A protozoan cell may be more complex than a single animal cell and have the same morphology and physiology of a typical eukaryotic cell.
- There are certain evolved structures as well like that of the **contractile vacuole** which is a fluid filled vacuole involved in the osmoregulation of certain protists.
- Many are symbionts that inhabit body fluids, tissues or cells of hosts, from mutualism to parasitism.
- Ciliates are a diverse group most of them being solitary fresh water inhabitants that use cilia to move and to feed.
- Dinoflagellates are all unicellular and mostly marine of greater ecological evolutionary and morphological interest.
- Abundant aquatic phytoplanktons suspend near the water surface and provide foundation of most marine and water food webs.
- Flagella and cilia are whiplike organelle that are identical structures. They are composed of nine sets of doublet microtubules arranged around two central single microtubules.
- Microfilaments can exists as single filaments, in bundles or networks made up of actin, a protein that exists in several forms constituting many functions in the animal species.
- Actin muscles cells, actin fibres are associated with another protein called myosin, and they together bring about contraction of muscles.
- The phylum Sarcomastigophora belongs to the Protista kingdom and it includes many unicellular or colonial, autotrophic, or heterotrophic organisms. It is characterized by flagellae, pseudopodia, or both, for locomotion. The common genera are **Giardia, Leishmania, Trichomonas, Trichonympha, Trypanosoma, Entamoeba**, etc. It also includes various types of symbiotic amoebae, of which most require other animals as host to survive, such as **Endamoeba** and **Entamoeba**.
• Phylum Labyrinthomorpha has non-amoeboid vegetative cells that are spindle shaped or spherical. These move in network of mucous tracks by gliding motion.
• Apicomplexa collectively called as sporozoans, they lack locomotory organs so have a spore forming stage in their life cycle. They are Inter or intra cellular parasites of animals. They are parasites distinguished by a unique apical complex collection of microtubules, microfibrils located at one end of cell plasmodium.
• Phylum Micospora is obligatory intracellular parasites lacking mitochondria. Many are pathogenic and have an important role in the biological control of certain insects.
• Phylum Ascetospora is parasitic protists that lack spore lacking polar caps or polar filaments. Ascetosporans found in tissues of molluscs.
• Mostly they are unicellular, aerobic and chemoheterotrophic in nature of kingdom Protista.
• Protozoa is not a coherent taxonomic group with a common ancestor but a collection of phylogenetically diverse species.
• *Entamoeba histolytica* lives in human large intestine and attacks the intestinal wall with enzymes, causing severe and often fatal diarrhea.
• Trophozoit Amoeba is the growing or feeding, most active form pathogenic to humans. The nucleus with a single chromatin enclosed by a delicate nuclear membrane. Ingested blood cells appear in the cytoplasm.
• The release of the parasite from the cyst is known as excystation.

10.6 KEY WORDS

• **Amoeba:** An amoeba, often called amoeboid, is a type of cell or unicellular organism which has the ability to alter its shape, primarily by extending and retracting pseudopods.
• **Protozoa:** Protozoa is an informal term for single-celled eukaryotes, either free-living or parasitic, which feed on organic matter such as other microorganisms or organic tissues and debris.
• **Excystation:** The release of the parasite from the cyst is known as excystation.
• **Ascetospora:** Parasitic protists that lack spore lacking polar caps or polar filaments.
• **Micospora:** Obligatory intracellular parasites lacking mitochondria.
10.7 SELF ASSESSMENT QUESTIONS AND EXERCISES

Short Answer Questions

1. What is a protozoa?
2. Give any five general characteristics of Protozoa.
3. Write about any two economic importance of Protozoa.
4. What are sporozoans?
5. Write a short note on Plasmodium.
6. What are protistan taxonomy?

Long Answer Questions

1. What are Protozoa? Write a detailed note on protozoa mentioning about its characteristics and economic importance.
2. Explain about actins in detail.
3. Explain with the help of a table different pathogenic Protozoa.
5. Discuss about Plasmodium in detail. Illustrate with the help of well labelled diagram.
7. Draw a well-labelled diagram for Entamoeba histolytica different stages.

10.8 FURTHER READINGS

UNIT 11 MOLECULAR TAXONOMY: CLASSIFICATION AND IDENTIFICATION

11.0 INTRODUCTION

The use of molecular genetics to study the evolution of relationship among individuals and species is based on the shared genetic attributes of the living and fossil organisms. Molecular taxonomy uses a stretch of nucleotide-sequence data to determine the evolutionary relationships of different organisms.

A comparison of the sequences of functionally homologous molecules from each organism to determine the number of differences between them. The greater the number of differences, the more distantly related the organisms are likely to be. Molecular taxonomy is the classification of organisms on the basis of the distribution and composition of chemical substances in them.

Molecular phylogenetic makes inferences of the evolutionary relationships that arise due to molecular evolution and results in the construction of a phylogenetic tree. In the light of development the enzymes termed type II restriction endonucleases have come to play a key role in all aspects. As a multigame the 16S ribosomal RNA gene widely present in all bacterial species organized as operons and codes for the RNA component of the 30S subunit of the bacterial ribosome.

In this unit, you will study about molecular taxonomy, bacterial identification using 16S rRNA sequencing in detail.
11.1 OBJECTIVES

After going through this unit, you will be able to:

- Explain molecular taxonomy
- Discuss bacterial identification using 16S rRNA sequencing

11.2 MOLECULAR TAXONOMY

Molecular taxonomy is the classification of organisms on the basis of the distribution and composition of chemical substances in them. Molecular genetic methods provide taxonomy with a powerful tool to identify or describe species, as they apply to all organisms and offer quantifiable characters. Molecular taxonomy is particularly effective in combination with other methods, usually with morphology. The advent of DNA cloning and sequencing methods have contributed immensely to the development of molecular taxonomy and population genetics over the last two decades. These modern methods have revolutionised the field of molecular taxonomy and population genetics with improved analytical power and precision.

Phylogeny of Bacteria

With every new species emerging the tree of life increases in complexity. Many of the conflicts that arise in defining morphological simplicity of Bacteria stem from differences in assumptions about the evolutionary process cannot be determined through morphological analysis and can only be deduced through molecular systematic. For years taxonomic study is based on morphological structures, phenotypical characters of individual organisms. Morphological systematics originated from comparative anatomy.

Taxonomists have a difficult time agreeing on the convention in their attempt to elucidate the phylogenetic relationships of prokaryotes due to lack of meaningful developmental stages. However, the development of techniques that allowed the sequencing of genetic material revolutionized the taxonomy of Bacteria.

Classification deals with the orderly arrangements of taxonomic units (taxa) Classification is often confused with identification. Genomic techniques to species identification is relatively new, yet evolving exponentially to serve as data used to phylogenetic trees as confirmatory evidences.

Therefore, classification precedes identification and can be categorized based on phonetic (non-evolutionary) and phylogenetic (evolutionary) relationships. However, phonetic resemblance is often an unreliable guide to genealogy.
There are several reasons why sequence databases can vary under morphological, cytological, and ecological and biochemical characters are the major criteria of the organisms of taxonomic importance and not accurately link correct relative placement of a bacterial sequence.

Comparison are commonly shown by phylogenetic trees and linear alignments.

Depending on the selective distance measure the dissimilarity or a similarity establishes a phylogenetic connection of certain biochemical characters like presence of an enzyme controlling a pathway, metabolic end products, the sequence of bases in DNA, the CG ratio and nucleic acid hybridisation.

The phylogenetic tree relationships are impacted when taxa under comparison is less closely related and more strongly affected by accurate 16S rRNA gene sequence identification of organisms.

The accurate sequence of a DNA molecule can help us identify several bacterial characteristics, for example staining reactions, presence and absence of spores, type of culture media, motility. It encodes regulating the function of a particular part of the accurate sequence for the isolate to be identified.

Comparing protein signatures, used as predictive models for more distantly related species database can be identified. It is an important method for evaluating position of each internal protein are characteristics of species origin in comparison to the sites identified from same sequence data. Evolutionary or phylogenetic classification, on the other hand, is not based on phonetic resemblances between species but on their genealogical relationships on isolating a particular stretch of DNA.

Amino acid sequencing form enough database of the number of substitutions in the polypeptide chain to infer the degree of relationship among the homologues of previously known genes of several taxa. Horizontal gene transfer and convergence are significant evolutionary forces among prokaryotes resulting in ecological adaptation of characters acquired by unrelated taxa.

The metagenomic approach is being applied to study microbial populations in many environments, the use of 16S rRNA gene sequences to infer relationship that span the diversity of living species as common genetic marker for genes conserved through billions of years of evolutionary divergence used for a number of reasons. These reasons include (i); its presence one to multiple copies in almost all Bacteria (ii); Stable function of the 16S rRNA gene over time making it easier to analyse random sequence changes are a more accurate measure of time (evolution); and (iii); the 16S rRNA gene available as large databases storing enormous amount of information for comparison.
With the abundance in the number of recognized taxa is directly attributable to the ease in using 16S rRNA as a tool to identify Bacteria and assist with differentiating between closely related bacterial species as opposed to complexity in manipulations involving unequivocally DNA-DNA hybridization investigations proposed for new species, for example unknown pathogenic strains.

One of the most attractive potential use of 16S rRNA informatics is to account for the identification for isolates with low acceptability to a biochemical profile, strain with ambiguous properties to the correct taxonomic unit rarely associated to human infectious diseases.

Strains with less than 97% similarity in 16S rRNA gene sequence represent a new bacterial species. With more than 97% similarity clustering with previous taxon is indicated expect in cases above 99% similarity in gene sequence represent DNA-DNA hybridisation (65 to 83%) at species level from 1to 14% of the isolates remaining unidentified after testing.

**Molecular Chronometers**

The concept of the ‘evolutionary clock’ and to the use of molecular sequences as chronometers was based on the fact that the sequence of a macromolecule used to determine the evolutionary time since the two organisms diverged.

It is a part of ribosome and protein synthesis so can be changed on a large scale without altering its function. Rate of change should reflect variability distance.

The variations of the deoxyribonucleic acid sequences occur more or less continuously to allow sequence conservation and to generate an alignment of characteristic pattern on an evolutionary time scale.

The majority of these change between environment and morphology are either deleterious or selectively neutral, and therefore do not alter the phenotype. The genotypic changes inferred by genetic markers used are the basis for inferring phylogenetic relationships. However, not all molecular chronometers as well as physiology and behavior are equally useful for reconstructing phylogenies. A useful chronometer organisms has to be ubiquitously distributed among organisms for inferring the phylogeny of all living. 16S has molecular chronometer present in all organisms. Its essential genes are less free to mutate but not loss over time. Non-essential gene reflect rate of evolution able to mutate without selective pressure of survival. They use conserved region to compare distantly related organism. Table 11.1 illustrates the summary of the methods presently employed in identification of the species.
Table 11.1 Summary of the Methods Presently Employed in Identification of the Species

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hybridization</td>
<td>Genetic materials from two different species is subjected to hybridize. Closely related species show higher percentage of hybridization.</td>
</tr>
<tr>
<td>DNA sequencing</td>
<td>DNA segments of two species are sequenced from one end to the other and the sequences of the two form the basis of establishing similarity or dissimilarity between them.</td>
</tr>
<tr>
<td>Restriction mapping</td>
<td>Segments of DNA are isolated from different species and subjected to restriction mapping. Closely related species will have more similar restriction map.</td>
</tr>
<tr>
<td>Chromosome banding</td>
<td>The chromosomes of different species are examined through microscope. Banding of chromosomes are also done for taxonomic purposes.</td>
</tr>
<tr>
<td>Amino acid sequencing</td>
<td>Like DNA sequencing protein sequencing is also done. The amino acid sequence of a given protein will be more similar between closely related species.</td>
</tr>
<tr>
<td>Immunological methods</td>
<td>Antibodies that recognize specific macromolecules, usually on the cell surface are tested on different species. Antibodies that recognize macromolecules form one species will often recognize closely related species, but not from distantly related species.</td>
</tr>
</tbody>
</table>

Check Your Progress

1. Define molecular taxonomy.
2. What does molecular genetic methods provide?
3. When are phylogenetic tree relationships impacted?
4. What represent a new bacterial species?

11.3 BACTERIAL IDENTIFICATION USING 16S RRNA SEQUENCING

Molecular phylogenetic makes inferences of the evolutionary relationships that arise due to molecular evolution and results in the construction of a phylogenetic tree. In the light of development the enzymes termed type II restriction endonucleases have come to play a key role in all aspects.

As a multigame the 16S ribosomal RNA gene widely present in all bacterial species organized as operons and codes for the RNA component of the 30S subunit of the bacterial ribosome.

Early studies using manual DNA-DNA hybridisation methods was replaced by informatics targeting on 16S rRNA genes as a measure of phylogenetic diversity.

Universal phylogenetic tree of the major phyla of Bacteria derived from comparative ribosomal RNA sequencing. During transcription 16S rRNA
Molecular Taxonomy: Classification and Identification

NOTES

Genes is exhibited as the most conserved component of all DNA in all cells to maintain critical functions. There are only 7 regions which are considered highly variable region most of it not subjected to mutation as shown in Figure 11.1. It is applicable as a target gene for sequencing DNA in diverse samples containing innumerable species.

Portions of the rDNA sequence from distantly related organisms are remarkably similar. This implies that the sequences from the distantly related organisms can be aligned making differences easy to measure. Gene that code for rRNA and rDNA have been extensively used to determine taxonomy phylogeny and to estimate rates of species divergence among Bacteria.

The conserved region of 16S along with the help of PCR primers makes it possible to amplify the gene from the single sample. While the sequencing of the variable regions mark evolutionary distance and relatedness of organisms by discriminating between different organism such as Bacteria, Archaea and microbial Eukarya. Conversely identification of viruses involves metagenomic sequencing due to lack of phylogenetic marker gene 16S. Figure 11.2 shows 16S rRNA structure and primer locations of full gene and sequence primer.

Fig. 11.1 Approximately 1.5 kb 16S rRNA Gene of E.coli showing the Nine Variable Regions as a Phylogenetic Marker Gene

Fig. 11.2 16S rRNA Structure & Primer Locations of Full Gene and Sequence Primer
In such instances 16S rRNA gene sequence data cannot provide a
definite answer since it cannot distinguish between recently diverged species.

Phenotypic characters are then considered for identification of species
with distance scores <0.5% to the next closest level. This could be due to
emergence of a novel taxa, species sharing identical 16S sequences, number
of nucleotide sequences in database very few or multiple genomovar present
for one species.

- 16S is the most rapidly evolving molecule and it has been useful in
  phylogenetic population studies emerging as a preferred genetic technique.

- This rapidly evolving DNA.

- Indicates the potential for coupling up with next generation sequencing
  PCR at low cost.

A future challenge is to translate information from 16S rRNA gene
sequencing to biochemical mechanisms as conventional 16S sequencing
methods have found sequencing of non-isolated organisms. Figure 11.3 shows
the schematic representation of the targeted and shotgun approach.

Schematic representation of the targeted and shotgun approach. (a) Prokaryotic 16S and eukaryotic 18S rRNA gene are used to characterize
metagenome by targeted sequencing. (b) Alignment of shotgun metagenomic
reads to reference genomes and/or genes.

**Restriction Fragment Length Polymorphism (RFLP)**

In Restriction Fragment Length Polymorphism (RFLP) technique organisms
are differentiated by analyzing the pattern derived from the cleavage of DNA
sequence (Refer Figure 11.4). If two organism differ in the distance between
the sites of cleavage through restriction endonuclease enzyme the length of
fragment will differ when DNA is digested. Any similarity of the patterns
generated in the strain can be used to differentiate species.
Restriction enzyme recognition sites are distinct short sequence palindromes. These can be visualised as distinct banding patterns on agarose gel electrophoresis.

normal™ strand and a mutated strand. The normal strand has 3 locations where the restriction enzyme MSTI cuts. The mutation on the other strand destroys one of the restriction sites. As a result, the mutated strand has one fewer bands on a gel.

**Fig.11.4  Restriction Fragment Length Polymorphism (RFLP) Technique**

The small red arrows pointing to the two different chromosome segments show the locations of the restriction enzyme recognition sites. After digestion and agarose gel electrophoresis, the banding patterns reflect the change by showing the loss of two shorter bands and the gain of a longer band.

**Developing RFLP Probes**

- The RFLP technique involves an enzymatic procedure for separation and identification of desired lengthy DNA fragments into short pieces.
- The digested DNA is size-fractionated to generate a characteristic pattern on a preparative agarose gel, and lengthy fragments are replicated into a plasmid vector.
- Plasmids are screened to check for any inserts present.
- Southern blots that facilitates the detection of DNA fragment among hundreds of other generated.
- The probes are screened for RFLPs, a method of hybridisation for bacterial systematic, binds to its complementary DNA sequence.
This band patterns varies in every organism and corresponds to a DNA fingerprint unique to an individual. Using restriction endonuclease enzymes fragments of DNA is detected by using restriction probes and cut at different sites known as restriction digest. An RFLP probe rapidly used in genome mapping

**Applications of RLFP**

- Forensic application: RLFP can be used in paternity cases or criminal cases to determine the source of DNA sample.
- Used to trace the disease status of the individual like detection of mutations.
- Used to measure the distance between RFLP loci measured.
- The course of evolution can be inferred to trace the cycle of epidemic pathogens and zoonotic viruses.

**Disadvantages**

With the advent of less expensive DNA profiling techniques PCR, RLFP is superseded since target DNA sequences can be amplified in a few hours with small quantity of sample. PCR has largely replaced RLFP as Isolation and accumulation of adequate DNA for analysis is cost dependent and labour intensive.

**Restriction Endonucleases:** Restriction endonucleases are enzymes that cleave DNA molecule depending on the particular enzyme used. Largely the shorter the recognition sequence 4-6 base pairs, greater number of fragments generated. Different sequences will give different length fragments. This will give greatest diversity in restriction product site. These enzymes are separated by gel electrophoresis from variety of bacterial genera and thought to be part of cells defenses against invading pathogens. Each fragment length represents an allele and can be processed further for genetic analysis.

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**Check Your Progress**

5. What is Restriction Fragment Length Polymorphism (RFLP)?
6. How RFLP probe is developed?
7. Give the applications of RFLP.

---

**11.4 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS**

1. Molecular taxonomy is the classification of organisms on the basis of the distribution and composition of chemical substances in them.
2. Molecular genetic methods provide taxonomy with a powerful tool to identify or describe species, as they apply to all organisms and offer quantifiable characters.

3. The phylogenetic tree relationships are impacted when taxa under comparison is less closely related and more strongly affected by accurate 16S rRNA gene sequence identification of organisms.

4. Strains with less than 97% similarity in 16S rRNA gene sequence represent a new bacterial species.

5. In Restriction Fragment Length Polymorphism (RFLP) technique organisms are differentiated by analysing the pattern derived from the cleavage of DNA sequence.

6. Developing RFLP probes:
   - The RFLP technique involves an enzymatic procedure for separation and identification of desired lengthy DNA fragments into short pieces.
   - The digested DNA is size-fractionated to generate a characteristic pattern on a preparative agarose gel, and lengthy fragments are replicated into a plasmid vector.
   - Plasmids are screened to check for any inserts present.
   - Southern blots that facilitates the detection of DNA fragment among hundreds of other generated.
   - The probes are screened for RFLPs, a method of hybridisation for bacterial systematic, binds to its complementary DNA sequence.

7. Applications of RFLP are as follows:
   - Forensic application: RFLP can be used in paternity cases or criminal cases to determine the source of DNA sample.
   - Used to trace the disease status of the individual like detection of mutations.
   - Used to measure the distance between RFLP loci measured.
   - The course of evolution can be inferred to trace the cycle of epidemic pathogens and zoonotic viruses.

11.5 SUMMARY

   - Molecular taxonomy is the classification of organisms on the basis of the distribution and composition of chemical substances in them.
   - Molecular genetic methods provide taxonomy with a powerful tool to identify or describe species, as they apply to all organisms and offer quantifiable characters.
Molecular taxonomy is particularly effective in combination with other methods, usually with morphology.

The advent of DNA cloning and sequencing methods have contributed immensely to the development of molecular taxonomy and population genetics over the last two decades.

Taxonomists have a difficult time agreeing on the convention in their attempt to elucidate the phylogenetic relationships of prokaryotes due to lack of meaningful developmental stages.

Classification deals with the orderly arrangements of taxonomic units (taxa).

Classification is often confused with identification. Genomic techniques to species identification is relatively new, yet evolving exponentially to serve as data used to phylogenetic trees as confirmatory evidences.

Classification precedes identification and can be categorized based on phonetic (non-evolutionary) and phylogenetic (evolutionary) relationships.

Phonetic resemblance is often an unreliable guide to genealogy. There are several reasons why sequence databases can vary under morphological, cytological, and ecological and biochemical characters are the major criteria of the organisms of taxonomic importance and not accurately link correct relative placement of a bacterial sequence.

The phylogenetic tree relationships are impacted when taxa under comparison is less closely related and more strongly affected by accurate 16S rRNA gene sequence identification of organisms.

The accurate sequence of a DNA molecule can help us identify several bacterial characteristics, for example staining reactions, presence and absence of spores, type of culture media, motility.

Comparing protein signatures, used as predictive models for more distantly related species database can be identified.

Evolutionary or phylogenetic classification, on the other hand, is not based on phonetic resemblances between species but on their genealogical relationships on isolating a particular stretch of DNA.

Amino acid sequencing form enough database of the number of substitutions in the polypeptide chain to infer the degree of relationship among the homologues of previously known genes of several taxa.

Horizontal gene transfer and convergence are significant evolutionary forces among prokaryotes resulting in ecological adaptation of characters acquired by unrelated taxa.

The metagenomic approach is being applied to study microbial populations in many environments, the use of 16S rRNA gene
sequences to infer relationship that span the diversity of living species as common genetic marker for genes conserved through billions of years of evolutionary divergence used for a number of reasons.

- With the abundance in the number of recognized taxa is directly attributable to the ease in using 16S rRNA as a tool to identify Bacteria and assist with differentiating between closely related bacterial species as opposed to complexity in manipulations involving unequivocally DNA-DNA hybridization investigations proposed for new species, for example unknown pathogenic strains.
- One of the most attractive potential use of 16S rRNA informatics is to account for the identification for isolates with low acceptability to a biochemical profile, strain with ambiguous properties to the correct taxonomic unit rarely associated to human infectious diseases.
- Strains with less than 97% similarity in 16S rRNA gene sequence represent a new bacterial species.
- With more than 97% similarity clustering with previous taxon is indicated expect in cases above 99% similarity in gene sequence represent DNA-DNA hybridisation (65 to 83%) at species level from 1to 14% of the isolates remaining unidentified after testing.
- The concept of the ‘evolutionary clock’ and to the use of molecular sequences as chronometers was based on the fact that the sequence of a macromolecule used to determine the evolutionary time since the two organisms diverged.
- 16S has molecular chronometer present in all organisms. Its essential genes are less free to mutate but not loss over time.
- Non-essential gene reflect rate of evolution able to mutate without selective pressure of survival. They use conserved region to compare distantly related organism.
- Universal phylogenetic tree of the major phyla of Bacteria derived from comparative ribosomal RNA sequencing.
- During transcription 16S rRNA genes is exhibited as the most conserved component of all DNA in all cells to maintain critical functions.
- The conserved region of 16S along with the help of PCR primers makes it possible to amplify the gene from the single sample.
- While the sequencing of the variable regions mark evolutionary distance and relatedness of organisms by discriminating between different organism such as Bacteria, Archaea and microbial Eukarya.
- In Restriction Fragment Length Polymorphism (RFLP) technique organisms are differentiated by analysing the pattern derived from the cleavage of DNA sequence.
• If two organism differ in the distance between the sites of cleavage through restriction endonuclease enzyme the length of fragment will differ when DNA is ingested.

• Restriction enzyme recognition sites are distinct short sequence palindromes. These can be visualised as distinct banding patterns on agarose gel electrophoresis.

• Restriction endonucleases are enzymes that cleave DNA molecule depending on the particular enzyme used. Largely the shorter the recognition sequence 4-6 base pairs, greater number of fragments generated.

11.6 KEY WORDS

• **Molecular taxonomy:** Molecular taxonomy is the classification of organisms on the basis of the distribution and composition of chemical substances in them.

• **Restriction endonucleases:** Restriction endonucleases are enzymes that cleave DNA molecule depending on the particular enzyme used.

• **Restriction Fragment Length Polymorphism (RFLP):** Restriction Fragment Length Polymorphism (RFLP) is a technique to differentiate organisms by analyzing the pattern derived from the cleavage of DNA sequence.

11.7 SELF ASSESSMENT QUESTIONS AND EXERCISES

**Short Answer Questions**

1. What is molecular taxonomy?
2. What is the phylogeny of bacteria?
3. What are molecular chronometers?
4. Define and expand the term RFLP and give its applications.
5. What is Restriction endonucleases?

**Long Answer Questions**

1. What is molecular taxonomy? Discuss in detail.
2. Explain the phylogeny of bacteria.
3. Explain with the help of table the methods that are currently employed in the identification of species.
4. Write in detail about Restriction Fragment Length Polymorphism (RFLP) giving its developing, applications and disadvantages also.

### 11.8 FURTHER READINGS


UNIT 12  PHYLOGENETIC TREES

12.0 INTRODUCTION

A phylogenetic tree or evolutionary tree is a branching diagram or ‘tree’ showing the evolutionary relationships among various biological species or other entities—their phylogeny based upon similarities and differences in their physical or genetic characteristics. All life on Earth is part of a single phylogenetic tree, indicating common ancestry.

In a rooted phylogenetic tree, each node with descendants represents the inferred most recent common ancestor of those descendants, and the edge lengths in some trees may be interpreted as time estimates. Each node is called a taxonomic unit. Internal nodes are generally called hypothetical taxonomic units, as they cannot be directly observed. Trees are useful in fields of biology such as bioinformatics, systematics, and phylogenetics. Unrooted trees illustrate only the relatedness of the leaf nodes and do not require the ancestral root to be known or inferred.

In this unit, you will study about phylogenetic tree its types and construction, molecular tools in assessing microbial diversity in detail.

12.1 OBJECTIVES

After going through this unit, you will be able to:

• Understand what phylogenetic tree is
• Discuss about types and construction of phylogenetic tree
• Explain the molecular tools in assessing microbial diversity
12.2 PHYLOGENETIC TREE: TYPES AND CONSTRUCTION

Phylogeny: The ancestral relation between species. Large efforts have been put in reconstructing tree life. Traditionally phylogenetic relations were inferred using morphological evidence similarly proceeding the same way with both living and fossil species. The ‘morphological’ evidence refers to all observable characters in the whole organism, as distinct from molecular characters.

Phylogenies are now increasing inferred from molecular sequence evidence. The phylogenetic inference is principally the same in both the types but the techniques used differ in many ways. This unit will begin with ‘cladistic’ techniques, which are used with morphological evidence. Then molecular evidence involving statistical procedures will be elucidated for correct inference.

Shared Characters used to Infer Phylogeny

The basic idea of phylogenetic is finding the genetic relationships between species to compare specific characters of the species, under the natural assumption that similar species are genetically close. Classic phylogenetics deals mainly with physical, or morphological features-size, color, number of legs, etc. Recent advances in sequencing technologies have created an exponentially increasing volume of DNA and protein sequence. Evidences from hard parts of the body such as vertebrate bones or mollusc shells constitute the evidence for the fossil species. For living species we have evidence from the soft parts. Certain characters that are not morphological in narrow sense but can be used for phylogenetic inference. For instance, reproductive and physiological characters are good evidences for phylogenetic inference. The techniques used with morphological characters are all cladistic.

Phylogenetic inference can be drawn from morphological characters using cladistic technique.

What is Phylogeny Good For?

- To understand the process of molecular evaluation as objects typically are proteins or nucleic acid sequences.
- To identify what is most conserved important in some class of sequences
- Tracing population history and multiple sequence alignment
- To estimate the time of divergence between various species to understand lineage they last shared a common ancestor and human origin.
Phylogenetic Trees

Phylogenetic trees are representation of phylogenetic information that shows relationships between species, it shares its most recent common ancestor with (Refer Figure 12.1).

![Phylogenetic Tree](image)

Fig. 12.1 Phylogenetic Tree

Types of Phylogenetic Trees

There are significant in showing the evolutionary relationship among the biological species that are believed to have a common ancestor. Each node is a taxonomic unit. Each node with the descendent represents the most recent common ancestor of the descendants. Edge lengths corresponds to time estimates. Root is the common ancestor of the species under study (Refer Figure 12.2).

![Phylogenetic Tree Terminology](image)

Fig. 12.2 Phylogenetic Tree Terminology
Phylogenetic Trees

Phylogenic Tree can be Rooted and Unrooted

A rooted tree is a tree in which one of the nodes is stipulated to be the root, and the direction of ancestral relationship is determined. An unrooted tree, shows how close or distant the species are, and has no pre-determined root and induces no hierarchy. Rooting an unrooted tree involves inserting a new node, which will function as the root node. This can be done by introducing an uncontroversial outgroup, a species that is distant from all the species of interest. The proposed root will be the direct predecessor of outgroup close enough to allow inference from sequence or trait data. It depicts only the relationship between organisms irrespective of the direction of the evolutionary time line. Unrooted trees can be generated from rooted trees by omitting the root indicating the origin of evolution.

![Phylogenetic Trees](image)

**Fig. 12.3 Unrooted Tree Representing the Relatedness of the Leaves without Assuming Ancestry**

An unrooted tree represents the relatedness of the leaves without assuming ancestry at all. Figure 12.3 (A); shows that A is more closely related to C than it is to either B or D, but does not specify the common ancestor for the four species or the direction of change. In contrast, the rooted tree Figure 12.3 (B), directed to a unique node does give a node that serves as the common ancestor and shows the development of the four species from this root.

The gradual change over time of the sequences of many rRNAs and proteins without destroying or severely altering their functions helps in determining their phylogenetic relationship. If sequences of similar molecules from two organisms differs, it means that they diverged very long time ago as there is an increase in changes with time.

The root of a tree signifies as the oldest point in the tree which represents the last common ancestor of all groups included in the tree unlike an unrooted tree that does not make any assumption about the common ancestor. Figure 12.4 illustrates phylogenetic tree. (a) unrooted tree; (b) rooted tree and Figure 12.5 represents an example of unrooted phylogenetic tree.
In rooted tree there exists a particular node called the root from which the unique path leads to any other node. The direction of each path corresponds to evolutionary time and the root is the common ancestor of all taxonomic unit.

In rooted evolutionary relationships are evident. In unrooted tree evolutionary relationships cannot be immediately assessed.

Branch length is the number of changes, for example nucleotide substitutions that have occurred along a branch. The total number of changes in a particular tree is called the tree length.
How to Construct a Phylogenetic Tree?

The distance of the lines is used to determine how closely two organisms are related to one another or how long ago they may have had a common ancestor. The line that connect all the other lines is the representation of the common ancestor that is being looked at for comparison.

**Step 1:** Choosing a genome region to study.

**Step 2:** Identifying and retrieving sets of homologous sequences from the same genome region of related individuals.

**Step 3:** Make a multiple alignment from base alignment or amino acid sequence (by using MUSCLE, BLAST, or other method).

**Step 4:** Check the multiple alignment if it reflects the evolutionary process.

**Step 5:** Choose the method to be used to determine the best fit nucleotide substitution model and calculate the distance or use the result depending on the method.

**Step 6:** Verify the result statistically. Visualization and editing of trees.

A tree can show edge lengths, indicating the genetic distance between the connected nodes. To determine the constant pace of the evolutionary process molecular clock is a good indicator of their relatedness. According to this assertion informational macromolecules evolving at constant rates for different lineages, representing the root as the mid-point of the longest span across the tree. On such a basis phylogenetic tree can be produced theoretically that is distance preserving tree presented along a time-axis-assigning to each node the time in which occurred in the history of evolution.

However, using these sequences to construct phylogenetic tree, it is important to ensure that each nucleotide or amino acid in each sequence is compared only with the corresponding homologous nucleotides or amino acids in the other sequences. This is done by aligning the sequence to one another to obtain a discrete character matrix in which each row represents one of the sequences and each column a set of homologous nucleotides or amino acids.

**There Are Two Types of Data Used for Building Phylogenetic Trees**

Character Based: Seeks the tree that accounts for estimated evolutionary distances of each character separately, i.e., the individual substitutions among the sequences, to determine the most likely ancestral relationships.

Each discrete character evolves independently from molecule sequences from individual data. The basic assumption is that characters at corresponding positions in a multiple sequence alignment are homologous among the sequences involved. Therefore the character states of the common ancestor can be traced from the dataset of a base in a specific position in a DNA sequence or amino acid in a protein.
They are more rapid and less intensive than character based. But the actual characters are discarded once the distance matrix is arrived. On the other hand character based makes use of all known evolutionary information based on individual substitutions among the sequences and to determine the most likely ancestral relationships.

Distance Based: Seeks the trees that account for estimated evolutionary distances. True evolutionary distances between sequences can be calculated from observed distances. The evolutionary distances can be used to construct a matrix of distances between all individual pairs of taxa. A node is bifurcating binary or dichotomous if has only immediate descendent lineages but multifurcating if it has more than two immediate descendent lineages. In a strictly bifurcating tree, each internal node is incident to exactly three branches, two derived and one ancestral (Refer Figure 12.6).

![Distance Based Tree](image)

**Fig. 12.6 Distance Based Tree**

A bifurcation is always interpreted as a speciation event. Two possible interpretation for a multifurcation in a tree. The polytomy represents two sequence of events whereby ancestral taxon gave rise to three or more descendent taxa simultaneously. The polytomy represents lack of resolution. The exact order of two or more bifurcation cannot be determined with the available data (Refer Figure 12.7).

![Multifurcation and Bifurcation](image)

**Fig. 12.7 Multifurcation and Bifurcation**
Genetic Distance

Genetic distance is number of evolutionary events marked as mutations occurring between species since their divergence. In order to evaluate we count the number of differences between two sequences. However, this may not always refer to the history of the sequence, as not all the events are recorded in current sequences. This could be replaced by the transition or transversion rate, along with different base frequencies between sites. All these models give similar results at low divergence.

The most popular methods can be classified into two main categories.

- Phenetic methods based on distance
- Cladistic methods based on characters

The former measures the pair-wise distance through dissimilarity between two genes. It helps in constructing tree from the resultant distance matrix. The latter evaluates all possible trees and seeks the one that optimizes the evolution.

Distance Based Methods: The amount of dissimilarity between pairs of sequence can be computed on the basis of sequence alignment.

Based on the pairwise distance score in the matrix, a phylogenetic tree can be constructed for all the taxa involved. The distance based method assume that all sequences involved are homologous and that the tree branches are additive, meaning that the distance between two taxa equals the sum of all branches lengths connecting them.

The most popular being unweighted pair group method with arithmetic mean UPGMA, NJ and those that optimises additivity of a distance tree. UPGMA Method:

This method follows a clustering procedure

- Assume initially each species is a cluster on its own
- Join closest two clusters and recalculate distance of the joint pair by taking the average.
- Repeat the process until all species are connected in single cluster.

This however does not aim to reflect revolutionary descent. It assign equal weight on the distance and assumes a randomised molecular clock.

Neighbor Joining Method: There is no assumption of molecular clock and the rate variation is adjusted among branches. Each pair is evaluated and the sum of all length of the branches of the resultant tree is calculated. The closest neighbours are the pairs that are with least sum. This method is relatively faster and gives better result than UPGMA. The only disadvantage is that of producing only one tree and neglects other possible trees.
Since errors in distance estimates are exponentially larger for longer distances, this method will yield a biased tree.

Maximum Parsimony: The criteria of the above method is that it finds the tree that explains the observed sequences with the fewest substitutions and evolutionary changes for all sequences to drive at a common ancestor during the divergence of the sequences. For each site in the alignment all possible trees are evaluated and are given a score based on the number of evolutionary changes needed to produce the observed sequence changes.

Example: Consider the following short sequences
1. ACTTT
2. AACAGT
3. AACGT
4. AATGT
5. AATTT

There are 105 possible rooted trees for 5 sequences

Which of the following trees explains the sequences with least number of substitutions?

Maximum Likelihood (ML): Seeks the tree that maximises the likelihood of the data. It calculates the likelihood for each tree and seeks the one that maximizes the likelihood of the data. ML uses each position in an alignment and evaluates all possible trees. It calculates the likelihood for each tree, at a given site, determined by evaluating the probability that the observed data was generated by a certain evolutionary model. This method is the slowest and most intensive though it gives the best results and generates most informative trees.

The Number of Phylogenetic Trees: An unrooted trees T with n leaves has 2n2 nodes and 2n 3 edges. A root can be added in any of the 2n 3 edges, thus producing 2n3 different rooted trees from T. For n=3 there are 3 ways of adding a root. Similarly there are 3 different ways of adding an extra edge with a new leaf to obtain an unrooted tree of 4 leaves.

Limitations to the Use of Trees

Phylogenetic Trees are meant to provide insight and not intended to represent an entire history of species. The output of the tree may be affected by several factors of gene transfer and protein function related to DNA degradation over time especially the evolutionary trees of the extinct organisms.

Polyphasic Taxonomy: Studying phylogeny based on both genotypic and phenotypic information ranging from molecular characteristics to ecological characters.
Numerical Taxonomy: Computer based approaches of grouping organisms is called Numerical taxonomy which is based on presence or absence of selected characters in the group of organisms clustered based on relatedness. It is method to express difference through evolutionary distance by estimating percent similarity to use measure of difference to create a tree. This method has great practical usefulness as well as being relatively unbiased in its approach. It has high degree of stability and predictability.

**Phylogenetic Trees**

- It seeks that either all internal nodes have degree 3, in which case T is called unrooted.
- In rooted T precisely one internal root node of degree 2
- In a Binary T every internal node v has degree 3 except if T is rooted.

**Leaves = Outer Branches**

Represent the taxa (sequences): Nodes = 1 2 3

Represent the relationships among the taxa (sequences), for example, Node 1 represent the ancestor seq from which seq A and seq B is derived.

Branches: the length of the branch represents the changes that occurred in the sequence prior to the next level of separation. External branches represents more recent diversions. Internal branches represents most ancient diversions.

**There are Two Kinds of Phylogenetic Trees**

- **Cladogram:** A group of species that shares its features from a common ancestor at (the root). There exists a particular root node. The paths from the root to the nodes. Root correspond to evolutionary time.
- **Phenogram:** Unrooted tree, a phylogenetic tree where related descendants – not able to specify the common ancestor (root). The nodes of the tree unable to indicate difference in evolutionary time.
**Remember:** A rooted tree is usually drawn with the root placed at the bottom, top or left of the figure.

- Rooted tree represents evolutionary connections over time.
- Unrooted trees depict only the relationship between organisms.

Notice that the two trees are made from the same data.

- Leaf nodes are numbered 1-5
- Internal nodes are numbered 6-8
- Rooted tree has additional node 9, which is one root

**Example:**

First question: How many trees are possible to construct in a given n species?

- If it’s a reasonable number, a tree-construction algorithm is immediately obvious:
  1. Enumerate every possible tree buildable from the n leaves.
  2. Score each of them, returning the one with the best score.

The solution:

Entering numbers into the above formulas, we get the following table:

<table>
<thead>
<tr>
<th># Leaves (n)</th>
<th># Unrooted Trees</th>
<th># Rooted Trees</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>105</td>
</tr>
<tr>
<td>6</td>
<td>105</td>
<td>945</td>
</tr>
<tr>
<td>8</td>
<td>10,395</td>
<td>135,135</td>
</tr>
<tr>
<td>10</td>
<td>2,027,025</td>
<td>34,459,425</td>
</tr>
</tbody>
</table>

In other words:

- This is an exponential function
- It is not a reasonable number
- We need to have a clever algorithm

**Distance-Based Methods:** We start by considering a couple of distance-based methods: UPGMA and Neighbor Joining.

We define the problem as follows:

- Given: An \( n \times n \) matrix \( M \) in which \( M_{ij} \) is the distance between objects \( i \) and \( j \).
- Do: Build an edge-weighted tree such that the distances between leaves \( i \) and \( j \) correspond to \( M_{ij} \).
Principle behind a distance based approach:

The vertical distance between any two leaves should correspond to the predefined distance.

UPGMA: Construction of a distance tree using clustering with the Unweighted Pair Group Method with Arithmatic Mean (UPGMA).

Unweighted Pair Group Method using Arithmetic Averages.

- Primarily pick two leaves/cluster and merge them
- Create new node in tree for merged cluster
- Distance $d_{ij}$ between clusters $C_i$ and $C_j$ is defined as:
  - (Average distance between pairs of elements from each cluster.)

The algorithm runs as follows:

- Assign each sequence to its own cluster
- Define one leaf for each sequence and place it at height 0

For more than two clusters

- Determine two clusters $i$, $j$ with smallest $d_{ij}$.
- Define a new cluster $C_k$ by merging $C_i$ and $C_j$.
- Define a node $k$ with children $i$ and $j$; place it at height ($\frac{1}{2}$ $d_{ij}$).
- Replace clusters $i$ and $j$ with $k$.
- Compute distance between $k$ and other clusters.

Join last two clusters, $i$ and $j$, by root at height ($\frac{1}{2}$ $d_{ij}$).

Fortunately, there’s a shortcut to calculating new distances:

- Given a new cluster $C_k$ formed by merging $C_i$ and $C_j$, the distance to another cluster $C_l$ is:

**UPGMA**

This is a simple distance method called UPGMA which stands for unweighted pair group method using arithmetic averages (Sokal & Michener 1958).
• Given a set of taxa $X$ and a distance matrix $D$, UPGMA produces a rooted phylogenetic tree $T$ with edge lengths.
• It operates by clustering the given taxa, at each stage merging two clusters and at the same time creating a new node in the tree.
• The tree is assembled bottom-up, first clustering pairs of leaves, then pairs of clustered leaves etc.
• Each node is given a height and the edge lengths are obtained as the difference of heights of its two end nodes.

Example $X = \{1; 2; 3; 4; 5\}$, distances given by distance in the plane:

**Neighbor Joining**

Neighbor Joining is another distance-based method for reconstructing phylogenetic trees from distance data.

• Like UPGMA:
  o Constructs a tree by iteratively joining subtrees.

• Unlike UPGMA:
  o Doesn’t make molecular clock assumption.
  o Produces unrooted trees.
  o Assumes additivity: distance between pair of leaves is sum of lengths of edges connecting them.

There are also two key differences in the merging process:
• How pair of subtrees to be merged is selected on each iteration.
• How distances are updated after each merge.

The two key differences are:
• How pair of subtrees to be merged is selected on each iteration.
• How distances are updated after each merge.

So far, each iteration we’ve chosen the two clusters that are closest together.
• But suppose the real tree (which is hidden from us) looks like this:

$$d_{AB} = 3$$
$$d_{AC} = 5$$
Problem Based Learning

Let us say you are trying to create a phylogenetic tree for five species, labeled A through E. Their distances from each other are given by the following:

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>8</td>
<td>8</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>3</td>
<td>8</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>D</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>E</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Build the tree using UPGMA.

Solution

Here’s how the problem starts out.

First we see which two leaves are closest to each other.

• There’s a tie, so we’re just going to pick A and E.
• We recalculate the distance matrix, so that AE is a member.
  o Its distances are the averages of those of A and of E.
  o In this case, that’s trivial.

```
  AE  B  C  D
  AE  0  8  8  5
  B    0  3  8
  C    0   8
  D    0
```

Repeating, we chose to merge B and C.

• Again, distances are easy to recompute.
Now AE is closest to D.

And merge the last two, making the tree’s root.

You may have noticed that in this example, the distances were remarkably well behaved.

- Averages were easy to calculate—too easy.
- Should be a tip-off that this is toy data.

Actually, this data was constructed using the molecular clock assumption.

- Divergence of elements is assumed to occur at the exact same rate at all points on the tree.
  - Evolution at a constant rate.”
- This assumption is not generally true. Selection pressures vary across:
Phylogenetic Trees

NOTES

- Time periods
- Organisms
- Genes within an organism
- Regions within a gene

- In other words, things evolve at different rates.
- If this does hold, then the data is said to be ultrametric.
  - Ultrametric data never really occurs in nature.
- Given ultrametric data, UPGMA will reconstruct the tree $T$ that is consistent with the data.

How can you tell ultrametric data?

- For any triplet of elements $i$, $j$, and $k$, one of two things will be true:
  1. The distances are all equal.
  2. Two are equal and the remaining one is smaller.
- For the matrix in the example, this was certainly true:
  
  $\begin{array}{cccc}
  A & B & C & D & E \\
  A & 0 & 8 & 8 & 5 & 3 \\
  B & 0 & 3 & 8 & 8 & \\
  C & 0 & 8 & 8 & & \\
  D & 0 & 5 & & & \\
  E & & & & & 0 \\
  \end{array}$

In general, for real data:

- UPGMA is not guaranteed to return the correct tree.
  - Differing rates of evolution can confound it.
  - Will do better with classic Darwinian evolution than with punctuated equilibrium.
- However, it may still be a reasonable heuristic.

Check Your Progress

1. What is phylogeny?
2. Why is phylogeny good for?
3. What is a phylogenetic tree?
4. Define the term cladogram.
5. What does neighbor joining mean?
12.3 MOLECULAR TOOLS FOR MICROBIAL DIVERSITY

Molecular Methods

Due to the inherent limitations of congenital phenotyping methods for detecting strains, as their mechanisms of resistance, molecular techniques that complement the information provided by these methods have been developed. Whole organisms that are only known from molecular sequences are now believed to be quantitatively significant in many environments. Molecular methods vary with respect to discriminatory powers, reproducibility, ease of use and interpretation.

Molecular methods have allowed characterization of many fresh bacterial isolates from natural environments. To obtain a congenital phenotypic description requires long fastidious work, and identification of novel isolates by the use of dichotomous keys or numerical taxonomy that do not warrant satisfactory identification of species already mentioned.

Identification of phylogenetic relationships can be derived from the degree of relatedness of their genomes. Methods using this approach comprise measurements of DNA relatedness over entire genome, comparison of ribotyping and comparative analysis of sequences of homologous genes.

Presently a direct comparison of rRNA sequences is probably the most powerful tool for identification of bacteria.

Comparative analysis of rRNA sequences not only provided phylogenetic framework which was lacking in microbial diversity, but also allowed the development of tools to address the vast microbial wealth. The ubiquity of rRNA molecules and comparative analysis of their sequences can be universally applied to infer relationships among organisms.

Essential rRNA domains are conserved across all phyla allowing the identification of tracts of sequences. The most commonly used form of competitive rRNA sequence analysis involves the construction of phylogenetic trees.

Genotyping Techniques

Polymerase Chain Reaction (PCR): The development of robust and simple DNA cloning techniques and PCR. Have allowed higher resolution analyses of more complex communities using rRNA sequence analysis. The presence of universally conserved sequences at the 5’ and 3’ ends allows the recovery of rRNA sequences and amplification of nearly complete rRNA genes from DNA extracted from natural samples. This is currently the most widely adopted method of. This reextracted DNA is subjected to rRNA sequence analysis.
The presence of universally conserved sequences at the 5’ and 3’ ends allow the recovery of rRNA sequences and amplification of nearly complete rRNA genes. This is currently the most widely adopted method of sequence retrieval from natural samples. The extracted DNA is subjected to PCR amplification using primers. The PCR product can be cloned by using commercially available designs.

DNA Hybridization: Colony hybridisation procedures using rRNA gene specific oligonucleotide probes of defined phylogenetic resolution may be used. Plasmids and restriction enzymes can be used to confirm the presence of cloned DNA.

This technique measures the similarity between the two DNAs.

Does not work well for comparing distantly related microorganisms.

Ribosomal RNA Sequence Analysis: rRNA genes, i.e., rDNA from an unknown is isolated, sequenced and compared to database entries. The rDNA can easily be isolated by using rDNA specific primers and PCR.

The amplified rDNA gene fragments are sequenced and compared to database entries, for example, Gene Bank or Ribosomal Database project.

- **Species level**
  - > 70% DNA re-association (DNA hybridization)
  - > 97% similarity between 16S rRNA sequences

- **Genus level**
  - > 20% - 30% DNA re-association (DNA hybridization)
  - 93% - 95% similarity in 16S rRNA sequences

- **Families**
  - 89 - 93 % similarity in 16S rRNA sequences

Family taxon is usually the highest level taxon used for prokaryotes.

Ribotyping: A technique used for bacterial identification.

- Genomic DNA is digested with restriction enzymes and then probed with an rRNA probe.
- Banding pattern is compared to a database.
- This technique is also known as molecular fingerprinting because a unique banding pattern appears for virtually any organism.

**Application of 16S/18S rRNA Sequences**

- **Fluorescent In-Situ Hybridization (FISH)**
  - Fluorescently labelled probes can enter permeabilized cells
  - Applied directly to cells in culture or a natural environment
  - Useful for non-culturable cells
• Microbial community analysis
  o Extraction of community nucleic acids
  o PCR amplify 16S/18S rRNA genes → rDNA clones
  o Sequence rDNA clones and generate phylogenetic tree
  o Can use quantitative PCR to determine relative abundance of specific organisms or groups

**Genetic Fingerprinting Techniques**

This technique can be used to characterize bacterial communities or single bacterial isolates. The G.F. of microbial communities provides a profile of the community diversity, based upon the physical separation of unique nucleic acid sequences.

Community analysis are relatively easy and rapid to perform and they allow simultaneous analysis of multiple samples enabling the comparison of genetic diversity from different habitats or their behavior over time.

**Check Your Progress**

6. How is identification of phylogenetic relationships derived?
7. What are the application of 16S/18S rRNA sequences?
8. What is genetic fingerprinting technique?

**12.4 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS**

1. Phylogeny is the ancestral relation between species.

2. Phylogeny is good for the reasons mentioned below:
   • To understand the process of molecular evaluation as objects typically are proteins or nucleic acid sequences.
   • To identify what is most conserved important in some class of sequences
   • Tracing population history and multiple sequence alignment
   • To estimate the time of divergence between various species to understand lineage they last shared a common ancestor and human origin.

3. Phylogenetic trees are representation of phylogenetic information that shows relationships between species, it shares its most recent common ancestor with.
4. Cladogram is a group of species that shares its features from a common ancestor at (the root). There exists a particular root node.

5. Neighbor joining is another distance-based method for reconstructing phylogenetic trees from distance data.

6. Identification of phylogenetic relationships can be derived from the degree of relatedness of their genomes.

7. Application of 16S/18S rRNA sequences are as follows:
   - Fluorescent In-Situ Hybridization (FISH)
   - Fluorescently labelled probes can enter permeabilized cells
   - Applied directly to cells in culture or a natural environment
   - Useful for non-culturable cells

8. Genetic fingerprinting techniques can be used to characterize bacterial communities or single bacterial isolates. The G.F. of microbial communities provides a profile of the community diversity, based upon the physical separation of unique nucleic acid sequences.

12.5 SUMMARY

- Traditionally phylogenetic relations were inferred using morphological evidence similarly proceeding the same way with both living and fossil species.
- The ‘morphological’ evidence refers to all observable characters in the whole organism, as distinct from molecular characters.
- Phylogenies are now increasingly inferred from molecular sequence evidence.
- The phylogenetic inference is principally the same in both types but the techniques used differ in many ways.
- The basic idea of phylogenetic is finding the genetic relationships between species to compare specific characters of the species, under the natural assumption that similar species are genetically close.
- Classic phylogenetics deals mainly with physical, or morphological features-size, color, number of legs, etc.
- Recent advances in sequencing technologies have created an exponentially increasing volume of DNA and protein sequence.
- Evidences from hard parts of the body such as vertebrate bones or mollusc shells constitute the evidence for the fossil species.
- A rooted tree is a tree in which one of the nodes is stipulated to be the root, and the direction of ancestral relationship is determined.
• An unrooted tree, shows how close or distant the species are, and has no pre-determined root and induces no hierarchy.

• Rooting an unrooted tree involves inserting a new node, which will function as the root node.

• Unrooted trees can be generated from rooted trees by omitting the root indicating the origin of evolution.

• In rooted tree there exists a particular node called the root from which the unique path leads to any other node. The direction of each path corresponds to evolutionary time and the root is the common ancestor of all taxonomic unit.

• In rooted evolutionary relationships are evident. In unrooted tree evolutionary relationships cannot be immediately assessed.

• Branch length is the number of changes, for example nucleotide substitutions that have occurred along a branch. The total number of changes in a particular tree is called the tree length.

• The evolutionary distances can be used to construct a matrix of distances between all individual pairs of taxa.

• A node is bifurcating binary or dichotomous if has only immediate descendent lineages but multifurcating if it has more than two immediate descendent lineages.

• In a strictly bifurcating tree, each internal node is incident to exactly three branches, two derived and one ancestral.

• A bifurcation is always interpreted as a speciation event. Two possible interpretation for a multifurcation in a tree.

• The polytomy represents two sequence of events whereby ancestral taxon gave rise to three or more decedent taxa simultaneously.

• Distance based methods is the amount of dissimilarity between pairs of sequence can be computed on the basis of sequence alignment.

• Maximum Likelihood (ML) seeks the tree that maximises the likelihood of the data. It calculates the likelihood for each tree and seeks the one that maximizes the likelihood of the data.

• Phylogenetic trees are meant to provide insight and not intended to represent an entire history of species.

• The output of the tree may be affected by several factors of gene transfer and protein function related to DNA degradation over time especially the evolutionary trees of the extinct organisms.

• Polyphasic taxonomy refers to the study of phylogeny based on both genotypic and phenotypic information ranging from molecular characteristics to ecological characters.
• Numerical taxonomy is computer based approaches of grouping organisms is called numerical taxonomy which is based on presence or absence of selected characters in the group of organisms clustered based on relatedness.

• Numerical taxonomy is a method to express difference through evolutionary distance by estimating percent similarity to use measure of difference to create a tree.

12.6 KEY WORDS

• **Phylogenetic tree**: Phylogenetic trees are representation of phylogenetic information that shows relationships between species, it shares its most recent common ancestor with.

• **Cladogram**: A group of species that shares its features from a common ancestor at (the root).

• **Distance based methods**: The amount of dissimilarity between pairs of sequence can be computed on the basis of sequence alignment.

• **Polyphasic Taxonomy**: Studying phylogeny based on both genotypic and phenotypic information ranging from molecular characteristics to ecological characters.

• **Numerical Taxonomy**: Computer based approaches of grouping organisms is called Numerical taxonomy which is based on presence or absence of selected characters in the group of organisms clustered based on relatedness.

• **Ribotyping**: A technique used for bacterial identification.

12.7 SELF ASSESSMENT QUESTIONS AND EXERCISES

**Short Answer Questions**

1. What is phylogeny?
2. What are the shared characters used to infer phylogeny?
3. What is phylogeny good for?
4. What is rooted and unrooted tree?
5. What is distance based phylogenetic tree?

**Long Answer Questions**

1. Write a detailed note on phylogenetic tree.
2. Draw a well-labelled diagram of phylogenetic tree.
3. Discuss about types and construction of phylogenetic tree.
4. What are the limitations to the use of phylogenetic tree?
5. Explain the molecular tools in assessing microbial diversity.

12.7 FURTHER READINGS

UNIT 13  METAGENOMICS: SEQUENCING METHODS, DATA ANALYSIS AND ITS APPLICATIONS

Structure
13.0 Introduction
13.1 Objectives
13.2 Metagenomics
   13.2.1 Sequencing Methods and Strategies
   13.2.2 Data Analysis and its Applications
13.3 Answers to Check Your Progress Questions
13.4 Summary
13.5 Key Words
13.6 Self Assessment Questions and Exercises
13.7 Further Readings

13.0 INTRODUCTION

Metagenomics is the genomic analysis of microorganisms by direct extraction and cloning of DNA from an assemblage of microorganisms. The development of metagenomics stemmed from the ineluctable evidence that as-yet-uncultured microorganisms represent the vast majority of organisms in most environments on the Earth. This evidence was derived from analyses of 16S rRNA gene sequences amplified directly from the environment, an approach that avoided the bias imposed by culturing and led to the discovery of vast new lineages of microbial life. Although the portrait of the microbial world was revolutionized by analysis of 16S rRNA genes, such studies yielded only a phylogenetic description of community membership, providing little insight into the genetics, physiology, and biochemistry of the members. Metagenomics provides a second tier of technical innovation that facilitates study of the physiology and ecology of environmental microorganisms. Novel genes and gene products discovered through metagenomics include the first bacteriorhodopsin of bacterial origin; novel small molecules with antimicrobial activity; and new members of families of known proteins, such as an Na]));\text{Li}^+\text{)}\text{H}^+\text{ antiporter, RecA, DNA polymerase, and antibiotic resistance determinants. Reassembly of multiple genomes has provided insight into energy and nutrient cycling within the community, genome structure, gene function, population genetics and microheterogeneity, and lateral gene transfer among members of an uncultured community. The application of metagenomic sequence information will facilitate the design of better culturing strategies to link genomic analysis with pure culture studies.}
In this unit, you will study about metagenomics, sequencing methods, data analysis and applications in detail.

13.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand what metagenomics is
- Discuss sequencing methods and strategies
- Explain data analysis and its applications

13.2 METAGENOMICS

Metagenomics is the study of genetic material recovered directly from environmental samples. The broad field may also be referred to as environmental genomics, ecogenomics or community genomics. While traditional microbiology and microbial genome sequencing and genomics rely upon cultivated clonal cultures, early environmental gene sequencing cloned specific genes (often the 16S rRNA gene) to produce a profile of diversity in a natural sample. Such work revealed that the vast majority of microbial biodiversity had been missed by cultivation-based methods. Recent studies use either ‘shotgun’ or PCR (Polymerase Chain Reaction) directed sequencing to get largely unbiased samples of all genes from all the members of the sampled communities. Because of its ability to reveal the previously hidden diversity of microscopic life, metagenomics offers a powerful lens for viewing the microbial world that has the potential to revolutionize understanding of the entire living world. As the price of DNA sequencing continues to fall, metagenomics now allows microbial ecology to be investigated at a much greater scale and detail than before.

Metagenomics represent a new approach in a genomic analysis. This method accesses the potential reservoir of novel genes in soil. To explore this reservoir, DNA from an environmental sample is extracted, cloned into an appropriate vector, and transformed into competent _E. coli_ cells. The resulting transformants in metagenomic libraries are screened for novel physiological, metabolic, and genetic features. Although time-consuming and labor-intensive, metagenomic is the most powerful environmental approach that offers possibilities to discover novel genes and novel biomolecules through the expression of genes from uncultivated and unknown bacteria in recipient host cell. Theoretically, a metagenomic database should contain DNA sequences for all the genes in the microbial community. However, often those genes cannot be expressed, folded, or excreted correctly in the corresponding host system.
The term metagenomics was first used by Jo Handelsman, Jon Clardy, Robert M. Goodman, Sean F. Brady, and others, and first appeared in publication in 1998. The term metagenome referenced the idea that a collection of genes sequenced from the environment could be analyzed in a way analogous to the study of a single genome. In 2005, Kevin Chen and Lior Pachter defined metagenomics as the application of modern genomics technique without the need for isolation and lab cultivation of individual specie.

Why Metagenomics?

Microbes run the world. It is that simple. Although we cannot usually see them, microbes are essential for every part of human life, indeed all life on Earth. Every process in the biosphere is touched by the seemingly endless capacity of microbes to transform the world around them. The chemical cycles that convert the key elements of life, carbon, nitrogen, oxygen, and sulphur into biologically accessible forms are largely directed by and dependent on microbes. All plants and animals have closely associated microbial communities that make necessary nutrients, metals, and vitamins available to their hosts. Through fermentation and other natural processes, microbes create or add value to many foods that are staples of the human diet. We depend on microbes to remediate toxins in the environment both the ones that are produced naturally and the ones that are the byproducts of human activities, such as oil and chemical spills. The microbes associated with the human body in the intestine and mouth enable us to extract energy from food that we could not digest without them and protect us against disease-causing agents.

These functions are conducted within complex communities intricate, balanced, and integrated entities that adapt swiftly and flexibly to environmental change. But historically, the study of microbes has focused on single species in pure culture, so understanding of these complex communities lags behind understanding of their individual members. We know enough, however, to confirm that microbes, as communities, are key players in maintaining environmental stability.

By making microbes visible, the invention of microscopes in the late 18th century made us aware of their existence. The development of laboratory cultivation methods in the middle 1800s taught us how a few microbes make their livings as individuals, and the molecular biology and genomics revolutions of the last half of the 20th century united this physiological knowledge with a thorough understanding of its underlying genetic basis. Thus, almost all knowledge about microbes is largely laboratory knowledge, attained in the unusual and unnatural circumstances of growing them optimally in artificial media in pure culture without ecological context. The science of metagenomics, only a few years old, will make it possible to
investigate microbes in their natural environments, the complex communities in which they normally live. It will bring about a transformation in biology, medicine, ecology, and biotechnology that may be as profound as that initiated by the invention of the microscope.

Role of Microbes:
- Microbes Modulate and Maintain the Atmosphere
- Microbes Keep Us Healthy
- Microbes Support Plant Growth and Suppress Plant Disease
- Microbes Clean Up Fuel Leaks

Contribution of Metagenomics in Advancements of Many Fields

Metagenomics offers a means of solving practical problems facing humanity. Cracking the secrets of some of Earth’s countless microbial communities will reveal ways to meet myriad challenges in biomedicine, agriculture, and environmental stewardship. These are among the most important potential contributions:

- **Earth Sciences**: The development of genome-based microbial ecosystem models to describe and predict global environmental processes, change, and sustainability.
- **Life Sciences**: The advancement of new theory and predictive capabilities in community-based microbial biology, ecology, and evolution.
- **Biomedical Sciences**: The description, on a global scale, of the role of the human microbiome (the collective genome of our symbionts) in health and disease in individuals and populations, and the development of novel diagnostic and treatment strategies based on this knowledge.
- **Bioenergy**: The development of microbial systems and processes for new bioenergy resources that will be more economical and environmentally sustainable and less vulnerable to disruption by world politics.
- **Bioremediation**: The development of tools for monitoring environmental damage at all levels (from climate change to leaking gas-storage tanks) and microbe-based (green) methods for restoring healthy ecosystems.
- **Biotechnology**: The identification and exploitation of the remarkably versatile and diverse biosynthetic capacities of microbial communities to generate beneficial industrial, food, and health products.
- **Agriculture**: The development of more effective and comprehensive methods for early detection of threats to Food Production (Crop and Animal Diseases) and Food Safety (monitoring and early detection
of Dangerous Microbial Contaminants) and the development of management practices that maximize the beneficial attributes of microbial communities in and around domestic plants and animals.

- **Biodefense and Microbial Forensics:** The development of more effective vaccines and therapeutics against potential bioterror agents, the deployment of genomic biosensors to monitor microbial ecosystems for known and potential pathogens, and the ability to precisely identify and characterize microbes that have played a role in war, terrorism, and crime events, thus contributing to discovering the source of the microbes and the party responsible for their use.

### 13.2.1 Sequencing Methods and Strategies

DNA sequencing is the process of determining the nucleic acid sequence – the order of nucleotides in DNA. It includes any method or technology that is used to determine the order of the four bases: Adenine (A), Guanine (G), Cytosine (C), and Thymine (T). The advent of rapid DNA sequencing methods has greatly accelerated biological and medical research and discovery.

Knowledge of DNA sequences has become indispensable for basic biological research, and in numerous applied fields, such as medical diagnosis, biotechnology, forensic biology, virology and biological systematics. The rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in the sequencing of complete DNA sequences, or genomes, of numerous types and species of life, including the human genome and other complete DNA sequences of many animal, plant, and microbial species. Figure 13.1 illustrates an example of the DNA sequence ladder formed by radioactive sequencing as compared to fluorescent peaks.

![Fig. 13.1 An Example of the DNA Sequencing](image-url)
The first DNA sequences were obtained in the early 1970s by academic researchers using laborious methods based on two-dimensional chromatography. Following the development of fluorescence-based sequencing methods with a DNA sequence, DNA sequencing has become easier and orders of magnitude faster.

Recovery of DNA sequences longer than a few thousand base pairs from environmental samples was very difficult until recent advances in molecular biological techniques allowed the construction of libraries in Bacterial Artificial Chromosomes (BACs), which provided better vectors for molecular cloning.

Primarily traditional genome organisation was based on the study of Prokaryotic organisms and the means of obtaining DNA sequence was called reverse genetics in which amino acid sequence of gene is back translated into a nucleotide sequence. Given the degeneracy of genetic code this method lead to two methods for direct sequencing of DNA. These were Chemical Cleavage Maxim-Gilbert method and the Sanger Chain Terminator method.

Some important applications of DNA sequencing are:

- The principle of delivering genes by locating DNA binding sites and cell membrane receptor sites for the use of viral vectors.
- Evolutionary biology has been brought to new light by inventing better predictive models for homology among species.
- Tailoring therapeutics depending on a person’s genetic make – detection and identification of genetic disorders, the rationale for developing treatments involving gene therapy.
- DNA profiling used for criminal investigations to identify suspects.
- Metagenomics, the study of genetic material recovered from environmental samples, allow the identification of organisms in water bodies, sewage.

Types of DNA Sequencing

There are two types of DNA sequencing performed:

- De Novo
- Resequencing

De Novo: The DNA is sequenced for the first time. There is absence of reference genomes to align reads to. De novo sequencing refers to sequencing a novel genome where there is no reference sequence available for alignment. Sequence reads are assembled as contigs, and the coverage quality of de novo sequence data depends on the size and continuity of the contigs, i.e., the number of gaps in the data. Next- Generation Sequencing (NGS) enables faster, more accurate characterization of any species compared to traditional methods, such as Sanger Sequencing. Illumina offers mate pair sequencing.
and long-read technology to complement shorter reads for comprehensive, accurate characterization of any species.

**Resequencing**: Resequencing on the other hand has a reference genome. Resequencing, especially with human samples, is one of the most popular applications of Next Generation Sequencing or NGS. It is used to determine the genomic variations of a sample in relation to a common reference sequence. The generated sequence is aligned to the reference sequence and mined for SNPs and CNVs as well as genomic rearrangements and indels.

**Early DNA Sequencing Approaches**

Early method of sequencing focused on protein sequencing. The amino acid sequence of a peptide has traditionally been determined by degradation. The advent of Recombinant DNA technology has facilitated the process of detecting the mRNA or messenger RNA and its sequence which can deduce a protein sequence faster.

Two DNA sequencing methods were developed independently by Maxam and Gilbert, and Sanger. Sanger sequencing was one of early methods used. Though the method was primarily based on capillary electrophoresis but even with automation and optimisation it was found to be too slow and costly. This lead to new technique that involved cyclic methods. This method fell under a family of techniques known as Next Generation Sequencing (NGS).

Sanger sequencing was developed by Frederick Sanger and his colleagues dominated the sequencing platform bringing successful completion to HGP (Human Genome Project). Although this technique has been replace by next generation methods it is still indispensable for small scale projects. In 1975 Sanger introduced method for DNA sequencing. The largest stretch of DNA that can be sequenced by this method are over a million times larger therefore it was a critical transition technique evolving in to the present day methods.

With the use of poly acrylamide gel, each genome was broken into small fragments for sequencing in order of increasing chain length.

Specific markers were used to join these fragments again because two fragments can only be shown to overlap if they have unique sequences that do not repeat themselves.

**Early DNA Sequencing Techniques**

Common challenges with Sanger method includes poor quality regions of the sequences due to primer binding. In cases where DNA fragments are cloned before sequencing the resulting fragments may contain parts of the cloning vector incorporated. However, PCR sequencing technology based on
pyrosequencing do away with clone vectors. The main obstacle is inadequate resolving power for separating large DNA fragments.

A sequencing can be done by different methods:
- Maxam-Gilbert Sequencing
- Chain Termination Methods
- Dye Terminator Sequencing
- Automation and Sample Preparation
- New Sequencing Methods

Maxam-Gilbert Sequencing

In 1976-1977, Allan Maxam and Walter Gilbert developed a DNA sequencing method based on chemical modification of DNA and subsequent cleavage at specific bases. With the enhancement of the Chain Termination method, the Maxam-Gilbert sequencing was least used in standard molecular biology due to its technical complexity.

The Maxam-Gilbert method is considered as general sequencing method, and is known as the ‘Chemical-Cleavage Protocol’ of degrading existing DNA chains, rather than synthesizing new ones. Maxam-Gilbert method, is based on use of selective reaction for cleavage of DNA at specific bases creating a single stranded DNA. With such four samples of DNA restriction fragment separate cleavage reactions a population of labelled cleavage products ending in known nucleotides is produced. These sequences are then mapped according to DNA size. A pure sample of radioactively end labelled DNA fragment is subjected to a chemical treatment that breaks each DNA molecule at places where one of the bases occur. This generates a population of labelled molecules whose sizes are determined by the sequence. Determination of different size samples cleaved with specific cleavage treatment yields complete sequence information. Using autoradiography, the labelled fragments can be detected and visualised as dark bands. The sequence of the original molecule can be inferred from a parallel electrophoretogram of the four samples indicating a radiolabelled DNA fragment.

However, much work remains to improve reliability of this technology due to a disadvantage of relying on toxic chemicals. It is rare to find clean cleavage products.

Chain Terminator Sequencing

The key element of Sanger method was a third form of ribose sugar - a dideoxynucleotide (ddNTP) which is an artificial molecule that lacks a hydroxyl group at both the 2nd and 3rd carbon used as DNA Chain Terminator since it cannot make a Phosphodiester Bond with the Nucleotide. This is what allows for a DNA strand to elongate. The incorporation of specific
ddNTP. In vitro would result in selective chain termination stopping the chain irreversibly.

A DNA primer is attached by hybridisation to the template strand and ddNTP are sequentially added to the primer strand by DNA polymerase continues to add base after base. The result is a set new chains all of different lengths in a mix of regular four dNTP monomers.

DNA elongation cannot occur with ddNTP. During DNA replication an incoming nucleoside triphosphate is linked by its 5-phosphate group to the 3-OH group of the last nucleotide of the growing chain. With ddNTP where there is no 3-OH group this reaction cannot take place.

Figures 13.2, 13.3 and 13.4 illustrates the process of Sanger Sequencing methodology with the help of a simple example recorded after the laboratory test.

**Fig. 13.2  Sanger Sequencing for Determining Position of ‘A’**
The Principle of DNA Sequencing

The structure of DNA gave two popular methods for DNA sequencing out of which for certain obvious reasons the Chain Termination Method is the most
commonly used method. This method involves the enzymatic synthesis of the radio labelled fragments from unlabeled DNA strands that can be separated from one another using polyacrylamide gel electrophoresis.

The chain terminator method enchases on the following two properties of DNA polymerase:

- The ability to synthesis complimentary copy of single stranded of DNA template.
- The ability to use 2’3’dideoxynucleotides as substrates.

Figure 13.5 illustrates the structure of dideoxyribonucleoside triphosphate.

![Fig. 13.5 Structure of Dideoxyribonucleoside Triphosphate](image)

The isolated DNA strands along with most critical component of DNA polymerase separates into four separate sequencing reactions with four different reaction mixtures ddATP, ddGTP, ddCTP or ddTTP, interrupting the replicating process at one of the four bases (Refer Figure 13.6).

![Fig. 13.6 DNA Polymerase Separated into ddATP, ddTTP, ddCTP or ddGTP, Interrupting the Replicating Process at One of the Four Bases](image)
A short oligonucleotide is annealed and joined to the same position on each template strand that will be complementary to the template DNA.

- A DNA Template: Fresh synthesised DNA fragments are heat denatured into single strands.
- A primer tagged with a radioactive molecule at its 3’end.
- DNA polymerase with four nucleotides and one dideoxynucleotide.
- A dark band in a lane indicates a DNA fragment that is the result of chain termination the relative distribution of which are read as the sequence.

Sanger’s method of gene sequencing is also known as Dideoxy Chain Termination Method. It generates nested set of labelled fragments from a template strand of DNA to be sequenced by replicating that template strand and interrupting the replication process at one of the four bases.

Four different reaction mixtures are produced that terminates in A, T, G or C, respectively.

The Steps of the Procedure for Clone DNA Strands into a Vector

**Step 1:** A DNA primer is attached by hybridization to the template strand and deoxynucleosides triphosphates (dNTPPs) are sequentially added to the primer strand by DNA polymerase.

**Step 2:** The primer is designed for the known sequences at 3’ end of the template strand.

**Step 3:** M13 sequences is generally attached to 3’ end and the primer of this M13 is made.
Step 4: The reaction mixture also contains dideoxynucleoside triphosphate (ddNTPs) along with usual dNTPs.

Step 5: If during replication ddNTPs is incorporated instead of usual dNTPs in the growing DNA strand then the replication stops at that nucleotide.

Step 6: The ddNTPs are analogue of dNTPs.

Step 7: The ddNTPs lacks hydroxyl group (-OH) at C3 of ribose sugar, so it cannot make phosphodiester bond with next nucleotide, thus terminates the nucleotide chain.

Step 8: Respective ddNTPs of dNTPs terminates chain at their respective site. For example ddATP terminates at A site. Similarly ddCTP, ddGTP and ddTTP terminates at C, G and T site, respectively.

Template Preparation: Copies of template to be sequenced with short stretches at 3’ end of the template strand that will bind to a standard primer as shown below in Figure 13.8.

Copies of the template strand must be sequenced with short known sequences at 3’ end of the template strand. A DNA primer preparation essential to initiate replication at 3’ end required. Single stranded cloning vector M13 serve as binding site of primer at 3’ end.

To generate a set of labelled fragments that terminate at C, G, T and respective ddNTP are added to four different reaction mixture. The synthesised fragments compose the set of fragments needed to determine the order of the base in the complimentary strand. Figure 13.9 illustrates the DNA sequencing through the Sanger method.
Electrophoresis and Gel Reading: The batches of reaction mixture loaded into four different wells. The autoradiogram is read from bottom to top to determine the order of bases of the complimentary strand with shortest at the bottom.

Dye Terminator Sequencing

Dye terminator is an improved variant of Sanger sequencing in which involves sequencing in single reaction than four dideoxynucleotide chain terminators labelled by fluorescent primers (Refer Figure 13.10). These fluorescent dye primers exploit fluorescent energy transfer to optimise emission and absorption properties of the labelled fragment.
These offer the advantage of specific terminated elongated DNA chains so that background bands are eliminated and results in extremely cleaned sequenced data. Owing to greater expediency and speed dye terminating sequencing have become an essential tools for large scale sequencing in single colour detection instruments harnessing dye labelled chain terminators ddNTPs. The only limitation being dye effects due to incorporation variability resulting in unequal peak heights and shape in DNA sequence trace. Currently altered DNA polymerase enzyme system and dyes have been put to use for eliminating ‘Dye Blobs’.

- Highly accurate long sequence reads.
- Easier to automate at low cost using fluorescently labelled ddNTPs and primers for the first high throughput DNA sequencing machine.
- Less radioactive.

**Automation and Sample Preparation**

Automated sequencing has been developed in order to sequence more DNA in shorter period of time. In this process all four ddNTP’s, each labelled with a different colour dye are made to react in a single tube. Automate dDNA sequencers generate up to 348 DNA samples at one time involving PCR based method. To score on the quality of each peak of capillary electrophoresis can be used for size separation, detection and recording of dye fluorescence of DNA sequences, and data output can be traced that can trim low-quality DNA traces located at the end of sequences. Similar to the Sanger’s method, DNA is separated on a gel, but they all run on the same lane as opposed to four different ones since these dyes fluoresce at different wavelength, one for each labelled ddNTP used (Refer Figure 13.11).

```
dye label
  chain termination with ddGTF
  5' -GAATTCCTAGCAAGTTCTGTTCTCTCTGTCGTGCTCTG3'
  3' -GGACTTACAGGAAAGAGTTCCAGCTGTTCTCTGTCGTGCTCTG5'

  chain termination with ddATF
  5' -GAATTCCTAGCAAGTTCTGTTCTCTCTGTCGTGCTCTG3'
  3' -GGACTTACAGGAAAGAGTTCCAGCTGTTCTCTGTCGTGCTCTG5'

  chain termination with ddTTP
  5' -GAATTCCTAGCAAGTTCTGTTCTCTCTGTCGTGCTCTG3'
  3' -GGACTTACAGGAAAGAGTTCCAGCTGTTCTCTGTCGTGCTCTG5'

  chain termination with ddCTP
  5' -GAATTCCTAGCAAGTTCTGTTCTCTCTGTCGTGCTCTG3'
  3' -GGACTTACAGGAAAGAGTTCCAGCTGTTCTCTGTCGTGCTCTG5'
```

**Fig. 13.11 Automated Sequencing**
Figure 13.12 below shows the results of gel electrophoresis for the dye labelled DNA in automated sequencing. The image on the left shows the gel for the four reactions run in different lanes, as opposed to the image on the right which shows a gel where all the DNA is run in one lane.

Since different wavelength of dyes are used, identification of each band is determined according to the wavelengths at which it fluoresces. The results are depicted in the form of a chromatogram where location of the nucleotide in the sequence is marked by the colored peaks representing the four bases blue for C, green for A, black for G and red for T (Refer Figure 13.13).

Data analysis was carried out in steps, such as template preparation, gel pouring and sequencing operation to detect any ambiguity.

**Advantages**

- High Resolution
- Read Length Extended
- Data Analysis Comprehensive
Large-Scale Sequencing Strategies

Current methods form the basic arsenal of tools used to study the body’s responses to microbial and other diseases and can directly sequence very short DNA fragments in a single reaction (Refer Figure 13.14).

**Figure 13.14 Genomic DNA Fragmented into Random Pieces and Cloned as a Bacterial Library**

DNA from individual bacterial clones is sequenced and the sequence is assembled by using overlapping regions. Large scale sequencing capitalizes on sequence complementarity and at sequencing very long DNA pieces, such as the whole genome. First DNA is randomly cut with restriction enzyme and then ligated to linker sequences that permits isolation of short DNA fragments. This process is exploited in the DNA amplification in *E. coli* after the fragment is cloned into DNA vector. Short distinct DNA purified from individual bacterial colonies are individually assembled into one long contiguous sequence. This method referred to as De Novo Sequencing is carried out without any pre-existing information about the DNA. Gaps in the
assembled sequence may be filled by primer walking. The different strategies have different tradeoffs in speed and accuracy.

**Next Generation Sequencing (NGS): Shotgun Sequencing**

This is a type of **De Novo** sequencing, i.e., can assemble an entire genome that has not been sequenced before. This is widely used in larger sequencing projects to analyses DNA sequences longer than 1000 base pairs on the principle of breaking up multiple sequences of the same genome in various places and reassemble them in overlapping regions. These short fragments are cloned.

- Greater Sequencing Throughput.
- Cost Effective and more Economical Sequencing.
- Assures High Fidelity.
- Generates Long Read Length.

The strategy objectives include,

- Reduces Reaction Volume
- Extends the Numbers of Sequencing Reactions
- Massively Parallel Sequencing

![Diagram of Next Generation Sequencing (NGS)](image)

*Fig. 13.15 Next Generation Sequencing (NGS)*
Most of the techniques of molecular detection rely on an in vitro cloning step to amplify individual DNA molecules on isolating specific segments of DNA and sequencing is no exception. With the drive to reduce the cost the increase in throughput of conventional methods can be attained with the use of microchips.

**High-Throughput Sequencing**

With the development of above technology new methods are emerging that challenge the supremacy of the dideoxy method. High-Throughput sequencing parallelize the sequencing process are intended to lower the cost of sequencing beyond what is possible with standard dye terminator methods. The impact of these technologies have been profound and very high throughput is achieved. However, the accuracy of the individual reads and its length is highly compromised upon when compared to Sanger sequencing. Nonetheless the assemblies of such comparative genome analysis has revealed interesting patterns And can be highly accurate because of high degree of sequence coverage obtainable. The first practical application in which sequence data is aligned with a reference genome sequence is essentially resequencing, in isolating segments with specific differences. These massive parallel strategies for collecting sequence data are not potential sources of biological resources to be explored for future use, but also outstrip our capacity and indicate the need for further extensive genetic, enzymatic and physiological analyses, before genomic data can be fully exploited.

![High-Throughput Sequencing Procedure](image)

**Procedure**

- Genomic DNA is fragmented by sonification.
- All sticky end fragments are blunt ended with DNA polymerase exonuclease activity.
- Different length fragments separated after 5’end phosphorylation.
- A library is created per each size in plasmids and transformed into *E. Coli* cells.
• Vector DNA purified and amplified.
• Each DNA strand is sequenced overlapping segments and generates long continuous stretches.

13.2.2 Data Analysis and its Applications

Polymerase Chain Reaction (PCR): Analysis of Data

The Polymerase Chain Reaction (PCR) eliminates the dependence on cells to make multiple copies of DNA, achieving the same result through relatively simple reactions outside the cell.

Most methods of DNA analysis, such as restriction enzyme digestion and agarose gel electrophoresis, or DNA sequencing require is designed to copy the entire genome that initiates at one or more origin sites of large amounts of a specific DNA fragment. Thus in the past, large amounts of DNA were produced by growing the host cells of a genomic library.

However, libraries take time and effort to prepare and DNA samples of interest and the process is laborious often resulting the end product in minute quantities. The Polymerase Chain Reaction (PCR) is in vitro laboratory technique that permits rapid amplification in the number of copies of specific DNA sequences for further analysis (Refer Figure 13.17).

![DNA Sequencing Showing DNA Primer](image_url)
However, while primers composed of RNA are normally used in cells, DNA primers are used for PCR over multiple cycles. The high temperature required to physically (rather than enzymatically) separate the DNA primers are preferable due to their stability with known sequences targeting a specific DNA region can be chemically synthesized. These DNA primers are similar to the DNA probes in nature of its function.

Next Generation Sequencing (NGS) has made great strides in sequencing technology as it enables sequencing of genes in a high throughput manner with low cost. One of the important aspects of NGS data is its usage in early disease diagnosis especially in cancer which was earlier not possible with conventional sequencing technologies, such as Sanger sequencing, NGS can identify all those mutations which cannot be identified using conventional sequencing technologies, because the analyzer can sequence the whole genome, exome or transcriptome. The exome sequencing is preferred, as a higher number of mutations are found to exist in the exome part of genes.

**Applications of NGS**

NGS technologies have many applications, such as DNA sequencing and assembly to determine an unknown genome without any preparation or search for variations among genome samples, RNA-sequencing to analyze gene expression and to predominantly identify DNA regions of DNA binding proteins, for example, transcription factors, etc. The most important application of NGS is in identifying mutations. While most of the NGS applications concentrate on identification of Single Nucleotide Variations (SNVs) or small insertions/deletions (indels), structural variation including translocations, bigger indels, and Copy Number Variation (CNV) can also be recognized from similar data. Single-cell sequencing is used for characterization of cancer heterogeneity. Cancer heterogeneity is caused due to different factors, such as tissue hierarchies, clonal evolution, rare cells and dynamic cell states.

**Check Your Progress**

1. What is the role of microbes?
2. What is DNA sequencing?
3. Give some applications of DNA sequencing.
4. Name the types of DNA sequencing performed.
5. How is procedure of high-throughput sequencing conducted?
6. What is Polymerase Chain Reaction (PCR)?
13.3 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Role of microbes:
   - Microbes modulate and maintain the atmosphere.
   - Microbes keep us healthy.
   - Microbes support plant growth and suppress plant disease.
   - Microbes clean up fuel leaks.

2. DNA sequencing is the process of determining the nucleic acid sequence – the order of nucleotides in DNA. It includes any method or technology that is used to determine the order of the four bases: Adenine, Guanine, Cytosine, and Thymine.

3. Some important applications of DNA sequencing are:
   - The principle of delivering genes by locating DNA binding sites and cell membrane receptor sites for the use of viral vectors.
   - Evolutionary biology has been brought to new light by inventing better predictive models for homology among species.
   - Tailoring therapeutics depending on a person’s genetic make – detection and identification of genetic disorders, the rationale for developing treatments involving gene therapy.

4. There are two types of DNA sequencing performed:
   - De Novo
   - Resequencing

5. Procedure of high-throughput sequencing is conducted as follows:
   - Genomic DNA is fragmented by sonification.
   - All sticky end fragments are blunt ended with DNA polymerase exonuclease activity.
   - Different length fragments separated after 5’end phosphorylation.
   - A library is created per each size in plasmids and transformed into *E. Coli* cells.
   - Vector DNA purified and amplified.
   - Each DNA strand is sequenced overlapping segments and generates long continuous stretches.

6. The Polymerase Chain Reaction (PCR) is in vitro laboratory technique that permits rapid amplification in the number of copies of specific DNA sequences for further analysis.
13.4 SUMMARY

- Metagenomics is the study of genetic material recovered directly from environmental samples.
- The broad field may also be referred to as environmental genomics, ecogenomics or community genomics.
- While traditional microbiology and microbial genome sequencing and genomics rely upon cultivated clonal cultures, early environmental gene sequencing cloned specific genes (often the 16S rRNA gene) to produce a profile of diversity in a natural sample.
- Recent studies use either ‘shotgun’ or PCR directed sequencing to get largely unbiased samples of all genes from all the members of the sampled communities.
- Metagenomics represent a new approach in a genomic analysis. This method accesses the potential reservoir of novel genes in soil.
- To explore this reservoir, DNA from an environmental sample is extracted, cloned into an appropriate vector, and transformed into competent \textit{E. coli} cells.
- The resulting transformants in metagenomic libraries are screened for novel physiological, metabolic, and genetic features.
- The term metagenomics was first used by Jo Handelsman, Jon Clardy, Robert M. Goodman, Sean F. Brady, and others, and first appeared in publication in 1998.
- By making microbes visible, the invention of microscopes in the late 18th century made us aware of their existence.
- The development of laboratory cultivation methods in the middle 1800s taught us how a few microbes make their livings as individuals, and the molecular biology and genomics revolutions of the last half of the 20th century united this physiological knowledge with a thorough understanding of its underlying genetic basis.
- DNA sequencing is the process of determining the nucleic acid sequence – the order of nucleotides in DNA.
- It includes any method or technology that is used to determine the order of the four bases: Adenine, Guanine, Cytosine, and Thymine.
- The advent of rapid DNA sequencing methods has greatly accelerated biological and medical research and discovery.
- Knowledge of DNA sequences has become indispensable for basic biological research, and in numerous applied fields, such as medical diagnosis, biotechnology, forensic biology, virology and biological systematics.
- The rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in the sequencing of complete DNA sequences, or genomes, of numerous types and species of life, including the human genome and other complete DNA sequences of many animal, plant, and microbial species.
- The first DNA sequences were obtained in the early 1970s by academic researchers using laborious methods based on two-dimensional chromatography.
- Recovery of DNA sequences longer than a few thousand base pairs from environmental samples was very difficult until recent advances in molecular biological techniques allowed the construction of libraries in Bacterial Artificial Chromosomes (BACs), which provided better vectors for molecular cloning.
- Primarily traditional genome organisation was based on the study of prokaryotic organisms and the means of obtaining DNA sequence was called reverse genetics in which amino acid sequence of gene is back translated into a nucleotide sequence.
- Resequencing, especially with human samples, is one of the most popular applications of Next Generation Sequencing (NGS).
- The generated sequence is aligned to the reference sequence and mined for SNPs and CNVs as well as genomic rearrangements and indels.
- The structure of DNA gave two popular methods for DNA sequencing out of which for certain obvious reasons the chain termination method is the most common method used.
- Another general sequencing method, known as the ‘Chemical Cleavage Protocol’ of degrading existing DNA chains, rather than synthesizing new ones.
- Maxam-Gilbert method, is based on use of selective reaction for cleavage of DNA at specific bases creating a single stranded DNA.
- Dye terminator is an improved variant of Sanger sequencing in which involves sequencing in single reaction than four dideoxynucleotide chain terminators labelled by fluorescent primers.
- Most of the techniques of molecular detection rely on an in vitro cloning step to amplify individual DNA molecules on isolating specific segments of DNA and sequencing is no exception.
- The polymerase chain reaction eliminates the dependence on cells to make multiple copies of DNA, achieving the same result through relatively simple reactions outside the cell.
- Most methods of DNA analysis, such as restriction enzyme digestion and agarose gel electrophoresis, or DNA sequencing require is designed...
to copy the entire genome that initiates at one or more origin sites of large amounts of a specific DNA fragment.

13.5 KEY WORDS

- **Metagenomics**: Metagenomics is the study of genetic material recovered directly from environmental samples.
- **De novo sequencing**: De novo sequencing refers to sequencing a novel genome where there is no reference sequence available for alignment.
- **DNA sequencing**: DNA sequencing is the process of determining the nucleic acid sequence – the order of nucleotides in DNA.
- **Polymerase Chain Reaction (PCR)**: The Polymerase Chain Reaction (PCR) is an in-vitro laboratory technique that permits rapid amplification in the number of copies of specific DNA sequences for further analysis.

13.6 SELF ASSESSMENT QUESTIONS AND EXERCISES

**Short Answer Questions**

1. What is metagenomics?
2. Write a short note on the areas where metagenomics can be used.
3. What is sequencing?
4. What is resequencing?
5. What are the different methods by which sequencing can be done?
7. Define Next Generation Sequencing (NGS).

**Long Answer Questions**

1. Discuss about metagenomics and its importance in detail.
2. What are the various methods of sequencing? Explain with the help of examples.
3. Explain the different types of DNA sequencing with the help of examples.
4. Briefly discuss about Next Generation Sequencing (NGS) with the help of examples.
5. Write a detailed note on dye terminator sequencing.
6. Explain high-throughput sequencing.
7. What is data analysis? Give its various applications.
13.7 FURTHER READINGS


UNIT 14 BACTERIAL AND VIRAL DISEASES

Structure
14.0 Introduction
14.1 Objectives
14.2 Bacterial Diseases
   14.2.1 Typhoid Fever
   14.2.2 Tuberculosis
   14.2.3 Leprosy
14.3 Viral Diseases
   14.3.1 Ebola Virus
   14.3.2 Hepatitis B Virus
   14.3.3 Acquired Immune Deficiency Syndrome (AIDS)
14.4 Answers to Check Your Progress Questions
14.5 Summary
14.6 Key Words
14.7 Self Assessment Questions and Exercises
14.8 Further Readings

14.0 INTRODUCTION

Bacterial disease, any of a variety of illnesses caused by Bacteria. Until the mid-20th century, bacterial pneumonia was probably the leading cause of death among the elderly. Improved sanitation, vaccines, and antibiotics have all decreased the mortality rates from bacterial infections, though antibiotic-resistant strains have caused a resurgence in some illnesses. Bacteria and viruses are different types of pathogens, organisms that can cause disease. Bacteria are larger than viruses and are capable of reproducing on their own. Viruses are much smaller than Bacteria and cannot reproduce on their own. Instead, viruses reproduce by infecting a host and using the host’s DNA repair and replication systems to make copies of itself. The symptoms of a bacterial or viral infection depend on the area of the body that is affected. Sometimes the symptoms of the two can be very similar.

Bacteria cause disease by secreting or excreting toxins, by producing toxins internally, which are released when the Bacteria disintegrate, or by inducing sensitivity to their antigenic properties. Other serious bacterial diseases include cholera, diphtheria, bacterial meningitis, tetanus, Lyme disease, gonorrhea, and syphilis. Typhoid is a bacterial infection that can lead to a high fever, diarrhea, and vomiting. It can be fatal. It is caused by the Bacteria Salmonella typhi. Tuberculosis (TB) is a potentially serious infectious disease that mainly affects your lungs. The Bacteria that cause tuberculosis are spread from one person to another through tiny droplets released into the
air via coughs and sneezes. Leprosy, also known as Hansen’s disease, is a chronic infectious disease caused by *Mycobacterium leprae*. The disease mainly affects the skin, the peripheral nerves, mucosal surfaces of the upper respiratory tract and the eyes. Leprosy is known to occur at all ages ranging from early infancy to very old age.

In this unit, you will study about bacterial and viral diseases like typhoid, Tuberculosis (TB) and leprosy and viral diseases like Ebola, Hepatitis B and HIV in detail.

### 14.1 OBJECTIVES

After going through this unit, you will be able to:

- Discuss about causative agents, transmission, prophylaxis and control of bacterial diseases like typhoid, Tuberculosis (TB) and leprosy
- Explain the causative agents, transmission, prophylaxis and control of viral diseases like Ebola, Hepatitis B and HIV

### 14.2 BACTERIAL DISEASES

Bacteria are microscopic, single-cell organisms that live almost everywhere. Bacteria live in every climate and location on earth. Some are airborne while others live in water or soil. Bacteria live on and inside plants, animals, and people. The word Bacteria has a negative connotation, but Bacteria actually perform many vital functions for organisms and in the environment. For example, plants need Bacteria in the soil in order to grow. The vast majority of Bacteria are harmless to people and some strains are even beneficial. In the human gastrointestinal tract, good Bacteria aid in digestion and produce vitamins. They also help with immunity, making the body less hospitable to bad Bacteria and other harmful pathogens. When considering all the strains of Bacteria that exist, relatively few are capable of making people sick.

A Bacterial infection is a proliferation of a harmful strain of Bacteria on or inside the body. Bacteria can infect any area of the body. Pneumonia, meningitis, and food poisoning are just a few illnesses that may be caused by harmful Bacteria. Bacteria come in three basic shapes: rod-shaped (bacilli), spherical (cocci), or helical (spirilla). Bacteria may also be classified as Gram-Positive or Gram-Negative. Gram-Positive Bacteria have a thick cell wall while Gram-Negative Bacteria do not. Gram staining, bacterial culture with antibiotic sensitivity determination, and other tests are used to identify bacterial strains and help determine the appropriate course of treatment.

Bacteria can be strictly pathogenic, which means that they will cause disease if they manage to overwhelm the human immune system. Other
Bacterial and Viral Diseases

NOTES

Bacteria only cause disease given the right circumstances, these are so-called opportunistic pathogens. Opportunistic pathogens normally do not cause infections in healthy humans but when the immune system is compromised or suppressed by for example cancer chemotherapy, other diseases (like HIV/AIDS) or malnutrition, the risk of infection increases. These infections often originate from the individual’s own bacterial flora, such as that on the skin or in the gut. Many bacterial pathogens can spread and infect via water and food, including *Salmonella*, *Campylobacter* and *E. coli*. Sometimes Bacteria are transmitted directly or indirectly from animals to humans and cause disease. Such infections are called zoonotic infections. Other Bacteria like *Neisseria gonorrhea* and *Chlamydia trachomatis* spread via sexual contacts.

Managing Bacterial Infections

The introduction of antibiotics to treat bacterial infections in combination with improved hygiene and sanitation, use of preventive vaccinations as well as increased knowledge about bacteria have greatly reduced deaths from bacterial diseases. However, antibiotic resistance among Bacteria is now threatening to again leave us without effective treatments for many common bacterial infections. Resistant Bacteria are now widespread in most parts of the world and more and more people die from bacterial infections because the antibiotics have stopped working.

14.2.1 Typhoid Fever

Typhoid fever is an infectious feverish disease caused by the bacterium *Salmonella typhi* (Refer Figure 14.1) and less commonly by *Salmonella paratyphi*. It is an acute generalized infection of the reticuloendothelial system, intestinal lymphoid tissue, and the gall bladder. The infection always comes from another human, either an ill person or a healthy carrier of the bacterium. Symptoms are often non-specific and clinically non-distinguishable from other febrile illnesses. However, clinical severity varies and severe cases may lead to serious complications or even death. It occurs predominantly in association with poor sanitation and lack of clean drinking water. According to the most recent estimates, between 11 and 21 million cases and 128 000 to 161 000 typhoid-related deaths occur annually worldwide. A similar but often less severe disease, paratyphoid fever, is caused by *Salmonella Paratyphi* A and B (or uncommonly Paratyphi C). The bacterium is passed on with water and foods and can withstand both drying and refrigeration. Causative agent: Caused by bacterium *Salmonella Typhi*. 
Mode of Transmission

- Ingestion of contaminated food or water
- Contact with an acute case of typhoid fever.
- Contact with a chronic asymptomatic cancer.
- Water is contaminated where inadequate sewerage systems and poor sanitation.
- Eating food or drinking beverages that handled by a person carrying the Bacteria.
- Salmonella enteritidis and Salmonella typhimurium are other Salmonella Bacteria, cause food poisoning and diarrhoea.

Signs and Symptoms

Early illness

Once signs and symptoms do appear, you’re likely to experience:

- Fever that starts low and increases daily, possibly reaching as high as 104.9 degree F (40.5 degree C)
- Headache
- Weakness and Fatigue
- Muscle Aches
- Sweating
- Dry Cough
- Loss of Appetite and Weight Loss
- Abdominal Pain
- Diarrhea or Constipation
- Rash
**Bacterial and Viral Diseases**

**NOTES**

- Extremely Swollen Abdomen

**Later Illness**

If you don’t receive treatment, you may:

- Become delirious.
- Lie motionless and exhausted with your eyes half-closed in what’s known as the typhoid state.

In addition, life-threatening complications often develop at this time. In some people, signs and symptoms may return up to two weeks after the fever has subsided.

**Members of the Genus Salmonella**

Rod shaped, flagellated, aerobic, Gram-Negative bacterium. Large numbers of fimbrial and non-fimbrial adhesions are present, mediate biofilm formation and contact to host cells. Secreted proteins involved in host cell invasion and intracellular proliferation. Infects cattle, poultry, cats, humans. Refrigeration and freezing substantially slow or halt their growth. Pasteurizing and food irradiation kill Salmonella for commercially produced food stuffs containing raw eggs.

**How does the Bacteria Cause Disease?**

Ingestion of contaminated food or water.

*Salmonella* Bacteria

↓

Invade small intestine and enter the bloodstream.

↓

They are carried by white blood cells in the liver, spleen, and bone marrow.
Multiply and reenter the bloodstream.

Bacteria invade gallbladder, biliary system, and the lymphatic tissue of the bowel and multiply in high numbers. Then pass into the intestinal tract and can be identified for diagnosis in cultures from the stool tested in the lab.

No symptoms: if only a mild exposure, people become ‘carriers’ of typhoid.

Poor appetite, headaches, generalized aches and pains, fever, lethargy, diarrhea.

Have a sustained fever as high. Chest congestion develops in many patients, abdominal pain and discomfort are common, constipation, mild vomiting, and slow heartbeat. Figure 14.2 shows the pathogenesis of *Salmonella typhi*.

![Fig. 14.2 Pathogenesis of Salmonella typhi](image)

**Time Frame**

Occurs gradually over a few weeks after exposure to the Bacteria. Without treatment the conditions can last for weeks or even a month or longer.

- **First Stage**: Typhoid fever: the beginning stage is characterized by high fever, fatigue, weakness, headache, skin rash on the chest and abdominal area.
- **Second Stage**: Second stage typhoid fever is characterized by weight loss, high fever, severe diarrhea and severe constipation. The abdominal region also appears distended.
**Bacterial and Viral Diseases**

- **Typhoid State:** If untreated the individual may be delirious or unable to stand and move, and the eyes may be partially open during this time.

**Symptoms Visible**

- Blood, Bone Marrow, or Stool Cultures Test
- Widal Test
- Slide Agglutination
- Antimicrobial Susceptibility Testing

**Widal Test:** A test involving agglutination of typhoid bacilli when they are mixed with serum containing typhoid fever; used to detect the presence of *Salmonella typhi* and *Salmonella paratyphi*.

**Prevention**

- First Type of Vaccine
  - Contains killed *Salmonella typhi* Bacteria.
  - Administered by a shot.
- Second Type of Vaccine: Contains live but weakened strain of the *Salmonella* Bacteria that causes typhoid fever.
- Taken Orally:
  - To get vaccinated while travelling to countries where typhoid is common.
  - To follow the booster doses.
  - Avoid risky food and drinks.

**14.2.2 Tuberculosis**

Tuberculosis is a chronic disease that is rampant around the world. As long time and multiple therapy medication is taken, adverse reactions are most common. Discontinuous medications either temporarily or permanently shows risks to the individuals and TB resistance become complex or has serious adverse effects.

Certain observations as:

- Monitoring the Weight.
- Patients: Genetic Disposition, Malnutrition.
- Awareness of TB among the people.
- Examining the Edema and Achieving the Pharmacoepidemiological studies.
**Causative Agent: *Mycobacterium tuberculosis***

*Mycobacterium* are slender rod Bacteria that are stained with differential stains (Ziehl-Neelsen). They can be grown in lipid rich culture mediums in 12-18 hrs.

It is a widespread and fatal infectious disease typically attacks the lungs and also other parts of the body. The contamination of the disease occurs through the sneezing, coughing and through the respiratory fluids through the air.

Initial droplet infection results in primary tuberculosis localized in the apices of lungs.

Primary infection remain silent primarily, but does progress to secondary stage gradually in a few. This can bring about extensive tissue necrosis, the specific immunity and allergy that develop in the course of action reflects T- lymphocyte function.

*Mycobacterium* is Gram Positive, although they do not take gram staining well. The lipid rich cell wall does not allow the alkaline stains to penetrate. Once the Bacteria have been stained they resist destaining even with HCl-alcohol. This property is known as acid fastness. It can be treated with two to four ant tubercular chemotherapeutics in either a short or a long regimen. Figure 14.3 shows *Mycobacterium tuberculosis.*

![Fig. 14.3 Mycobacterium tuberculosis](image)

**Symptoms of Tuberculosis**

Although your body may harbor the Bacteria that cause Tuberculosis (TB), your immune system usually can prevent you from becoming sick. For this reason, doctors make a distinction between:

- **Latent TB:** In this condition, you have a TB infection, but the Bacteria remain in your body in an inactive state and cause no symptoms. Latent
TB, also called inactive TB or TB infection, isn’t contagious. It can turn into active TB, so treatment is important for the person with latent TB and to help control the spread of TB. An estimated 2 billion people have latent TB.

- **Active TB:** This condition makes you sick and in most cases can spread to others. It can occur in the first few weeks after infection with the TB Bacteria, or it might occur years later.

**Signs and Symptoms of Active TB Include:**

- Coughing that lasts Three or More Weeks
- Coughing up Blood
- Chest Pain, or Pain With Breathing or Coughing
- Unintentional Weight Loss
- Fatigue
- Fever
- Night Sweats
- Chills
- Loss of Appetite

**TB Risk Factors**

Anyone can get TB, but certain people should be tested for TB infection because they are at higher risk for being infected with TB Bacteria, including:

- People who have spent time with someone who has TB disease.
- People from a country where TB disease is common in most countries in Latin America, the Caribbean, Africa, Asia, Eastern Europe, and Russia.
- People who live or work in high-risk settings, for example correctional facilities, long term care facilities or nursing homes, and homeless shelters.
- Healthcare workers who care for patients at increased risk for TB disease.
- Infants, children and adolescents exposed to adults who are at increased risk for latent tuberculosis infection or TB disease.

Some of the other risk factors are as follows:

**Weak Immune System**

A healthy immune system often successfully fights TB Bacteria, but your body can’t mount an effective defense if your resistance is low. A number of diseases, conditions and medications can weaken your immune system, including:
- HIV/AIDS
- Diabetes
- Severe Kidney Disease
- Certain Cancers
- Cancer Treatment, such as Chemotherapy
- Drugs to Prevent Rejection of Transplanted Organs
- Some Drugs used to Treat Rheumatoid Arthritis, Crohn’s Disease and Psoriasis
- Malnutrition
- Very Young or Advanced Age

**Traveling or Living in Certain Areas**

The risk of contracting tuberculosis is higher for people who live in or travel to areas that have high rates of tuberculosis and drug-resistant tuberculosis, including:

- Africa
- Eastern Europe
- Asia
- Russia
- Latin America
- Caribbean Islands

**Poverty and Substance Used**

- Lack of Medical Care: If you receive a low or fixed income, live in a remote area, have recently immigrated to the United States, or are homeless, you may lack access to the medical care needed to diagnose and treat TB.
- Substance Usage: Use of IV drugs or excessive alcohol weakens your immune system and makes you more vulnerable to tuberculosis.
- Tobacco Usage: Using tobacco greatly increases the risk of getting TB and dying of it.

**Where you Work or Live**

- Health Care Work: Regular contact with people who are ill increases your chances of exposure to TB Bacteria. Wearing a mask and frequent hand-washing greatly reduce your risk.
- Living or working in a residential care facility. People who live or work in prisons, homeless shelters, psychiatric hospitals or nursing homes...
are all at a higher risk of tuberculosis. That is because the risk of the disease is higher anywhere there is overcrowding and poor ventilation.

- Living in or emigrating from a country where TB is common. People from a country where TB is common may be at high risk of tuberculosis infection.
- Living with someone infected with TB. Living with someone who has TB increases your risk.

**Pathogenesis**

Most of the time there are no symptoms found in individuals affected by tuberculosis, i.e., asymptomatic. The infection sets in when the Bacteria reach the pulmonary alveoli of the lungs which invades and undergo replication within endosomes of alveolar macrophages. The macrophages envelopes the whole Bacteria forming a phagosome. This combines with lysosome to destroy Bacteria by forming phagolysosome. But the *M. tuberculosis* has a thick waxy mycolic acid capsules that protects it from toxic substances. The host immune respond against the Bacteria after macrophages undergone phagocytosis. With receptor cells they develop innate immune response to increase the production of pro-inflammatory cytokines to bring about the anti-microbial activity against the pathogen. Figure 14.4 shows some of the possible causes for pulmonary Tuberculosis.

![Fig. 14.4 Some of the Possible Causes for Pulmonary Tuberculosis](image)

As shown the primary TB that develops post infection is clinically silent. Years later if secondary TB develops, may spread to the entire bronchial system or other organ system.
The main source of infection is the human carrier. There are no healthy carriers. Transmission of the disease is direct, by droplet infection. Indirect transmission is between dust and milk. The incubation period for Tuberculosis is 4-12 weeks.

**Prophylaxis:** An active live vaccine BCG (lyophilized bovine TB of the Calmette-Guerin type) is available that can reduce the risk. In places with low level prevalence the BCG (Bacillus Calmette–Guérin) vaccination is no longer recommended. Preventive Chemotherapy of latent tuberculosis bacterial infection has proved effective in high risk persons.

Monteux testing confirms the exposure of the patient to the infective bacilli. Ziehl-Neelsen staining by culture gives best results for the appearance of pathogen. PCR is highly sensitive for the treatment of TB.

- BCG Vaccine
- Radiology
- Microbial Culture

**Complications**

Without treatment, tuberculosis can be fatal. Untreated active disease typically affects your lungs, but it can spread to other parts of your body through your bloodstream. Examples of tuberculosis complications include:

- **Spinal Pain:** Back pain and stiffness are common complications of tuberculosis.
- **Joint Damage:** Tuberculosis arthritis usually affects the hips and knees.
- **Swelling of the membranes that cover your brain (meningitis).** This can cause a lasting or intermittent headache that occurs for weeks. Mental changes also are possible.
- **Liver or Kidney Problems:** Your liver and kidneys help filter waste and impurities from your bloodstream. These functions become impaired if the liver or kidneys are affected by tuberculosis.
- **Heart Disorders:** Rarely, tuberculosis can infect the tissues that surround your heart, causing inflammation and fluid collections that may interfere with your heart’s ability to pump effectively. This condition, called cardiac tamponade, can be fatal.

**Some Key Facts about Tuberculosis (TB)**

- Tuberculosis (TB) is one of the top 10 causes of death worldwide.
- In 2017, 10 million people fell ill with TB, and 1.6 million died from the disease (including 0.3 million among people with HIV).
- TB is a leading killer of HIV-positive people.
In 2017, an estimated 1 million children became ill with TB and 230,000 children died of TB (including children with HIV associated TB).

Multidrug-Resistant TB (MDR-TB) remains a public health crisis and a health security threat. WHO (World Health Organisation) estimates that there were 558,000 new cases with resistance to rifampicin – the most effective first-line drug, of which - 82% had MDR-TB.

Globally, TB incidence is falling at about 2% per year. This needs to accelerate to a 4–5% annual decline to reach the 2020 milestones of the End TB Strategy.

An estimated 54 million lives were saved through TB diagnosis and treatment between 2000 and 2017.

Ending the TB epidemic by 2030 is among the health targets of the Sustainable Development Goals.

**14.2.3 Leprosy**

Leprosy is a disease mainly caused by the Bacteria *Mycobacterium leprae* (Refer Figure 14.5), (Hensen 1973). It is mainly a Granulomatous disease affecting: peripheral nerves and mucosa of the upper-respiratory tract. Granulomatous - refers to granulomas which are lesions of epithelioid macrophages. The disease develops slowly (from six months to 40 years) and results in skin lesions and deformities, most often affecting the cooler places on the body (for example, eyes, nose, earlobes, hands, feet, and testicles). The skin lesions and deformities can be very disfiguring and are the reason that historically people considered infected individuals outcasts in many cultures. Although human-to-human transmission is the primary source of infection, three other species can carry and (rarely) transfer *M. leprae* to humans: chimpanzees, mangabeys monkeys, and nine-banded armadillos. The disease is termed a chronic granulomatous disease, similar to tuberculosis, because it produces inflammatory nodules (granulomas) in the skin and peripheral nerves over time. However, leprosy is actually not that contagious. You can catch it only if you come into close and repeated contact with nose and mouth droplets from someone with untreated leprosy. Children are more likely to get leprosy than adults.

Leprosy is caused by a slow-growing type of Bacteria called *Mycobacterium leprae* (*M. leprae*). Leprosy is also known as Hansen’s disease, after the scientist who discovered *M. leprae* in 1873.
Different Classifications of Leprosy

There are multiple forms of leprosy described in the literature. The forms of leprosy depend on the person’s immune response to \textit{M. leprae}. A good immune response can produce the so-called tuberculoid form of the disease, with limited skin lesions and some asymmetric nerve involvement. A poor immune response can result in the lepromatous form, characterized by extensive skin and symmetric nerve involvement. Some patients may have aspects of both forms. Currently, two classification systems exist in the medical literature: the WHO system and the Ridley-Jopling system. The Ridley-Jopling system is composed of six forms or classifications, listed below according to increasing severity of symptoms:

- **Indeterminate Leprosy:** A few hypopigmented macules; can heal spontaneously, this form persists or advances to other forms.

- **Tuberculoid Leprosy:** A few hypopigmented macules, some are large and some become anesthetic (lose pain sensation); some neural involvement in which nerves become enlarged; spontaneous resolution in a few years, persists or advances to other forms; cell-mediated immune response appears in this classification but is almost absent in lepromatous leprosy.

- **Borderline Tuberculoid Leprosy:** Lesions like tuberculoid leprosy but smaller and more numerous with less nerve enlargement. This form may persist, revert to tuberculoid leprosy, or advance to other forms.

- **Mid-Borderline Leprosy:** Many reddish plaques that are asymmetrically distributed, moderately anesthetic, with regional adenopathy (swollen lymph nodes). The form may persist, regress to another form, or progress.
Bacterial and Viral Diseases

NOTES

Self-Instructional Material

- **Borderline Lepromatous Leprosy**: Many skin lesions with macules (flat lesions) papules (raised bumps), plaques, and nodules, sometimes with or without anesthesia; the form may persist, regress or progress to lepromatous leprosy.

- **Lepromatous Leprosy**: Early lesions are pale macules (flat areas) that are diffuse and symmetric. Later medical professionals can find many *M. leprae* organisms in the lesions. Alopecia (hair loss) occurs. Often patients have no eyebrows or eyelashes. As the disease progresses, nerve involvement leads to anesthetic areas and limb weakness. Progression leads to aseptic necrosis (tissue death from lack of blood to area), lepromas (skin nodules), and disfigurement of many areas, including the face. The lepromatous form does not regress to the other less severe forms. Histoid leprosy is a clinical variant of lepromatous leprosy that presents with clusters of histiocytes (a type of cell involved in the inflammatory response) and a grenz zone (an area of collagen separating the lesion from normal tissue) seen in microscopic tissue sections.

Globally, health care professionals use the Ridley-Jopling classification in evaluating patients in clinical studies. However, the WHO classification system is more widely used. It has only two forms or classifications of leprosy. The 2009 WHO classifications depend on the number of skin lesions as follows:

- **Paucibacillary Leprosy**: Skin lesions with no bacilli (*M. leprae*) seen in a skin smear.

- **Multibacillary Leprosy**: Skin lesions with bacilli (*M. leprae*) seen in a skin smear.

Figure 14.6 (A) and (B) illustrates different pictures of people suffering from leprosy.

(A)
Spreading of Leprosy

Researchers suggest that *M. leprae* spreads person to person by nasal secretions or droplets from the upper respiratory tract and nasal mucosa. However, the disease is not highly contagious like the flu. They speculate that infected droplets reach other peoples’ nasal passages and begin the infection there. Some investigators suggest the infected droplets can infect others by entering breaks in the skin. *M. leprae* apparently cannot infect intact skin. Rarely, humans get leprosy from the few animal species mentioned above. Occurrence in animals makes it difficult to eradicate leprosy from endemic sources. Medical researchers are still investigating routes of transmission for leprosy. Recent genetic studies have demonstrated that several genes (about seven) are associated with an increased susceptibility to leprosy. Some researchers now conclude that susceptibility to leprosy may be partially inheritable. The incubation period for leprosy varies from about six months to 20 years.

The transmission of leprosy is thought to occur through the respiratory track.

Infected individuals discharge bacilli through their nose that can infect a healthy individual, the main reservoir is human. Risk group: children or people living in the endemic areas, in poor conditions, with insufficient diet, or have a disease that affects immunity.
Bacterial and Viral Diseases

Signs and Symptoms

Symptoms mainly affect the skin, nerves, and mucous membranes (the soft, moist areas just inside the body’s openings).

The disease can cause skin symptoms, such as:

- Discolored patches of skin, usually flat, that may be numb and look faded (lighter than the skin around)
- Growths (nodules) on the skin
- Thick, stiff or dry skin
- Painless ulcers on the soles of feet
- Painless swelling or lumps on the face or earlobes
- Loss of eyebrows or eyelashes

Symptoms Caused by Damage to the Nerves are:

- Numbness of affected areas of the skin
- Muscle weakness or paralysis (especially in the hands and feet)
- Enlarged nerves (especially those around the elbow and knee and in the sides of the neck)
- Eye problems that may lead to blindness (when facial nerves are affected)

Symptoms caused by the disease in the mucous membranes are:

- A Stuffy Nose
- Nosebleeds

Since Hansen’s disease affects the nerves, loss of feeling or sensation can occur. When loss of sensation occurs, injuries, such as burns may go unnoticed. Because you may not feel the pain that can warn you of harm to your body, take extra caution to ensure the affected parts of your body are not injured.

If left untreated, the signs of advanced leprosy can include:

- Paralysis and crippling of hands and feet
- Shortening of toes and fingers due to reabsorption
- Chronic non-healing ulcers on the bottoms of the feet
- Blindness
- Loss of eyebrows
- Nose disfigurement

Other complications that may sometimes occur are:

- Painful or tender nerves
- Redness and pain around the affected area
- Burning sensation in the skin

Table 14.1 Types of Leprosy

<table>
<thead>
<tr>
<th></th>
<th>Tuberculoid</th>
<th>Borderline Tuberculoid</th>
<th>Borderline Lepromatous</th>
<th>Borderline</th>
<th>Lepromatous</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Skin</strong></td>
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<tr>
<td>Infiltrated</td>
<td>Defined plaques, irregular plaques, healing centers</td>
<td>Polymorphic, partially raised edge, satellitae</td>
<td>Papular, nodules, punched-out centres</td>
<td>Diffuse thickening</td>
<td>Diffuse thickening</td>
</tr>
<tr>
<td>Lesions</td>
<td>Single, small</td>
<td>Several, any size</td>
<td>Multiple, all sizes, bizarre</td>
<td>Innumerable, small</td>
<td>Innumerable, confluent</td>
</tr>
<tr>
<td>Macular</td>
<td>Solitary, enlarged nerves</td>
<td>Irregular enlargement of several large nerves, asymmetrical pattern</td>
<td>Many nerves involved symmetrical pattern</td>
<td>Leprous thickening, asymmetrical anaesthesia, and paresis</td>
<td>None</td>
</tr>
<tr>
<td>Nerve Lesions</td>
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**Morphology and Culture**

The Gram-Positive, Acid-Fast Rods that are identical to Tuberculosis Bacteria. These intracellular, aerobic organisms cannot be grown on nutrient mediums or in cell cultures.

**Pathogenesis:** The host organism attempts to localize and isolate infection foci by forming granulomas. Leprous granulomas are histopathologically identical to tuberculosis granulomas. High counts of leprosy Bacteria are often found in macrophages of the granulomas.

**Immunity:** the immune defenses mobilized against a leprosy infection are strictly of the cellular type.

**Clinical Features**

- Skin lesions, typically anaesthetic at the tuberculoid end of the spectrum
- Thickened peripheral nerves
- Acid-fast bacilli on skin smears or biopsy
- Acid-fast is a property of Mycobacteria in which they a resistant to decolorization by acids during staining
- This is a helpful diagnostic tool for *M. tuberculosis* and *M. leprae*

**Diagnosis:** Is clinical, by finding signs of leprosy and supported with the use of acid-fast bacilli smear. There is no serological tests so the definite sign is the peripheral nerve thickening and tenderness.
Diagnosis of Leprosy

- Examine Skin
- Check for Patches
- Test for Sensation
- Count the Number of Patches
- Look for Damage to Nerves

*M. leprae* is unable to grow in-vitro. This is thought to be due to the fact that it no longer has the genes needed for independent growth. Due to its inability to grow on agar, nude mice and nine banded armadillo are used as animal models.

Treatment

**Skin Lesions:** Tuberculoid Leprosy. Patient lymphocytes respond to *M. leprae* in vitro. Skin tests with lepromin elicit a strong positive response. They also have a Th1-type response producing interleukin – 2 and interferons -y. These strong cell – mediated responses clear antigens, but cause local tissue destruction.

**Lepromatous Leprosy:** Patients in this case do not mount a normal cell mediated response to *M. lepare*, and in fact their lymphocytes do not respond to *M. leprae* in vitro.

Leprosy meets the demanding criteria for elimination:

- Practical and simple diagnostic tools, can be diagnosed on clinical signs alone.
- The availability of an effective intervention to interrupt its transmission, multidrug therapy.
- Only one reservoir for infection: Humans.

Signs of Leprosy

- Pale or Slightly Reddish Patch
- Loss of Sensation in the Patch
- Signs of Damage to the Nerves
  - Definite Loss of Sensation in Hands/Feet
  - Weakness of Muscles in Hands/Feet/Face
  - Visible Deformity of Hands/Face/Feet
Check Your Progress

1. What is typhoid fever?
2. What are the modes of transmission of typhoid fever?
3. What is Tuberculosis?
4. What is paucibacillary leprosy and multibacillary leprosy?
5. Give some of the signs and symptoms of leprosy.

14.3 VIRAL DISEASES

Viruses are small particles of genetic material (either DNA or RNA) that are surrounded by a protein coat. Some viruses also have a fatty envelope covering. They are incapable of reproducing on their own. Viruses depend on the organisms they infect (hosts) for their very survival. Viruses get a bad rap, but they also perform many important functions for humans, plants, animals, and the environment. For example, some viruses protect the host against other infections. Viruses also participate in the process of evolution by transferring genes among different species. In biomedical research, scientists use viruses to insert new genes into cells.

When most people hear the word virus, they think of disease-causing (pathogenic) viruses, such as the Common Cold, Influenza, Chickenpox, Human Immunodeficiency Virus (HIV), and others. Viruses can affect many areas in the body, including the reproductive, respiratory, and gastrointestinal systems. They can also affect the liver, brain, and skin. Research reveals that that viruses are implicated in many cancers as well.

A viral infection is a proliferation of a harmful virus inside the body. Viruses cannot reproduce without the assistance of a host. Viruses infect a host by introducing their genetic material into the cells and hijacking the cell’s internal machinery to make more virus particles. With an active viral infection, a virus makes copies of itself and bursts the host cell (killing it) to set the newly-formed virus particles free. In other cases, virus particles bud off the host cell over a period of time before killing the host cell. Either way, new virus particles are then free to infect other cells. Symptoms of the viral illness occur as a result of cell damage, tissue destruction, and the associated immune response.

14.3.1 Ebola Virus

Ebola virus is one of six known viruses within the genus Ebola virus. Four of the six known Ebola viruses, including EBOV, cause a severe and often fatal hemorrhagic fever in humans and other mammals, known as Ebola Virus Disease.
Ebola virus has caused the majority of human deaths from EVD, and is the cause of the 2013–2015 Ebola virus epidemic in West Africa, which resulted in at least 28,616 suspected cases and 11,310 confirmed deaths.

Ebola virus and its genus were both originally named for Zaire, the country where it was first described, and was at first suspected to be a new strain of the closely related Marburg virus. The virus was renamed Ebola virus in 2010 to avoid confusion. Ebola virus is the single member of the species Zaire Ebola virus, which is the type species for the genus Ebola virus, family Filoviridae, order Mononegavirales. The natural reservoir of Ebola virus is believed to be bats, particularly fruit bats, and it is primarily transmitted between humans and from animals to humans through body fluids.

Ebola Virus Disease (EVD) is a rare and deadly disease most commonly affecting people and non-human primates (monkeys, gorillas, and chimpanzees). It is caused by an infection with a group of viruses within the genus Ebola virus:

- Ebola Virus (species Zaire Ebola virus).
- Sudan Virus (species Sudan Ebola virus).
- Bundibugyo Virus (species Bundibugyo Ebola virus).
- Reston Virus (species Reston Ebola virus).
- Bombali Virus (species Bombali Ebola virus).

**Structure**

This virus belongs to the Filovirus family, and structurally resembles a length of thread. Ebola Virus are cylindrical/tubular, contain viral envelope, matrix, and nucleocapsid components. The virus appears in a long, filamentous form, in the *shepherd crook* appearance. Figure 14.7 illustrates the structure of Ebola.

![Structure of Ebola](image)
They have virally encoded GP-glycoprotein with spikes projecting from the lipid bilayer surface. In the process called GP are Glycosylation-Proteins that contain carbohydrate chains (glycans) get covalently attached to their polypeptide side chains.

The glycoprotein GP is the available habitat of the Ebola virus surface and was responsible for attaching and entering new host cells. The outer viral envelope of the virion is derived by budding from domains of host cell membrane. GP spikes have been inserted during their biosynthesis. Figure 14.8 shows the structure of Ebola virus.

![Fig. 14.8 Structure of Ebola Virus](image)

Ebola Virus have a negative –sense, non-segmented single stranded linear RNA genome. The 3’ Virus is not polyadenylated and the 5’ end is not capped.

It encodes seven structural proteins; nucleoprotein NP, polymerase cofactor, transcription activator and RNA polymerase. Figure 14.9 illustrates the genome of Ebola virus.

![Fig. 14.9 Genome of Ebola Virus](image)
Replication of Ebola Virus: Ebola virus do not replicate through any kind of cell division. But rather use a combination of host and virally encoded enzymes. These are used to produce multiple copies of viruses. These then self-assemble into viral macromolecules inside the host cells.

Attachment: Virion attachment is through host receptors through GP–glycoprotein which is endocytosed into vesicles in the host cell.

Viral Entry (Penetration): The virions enter early endosomes by Macropinocytosis or clathrin-mediated endocytosis.

- **Macropinocytosis:** Parts of the host plasma membrane protrude out from the cell and form invaginations where the virus utilizes glycoproteins in order to attach to the surface of the plasma membrane. This is a process in which eukaryotic host cells form macropinosomes. These small bodies are segments of plasma membranes that extend out from the cell in order to incorporate the virus into the cell. Various growth factors get activated and participate with the intake of cellular molecules of extracellular fluid.

- **Clathrin Mediated Endocytosis:** Similar to macropinocytosis this is another way of entering the host cell. The plasma membrane forms invaginations that engulf the cell. This method is different however in a way that the proteins and clathrin help in the attachment of virus on the surface of the host membrane. Glycoproteins are used to attach the virus to the cell surface through lysosomes with endosomes and escape into the cytoplasm.

To penetrate the cell, the viral membrane fuses with vesicle membrane, and the nucleocapsid is released into the cytoplasm. In some culture cell, GP highly expressed in dendritic cells. Fusion of virus membrane with the vesicle membrane is triggered by either low pH or NPC1 binding.

- **Sequential Transcription:** During transcription, the RNA genome is transcribed into seven monocistronic mRNA whose length is determined by highly conserved start and stop signals.

- **Replication:** The transcription process begins with the binding of the polymerase complex to a single binding site located within the leader region of the genome. The complex slides along the RNA template and sequentially transcribes the individual genes in their 3’ to 5’ order. Monocistronic mRNA from the template of antisense genome mRNA which is rapidly encapsidated and is translated into individual viral proteins. As viral protein level rise, there is transformation that occurs from translation to replication.

- **Budding:** The newly formed nucleocapsids and envelope proteins assemble at the host cells plasma membrane, budding occurs, destroying the cell. These viruses recruit components of the endosomal
sorting complex required for transport to mediate host assisted viral budding. These are used for functions like membrane remodeling like autophagy or cytokinesis.

- **Release:** Finally, the virion is released.

**Symptoms:** Symptoms may appear somewhere from 2-21 days after exposure to Ebola. An affected person will develop a fever, headache, joint and muscle pain, a sore throat, and intense muscle weakness.

Recovery from Ebola infection develop antibodies that last for at least 10 years:

- High Fever, i.e., usually higher than 38.3 °C (100.9 °F)
- Muscle and Joint Aches
- Headache
- Sore Throat and Shortness of Breath
- Chest Pain and Cough
- Red Eyes
- Weakness
- Swelling
- Severe Weight Loss
- Chills
- Confusion
- Fatigue
- Nausea and Vomiting
- Diarrhea (may be Bloody)
- Internal and External Bleeding
- Bleeding, usually from the Eyes
- Stomach Pain
- Hiccups
- Raised Rash
- Kidneys and Liver Failure

### 14.3.2 Hepatitis B Virus

Hepatitis B is a liver infection caused by the Hepatitis B Virus (HBV) (Refer Figure 14.10). Hepatitis B is transmitted when blood, semen, or another body fluid from a person infected with the Hepatitis B virus enters the body of someone who is not infected. This can happen through sexual contact; sharing needles, syringes, or other drug-injection equipment; or from mother to baby
at birth. For some people, Hepatitis B is an acute, or short-term, illness but for others, it can become a long-term, chronic infection. Risk for chronic infection is related to age at infection: approximately 90% of infected infants become chronically infected, compared with 2%–6% of adults. Chronic Hepatitis B can lead to serious health issues, like cirrhosis or liver cancer. The best way to prevent Hepatitis B is by getting vaccinated.

The Hepatitis B virus is a DNA virus, meaning that its genetic material is made up of deoxyribonucleic acids. It belongs to a family of viruses known as *Hepadnaviridae*. The virus is primarily found in the liver but is also present in the blood and certain body fluids. Hepatitis B virus consists of a core particle (central portion) and a surrounding envelope (outer coat). The core is made up of DNA and the core antigen (HBCAg). The envelope contains the surface antigen (HBsAg). These antigens are present in the blood and are markers that are used in the diagnosis and evaluation of patients with suspected Viral Hepatitis.

The Hepatitis B Virus reproduces in liver cells, but the virus itself is not the direct cause of damage to the liver. Rather, the presence of the virus triggers an immune response from the body as the body tries to eliminate the virus and recover from the infection. This immune response causes inflammation and may seriously injure liver cells. Therefore, there is a balance between the protective and destructive effects of the immune response to the Hepatitis B Virus. Figure 14.11 shows the three Hepatitis B surface antigen HBsAg containing forms that can be identified in serum from Hepatitis B virus HBV carriers.

The virus is a DNA virus and belongs to the Hepadnaviridae family. It is a viral infection that attacks the liver and can cause both acute and chronic disease.

Group VII: (dsDNA-RT).

Family: Hepadnaviridae
Genus: *Orthohepadnavirus*
Species: *Hepatitis B Virus*

The above Figure 14.11 shows three Hepatitis B Surface Antigen HBsAg containing forms that can be identified in serum from Hepatitis B Virus HBV Carriers. The core contains the partially double stranded viral DNA genome.

A soluble antigen, termed Hepatitis B e antigen (HBeAg), may be released from core particles by treatment with strong detergent. HBcAg, Hepatitis B core antigen.

Icosahedral nucleocapsid makes the core and an outer envelope composed of lipid and protein or the surface antigen or HBsAg. Inner protein shell forming the core particle or HBcAg contains the viral DNA and enzymes used in viral replication called DNA polymerase. HBeAg (Hepatitis) is the antigenic determinant closely associated with the nucleocapsid of HBV. It also circulates as a soluble protein in serum.

**Genome of Hepatitis B Virus**

Genome is partially double stranded DNA that forms a covalently closed circle with 5' end of the minus strand which is linked to the viral DNA polymerase.

This genome is 3020-3320 nucleotides long for the full length strand.

It is compact with four overlapping reading frames running in one direction and no non-coding regions. Figure 14.12 shows the genetic
organisation of the Hepatitis B Virus Genome. Four open reading frames encoding seven peptides are indicated by large arrows.

**Fig. 14.12 Genetic Organisation of the Hepatitis B Virus Genome**

**Symptoms of Hepatitis B**

**Incubation Period:** 75 days on average, but can vary from 30 to 180 days.

- Fever
- Fatigue
- Dark Urine
- Loss of Appetite
- Nausea, Vomiting
- Joint Pain
- Clay Colored Bowel Movements
- Jaundice
- Pain in the Upper Right Abdomen due to Inflamed Liver
- People with Chronic Hepatitis B develop Serious Liver Conditions

**Transmission**

- Through blood and infected bodily fluids as well as through saliva, menstrual vaginal and seminal fluids.
• Through direct blood to blood contact and from an infected women to her newborn during the delivery process.
• Infection can occur during medical, surgical and dental procedures.
• Rarely Hepatitis B can be transmitted through transfused blood products, donated livers and other organs.

Figure 14.13 shows the Hepatitis B pathophysiology diagram.

![Hepatitis B Pathophysiology Diagram](image)

- The infectious virion attaches to cells and sheds its coat.
- The partially double stranded viral genome is converted to covalently circular double stranded DNA cccDNA.
- The cccDNA serves as template of pregenome RNA becomes encapsidated with newly synthesised HBcAg.
- The negative-strand DNA are synthesised by reverse transcription by viral polymerase forming the internal core.
- The polymerase synthesize the positive DNA strand.
- Cores may be reimported into the nucleus and initiate cell.

**Pathogenesis**

Reactions involved in liver cell injury during HBV infections. The HLA class I restricted cytotoxic T-cell response directed at HBcAg/HBeAg on HBV – infected hepatocytes. A second mechanism is a direct Cytopathic effect of HBcAg expression in infected hepatocytes. A third mechanism is high level expression and inefficient secretion of HBsAg. In acute condition there are
signs of inflammation in the portal tracts, the infiltrate is lymphocytic. In liver parenchyma, infected hepatocytes form acidophilic bodies when they die.

In chronic Hepatitis, damage covers from the portal tracts. As the disease progresses fibrosis and cirrhosis develops. Chronic liver damage results from immune mediated destruction of hepatocytes expressing viral antigens. These reactions may cause damage as immune responses are induced to various liver-specific antigens.

**Diagnosis of Hepatitis B**

Diagnosis is based on clinical, biochemical, histological and serological findings. HBV DNA can be quantified in serum or plasma using PCR (Polymerase Chain Reaction) assays. Presence of HBsAg and Immunoglobulin M (IgM) antibody with core antigen HBcAg is responsible for causing infection. Presence of e antigen shows excessive replication of the virus and high contagious state. HBsAg is the indicator of chronic liver disease and liver cancer.

**Treatment of Hepatitis B**

Interferon alpha is the first line treatment option for patients without cirrhosis. This helps in suppressing HBV replication, prevent the progression of liver cirrhosis due to anti-viral and immune-modulatory effects. The Hepatitis B can be controlled by a vaccine given as 3-4 shots over a 6 months period.

**Plasma Derived Vaccines**

**Recombinant DNA Derived Vaccines:** The HBsAg is expressed with morphological characteristics of free surface antigen in plasma. This is as powerful as the plasma derived vaccines. This vaccine can also combine with other vaccines like BCG, Measles, Rubella, etc.

**14.3.3 Acquired Immune Deficiency Syndrome (AIDS)**

HIV stands for Human Immunodeficiency Virus. It is the virus that can lead to acquired immunodeficiency syndrome, or AIDS, if not treated. Unlike some other viruses, the human body can’t get rid of HIV completely, even with treatment. So once you get HIV, you have it for life.

HIV attacks the body’s immune system, specifically the CD4 cells (T cells), which help the immune system fight off infections. Untreated, HIV reduces the number of CD4 cells (T cells) in the body, making the person more likely to get other infections or infection-related cancers. Over time, HIV can destroy so many of these cells that the body cannot fight off infections and disease. These opportunistic infections or cancers take advantage of a very weak immune system and signal that the person has AIDS, the last stage of HIV infection.
No effective cure currently exists, but with proper medical care, HIV can be controlled. The medicine used to treat HIV is called antiretroviral therapy or ART. If taken the right way, every day, and this medicine can dramatically prolong the lives of many people infected with HIV, keep them healthy, and greatly lower their chance of infecting others. Before the introduction of ART in the mid-1990s, people with HIV could progress to AIDS in just a few years. Today, someone diagnosed with HIV and treated before the disease is far advanced can live nearly as long as someone who does not have HIV.

AIDS is the most severe phase of HIV Infection. People with AIDS have such badly damaged immune systems that they get an increasing number of severe illnesses, called opportunistic infections.

No effective cure currently exists for HIV. But with proper medical care, HIV can be controlled. Treatment for HIV is called antiretroviral therapy or ART. If taken the right way, every day, ART can dramatically prolong the lives of many people infected with HIV, keep them healthy, and greatly lower their chance of infecting others. Before the introduction of ART in the mid-1990s, people with HIV could progress to AIDS (the last stage of HIV infection) in a few years. Today, someone diagnosed with HIV and treated before the disease is far advanced can live nearly as long as someone who does not have HIV.

Causative agent for HIV (Human Immunodeficiency Virus) virus is Acquired Immuno-Deficiency Syndrome (AIDS), it is a Retrovirus. AIDS is a set of symptoms that occur when the virus transmitted via body fluid has destroyed the immune system leaving the victim susceptible to other life threatening infections. There are two major strains of HIV. HIV 1 causes majority of functions worldwide and more easily transmitted HIV 2 which is restricted to West Africa. It is RNA virus with reverse transcriptase for a copy of viral DNA, which is then transcribed into viral RNA. Some retroviruses can become incorporated into host genomes. This may occur with HIV as an example of viral latency. A second enzyme integrase, helps to splice the viral DNA into host DNA genome. Another enzyme a protease, helps cut the new viral proteins coded for by the viral nucleic acid into viral capsids. These bud from the cells acquiring their envelope as virus leaves host cells. AIDS manifests as a reduction of T-helper cells after an average incubation of ten years. The collapse of cellular immunity results in occurrence of opportunistic infections as well as lymphomas and Kaposi sarcoma.

**Origin of HIV:** Virus originated in West Africa. The infection was localised to a small area. Development of rapid and widespread travel contributed to its spread.
Primary Infection (Acute HIV)

Most people infected by HIV develop a flu-like illness within a month or two after the virus enters the body. This illness, known as primary or acute HIV infection, may last for a few weeks. Possible signs and symptoms include:

- Fever
- Headache
- Muscle Aches and Joint Pain
- Rash
- Sore Throat and Painful Mouth Sores
- Swollen Lymph Glands, mainly on the Neck

What is a Retrovirus?

During the time of protein formation the DNA is used as a template to make another nucleic acid called RNA in a process called transcription. The information on the RNA is used to assemble the sequence of polypeptides that make up the particular protein – translation.
Reverse transcription: Retroviruses carry out transcription in reverse with the help of enzyme called reverse transcriptase. Incorporation of the DNA derived from the viral genome in the host cell genome is a precondition for viral replication. Some viruses are also capable of oncogenic cell transformation. Due to the presence of RNA genome to bring about this change it is called oncornaviruses. This transcribes the viral RNA into DNA that can be inserted into the genome of the host cell where it stays for the lifetime of the cell. The cell synthesises viral RNA and proteins, allowing the virus to multiply inside the host cells.

**Transmission of HIV**

HIV is labile in a free state and cannot enter through the intact body surfaces. It has been detected in body fluids like blood, cervical secretions, breast milk, etc. or from infected mothers to offspring. Damage to the host: HIV specifically damages T lymphocytes, T4 as they have an antigen CD4 on the surface of the cell. The CD4 receptors normally involved in antigen recognition, a co-receptor are the site of attachment for HIV the viral envelope fuses with the cell membrane and releases the viral enzyme with to strands of RNA of the HIV genome. In the acute stage, rapid viral replication takes place which damages the CD4 cells. Many people will have symptoms similar to other viral infection, such as large lymph nodes, muscle aches. The immune response keeps a check on viral replication. A steady state of viral replication is then which is then reached, which is the chronic stage. High CD4 levels holds secondary infection in check. The damage to CD cells leads to an imbalance in the ratio of (Helper/Suppressor/Cytotoxic Cell) Lymphocytes. This leads to a further suppression of immune response. The T helper cells regulate the B cells, as well as macrophages, these immune functions are also damaged.

- The nucleocapsid containing RNA and reverse transcriptase inserts into the cell.
- Complementary single stranded DNA molecules are made using the RNA as a template.
- The cDNA hybridises to make double stranded DNA.
- The viral DNA integrates into the host genome.
- Viral RNA and proteins are synthesised.
- New HIV particles are assembled.
- HIV leaves the cell by budding acquiring its envelope in the process.
- This disrupts the cell membrane leading to cell lysis.

Although in the US, the HIV infection and resulting aids cases have been predominantly rampant due to gay community. There has been an increase in the percentage of heterosexual transmission. This is due to the
spread of HIV contaminated needles by IV drug users. There is also an increase in the spread of HIV from infected mothers to foetuses. In less developed countries there is an increase in the spread of HIV infection by heterosexuals. Figure 14.15 HIV Cell Microbiology.

Measure of the number of CD4 lymphocytes present in the blood is help monitor the progression of the disease. A low number would indicate that the infection is more serious. Measure of the viral load have aided in the means of therapies to hold viral replication in check by means of quantitative RT-PCR. Figure 14.16 illustrates the HIV Transmission Category.

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**Fig. 14.15 HIV Cell Microbiology**

**Fig. 14.16 HIV Transmission Category**
Virus level are high during the initial acute infection and decrease until the later phases of HIV disease and AIDS. Antibody levels gradually rise and remain relatively high throughout phases III and IV. T-cell numbers remain relatively normal until the later phases of HIV disease and full blown AIDS.

The designation of aids related complex, ARC, has been used in the past to type the beginnings of the aids disease. This type of artificial separation is not in much use these days. Early signs, in addition to viral load and the lower T4 cells levels develops into later symptoms into two categories as below:

**Infectious Diseases:**
- Opportunistic Infections
- Mycobacterial Infections, such as TB
- Viral Infection, such as Serpes
- Oncological Manifestations

**Diagnosis:** HIV infections are routinely detected by serology (antibodies or viral antigens).

**Therapy:** Inhibitors of reverse transcriptase and protease.

**Prevention:** Exposure prophylaxis when contacted with blood. Post exposure prophylaxis and prophylaxis in pregnancy with chemotherapeutics pathogen. The retroviridae family is the classification group for all RNA viruses with reverse transcription of RNA to DNA in their reproductive cycles. Only zoopathic retroviruses were known for many years that cause many tumours. The H1 virus can persist in a latent state in CD4+ T lymphocytes, macrophages, and the Langerhans cells. Viral replication continues throughout this period, especially in lymphoid organs. The drop in CD4+ lymphocytes and the rise in the virus count in peripheral blood is followed by lymphadenopathy stage. This sets the pace of opportunistic infections combined with Kaposi sarcoma. Similar neurological symptoms may also be induced HIV-induced immunosuppression.

**HIV Replication**

CD4 is the main receptor for HIV with a number of other active co-receptors depending on the viral strain involved. HIV is then taken in by the cell. After uncoating reverse transcription takes place. The interaction of the many different contributing control gene is responsible for the long latency period and subsequent viral replication. Replication of HIV takes the form of a lytic cycle, i.e., it results in the destruction of host cells. Besides cell destruction apoptosis also appears to play an important role in elimination of CD4+ cells.
Figure 14.17 illustrates the steps occur in the latent period.

The virus is adsorbed and endocytosed. The RNA are uncoated. The nucleocapsid containing RNA and reverse transcriptase inserted into the cell catalyzes the Complementary single stranded DNA are made using the RNA as a template, ssDNA. This single strand hybridized for synthesis of double strand of DNA. dsDNA is inserted into the host chromosome as a provirus,
After a latent period various immune activator stimulate the infected cells, causing reactivation of the provirus genes and production of viral mRNA (Refer Figure 14.18). The viral DNA integrates into the host genome. Viral RNA and proteins are synthesised. New HIV particles are assembled (Refer Figure 14.19).
HIV mRNA is translated by the cells into all virus components like capsid, reverse transcriptase. HIV leaves the cell by budding and acquiring its envelope in the process. This disrupts the cell membrane. Budding of mature viruses after assembling all the parts bring about lysis of the infected cell.

**Recommended Therapy:** Currently the recommended therapy is HARRT; HAART is the acronym for ‘Highly Active Anti-Retroviral Therapy’, a term coined in the late 1990s to describe the effectiveness of combination drug therapies used to treat HIV. This consists of triple therapy, including two nucleoside analogues and a protease inhibitor.

**Prevention**
- No Vaccine
- Education as a Tool

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**Check Your Progress**

6. What are viruses?
7. What is Ebola virus?
8. Give some of the symptoms of Hepatitis B.
9. How did HIV originated?

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**14.4 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS**

1. Typhoid fever is an infectious feverish disease caused by the bacterium *Salmonella typhi* and less commonly by *Salmonella paratyphi*.

2. Following are some of the mode of transmission of typhoid fever:
   - Ingestion of contaminated food or water
   - Contact with an acute case of typhoid fever.
   - Contact with a chronic asymptomatic carrier.
   - Water is contaminated where inadequate sewerage systems and poor sanitation.

3. Tuberculosis is a chronic disease that is rampant around the world. As long time and multiple therapy medication is taken, adverse reactions are most common.

4. Paucibacillary leprosy is skin lesions with no bacilli (*M. leprae*) seen in a skin smear and multibacillary leprosy is skin lesions with bacilli (*M. leprae*) seen in a skin smear.

5. Some of the signs and symptoms of leprosy are: Symptoms mainly affect the skin, nerves, and mucous membranes (the soft, moist areas just inside the body’s openings):
• Discolored patches of skin, usually flat, that may be numb and look faded (lighter than the skin around).
• Growths (nodules) on the skin.
• Thick, stiff or dry skin.

6. Viruses are small particles of genetic material (either DNA or RNA) that are surrounded by a protein coat. Some viruses also have a fatty envelope covering.

7. Ebola virus is one of six known viruses within the genus Ebola virus. Four of the six known Ebola viruses, including EBOV, cause a severe and often fatal hemorrhagic fever in humans and other mammals, known as Ebola Virus Disease (EVD). Ebola virus has caused the majority of human deaths from EVD, and is the cause of the 2013–2015 Ebola virus epidemic in West Africa, which resulted in at least 28,616 suspected cases and 11,310 confirmed deaths.

8. Following are some of the symptoms of Hepatitis B:
   Incubation Period: 75 days on average, but can vary from 30 to 180 days.
   • Fever
   • Fatigue
   • Dark Urine
   • Loss of Appetite
   • Nausea, Vomiting
   • Joint Pain
   • Clay Colored Bowel Movements

9. HIV Virus originated in West Africa. The infection was localised to a small area. Development of rapid and widespread travel contributed to its spread.

14.5 **SUMMARY**

• Bacteria are microscopic, single-cell organisms that live almost everywhere.
• Bacteria live in every climate and location on earth. Some are airborne while others live in water or soil.
• Bacteria live on and inside plants, animals, and people. The word bacteria has a negative connotation, but Bacteria actually perform many vital functions for organisms and in the environment.
• A bacterial infection is a proliferation of a harmful strain of Bacteria on or inside the body.
• Bacteria can infect any area of the body. Pneumonia, meningitis, and food poisoning are just a few illnesses that may be caused by harmful Bacteria.
• Bacteria come in three basic shapes: rod-shaped (bacilli), spherical (cocci), or helical (spirilla). Bacteria may also be classified as Gram-
Bacterial and Viral Diseases

Notes

Positive or Gram-Negative. Gram-Positive Bacteria have a thick cell wall while Gram-Negative Bacteria do not.

- Gram staining, Bacterial culture with antibiotic sensitivity determination, and other tests are used to identify bacterial strains and help determine the appropriate course of treatment.
- Bacteria can be strictly pathogenic, which means that they will cause disease if they manage to overwhelm the human immune system. Other Bacteria only cause disease given the right circumstances, these are so-called opportunistic pathogens.
- Opportunistic pathogens normally do not cause infections in healthy humans but when the immune system is compromised or suppressed by for example cancer chemotherapy, other diseases (like HIV/AIDS) or malnutrition, the risk of infection increases.
- Typhoid fever is an infectious feverish disease caused by the bacterium Salmonella typhi and less commonly by Salmonella paratyphi. It is an acute generalized infection of the reticulo-endothelial system, intestinal lymphoid tissue, and the gall bladder.
- Tuberculosis is a chronic disease that is rampant around the world. As long time and multiple therapy medication is taken, adverse reactions are most common.
- Mycobacterium are slender rod Bacteria that are stained with differential stains (Ziehl-Neelsen).
- Primary infection remain silent primarily, but does progress to secondary stage gradually in a few.
- Mycobacterium is Gram Positive, although they do not take gram staining well. The lipid rich cell wall does not allow the alkaline stains to penetrate.
- Most of the time there are no symptoms found in individuals affected by tuberculosis, i.e., asymptomatic.
- The infection sets in when the Bacteria reach the pulmonary alveoli of the lungs which invades and undergo replication within endosomes of alveolar macrophages.
- The macrophages envelopes the whole Bacteria forming a phagosome. This combines with lysosome to destroy Bacteria by forming phagolysosome. But the M. Tuberculosis has a thick waxy mycolic acid capsules that protects it from toxic substances. The host immune respond against the Bacteria after macrophages undergo phagocytosis.
- Tuberculosis (TB) is one of the top 10 causes of death worldwide.
- In 2017, 10 million people fell ill with TB, and 1.6 million died from the disease (including 0.3 million among people with HIV).
• In 2017, an estimated 1 million children became ill with TB and 230,000 children died of TB (including children with HIV associated TB).

• Globally, TB incidence is falling at about 2% per year. This needs to accelerate to a 4–5% annual decline to reach the 2020 milestones of the End TB Strategy.

• Leprosy is a disease mainly caused by the Bacteria *Mycobacterium leprae* (Hensen 1973). It is mainly a Granulomatous disease affecting: peripheral nerves and mucosa of the upper-respiratory tract.

• Leprosy is caused by a slow-growing type of Bacteria called *Mycobacterium leprae* (*M. leprae*). Leprosy is also known as Hansen’s disease, after the scientist who discovered *M. leprae* in 1873.

• There are multiple forms of leprosy described in the literature. The forms of leprosy depend on the person’s immune response to *M. leprae*.

• A good immune response can produce the so-called tuberculoid form of the disease, with limited skin lesions and some asymmetric nerve involvement.

• Paucibacillary Leprosy is the skin lesions with no bacilli (*M. leprae*) seen in a skin smear.

• Multibacillary Leprosy is the skin lesions with bacilli (*M. leprae*) seen in a skin smear.

• Researchers suggest that *M. leprae* spreads person to person by nasal secretions or droplets from the upper respiratory tract and nasal mucosa.

• The transmission of leprosy is thought to occur through the respiratory track of infected individuals discharge bacilli through their nose that can infect a healthy individual, the main reservoir is human.

• Risk group: children or people living in the endemic areas, in poor conditions, with insufficient diet, or have a disease that affects immunity.

• *M. leprae* is unable to grow in vitro. This is thought to be due to the fact that it no longer has the genes needed for independent growth. Due to its inability to grow on agar, nude mice and nine banded armadillo are used as animal models.

• Viruses are small particles of genetic material (either DNA or RNA) that are surrounded by a protein coat.

• Some viruses also have a fatty envelope covering. They are incapable of reproducing on their own.

• Viruses depend on the organisms they infect (hosts) for their very survival. Viruses get a bad rap, but they also perform many important functions for humans, plants, animals, and the environment.
When most people hear the word virus, they think of disease-causing (pathogenic) viruses, such as the Common Cold, Influenza, Chickenpox, Human Immunodeficiency Virus (HIV), and others.

Viruses can affect many areas in the body, including the reproductive, respiratory, and gastrointestinal systems. They can also affect the liver, brain, and skin. Research reveals that that viruses are implicated in many cancers as well.

A viral infection is a proliferation of a harmful virus inside the body. Viruses cannot reproduce without the assistance of a host.

Viruses infect a host by introducing their genetic material into the cells and hijacking the cell’s internal machinery to make more virus particles.

Ebola virus is one of six known viruses within the genus Ebola virus. Four of the six known Ebola viruses, including EBOV, cause a severe and often fatal hemorrhagic fever in humans and other mammals, known as Ebola Virus Disease (EVD).

Ebola virus has caused the majority of human deaths from EVD, and is the cause of the 2013–2015 Ebola virus epidemic in West Africa, which resulted in at least 28,616 suspected cases and 11,310 confirmed deaths.

Ebola Virus Disease (EVD) is a rare and deadly disease most commonly affecting people and non-human primates (monkeys, gorillas, and chimpanzees).

Hepatitis B is a liver infection caused by the Hepatitis B Virus (HBV).

The Hepatitis B virus is a DNA virus, meaning that its genetic material is made up of deoxyribonucleic acids. It belongs to a family of viruses known as Hepadnaviridae.

HIV stands for Human Immunodeficiency Virus. It is the virus that can lead to Acquired Immuno-Deficiency Syndrome or AIDS if not treated. Unlike some other viruses, the human body cannot get rid of HIV completely, even with treatment. So once you get HIV, you have it for life.

HIV attacks the body’s immune system, specifically the CD4 cells (T cells), which help the immune system fight off infections.

Untreated, HIV reduces the number of CD4 cells (T cells) in the body, making the person more likely to get other infections or infection-related cancers.

Over time, HIV can destroy so many of these cells that the body cannot fight off infections and disease.

The medicine used to treat HIV is called HAART (Highly Active Anti-Retroviral Therapy).
- If taken the right way, every day, and this medicine can dramatically prolong the lives of many people infected with HIV, keep them healthy, and greatly lower their chance of infecting others.
- AIDS is the most severe phase of HIV infection. People with AIDS have such badly damaged immune systems that they get an increasing number of severe illnesses, called opportunistic infections.
- HIV is labile in a free state and cannot enter through the intact body surfaces.
- It has been detected in body fluids like blood, cervical secretions, breast milk, etc., or from infected mothers to offspring.

### 14.6 KEY WORDS

- **Bacteria**: Bacteria are microscopic, single-cell organisms that live almost everywhere.
- **Bacterial infection**: A bacterial infection is a proliferation of a harmful strain of Bacteria on or inside the body.
- **Paucibacillary leprosy**: Skin lesions with no bacilli (\(M. \ leprae\)) seen in a skin smear.
- **Multibacillary leprosy**: Skin lesions with bacilli (\(M. \ leprae\)) seen in a skin smear.
- **Virus**: Viruses are small particles of genetic material (either DNA or RNA) that are surrounded by a protein coat.
- **Viral infection**: A viral infection is a proliferation of a harmful virus inside the body. Viruses cannot reproduce without the assistance of a host.
- **Hepatitis B**: Hepatitis B is a liver infection caused by the Hepatitis B Virus (HBV).
- **HAART**: HAART is the acronym for ‘Highly Active Anti-Retroviral Therapy’, a term coined in the late 1990s to describe the effectiveness of combination drug therapies used to treat HIV. This consists of triple therapy, including two nucleoside analogues and a protease inhibitor.

### 14.7 SELF ASSESSMENT QUESTIONS AND EXERCISES

**Short Answer Questions**

1. What are bacterial diseases?
2. Give the symptoms of typhoid fever.
3. How is bacterial disease caused?
4. Give some of the key facts about tuberculosis.
5. What are viral diseases?
7. Give the symptoms of HIV.

**Long Answer Questions**

1. Describe in detail about typhoid fever mentioning about its signs and symptoms, prevention and cure methods also.
2. What is tuberculosis? Give the causative agents, symptoms, risk factors and preventative methods of tuberculosis.
3. Explain about leprosy, its different classifications, mode of transmission, symptoms and treatment methods.
4. Discuss about Ebola virus, its structure, symptoms in detail. Give the relevant diagrams also.
5. Explain about Hepatitis B Virus in detail. Draw well labelled diagrams to explain the same.
6. Discuss in detail about HIV virus.

**14.8 FURTHER READINGS**


