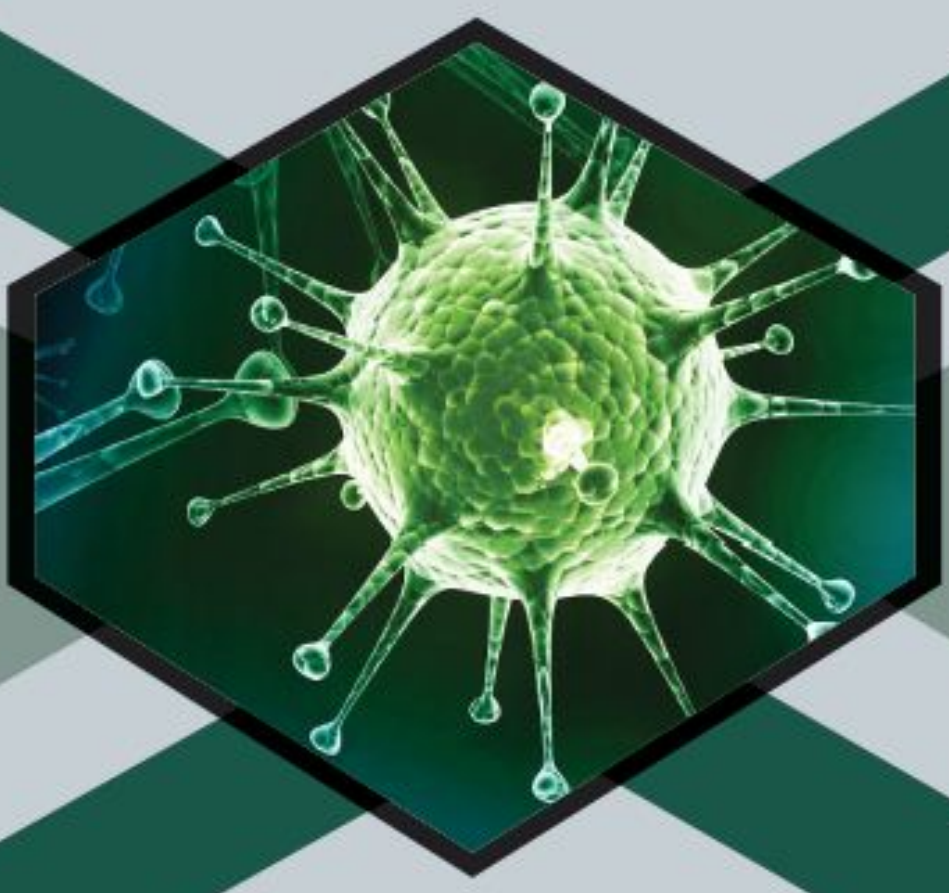
AS PER PCI REGULATIONS
SECOND YEAR B. PHARM. | SEMESTER-III

PHARMACEUTICAL MICROBIOLOGY

Dr. KUNTAL DAS



NIRALI PRAKASHAN
ADVANCEMENT OF KNOWLEDGE

A Text Book of

PHARMACEUTICAL MICROBIOLOGY

As Per PCI Regulations

SECOND YEAR B. PHARM., SEMESTER III

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Dedicated to



My beloved

Parents, Wife and Son

Preface

This is the first edition and first time the attempt has been made to write a Pharmaceutical microbiology text book as per the new syllabus prescribed by Pharmacy Council of India for Semester - III. There are many text books on microbiology already available in market by famous authors. Drug safety is a major focus of pharmaceutical microbiology. Pathogenic bacteria, fungi (yeasts and moulds) and toxins produced by microorganisms are all possible contaminants hence it is require for Quality Assurance and Quality Control on protecting pharmaceutical and healthcare products from spoilage by microorganisms. Most of the information in this book has presented in a very simple manner, with tables, figures and wherever necessary with diagrams. The aim of this book is made easy for understandable to the student. This book is as per the revised syllabus prescribed by the Pharmacy Council of India under Regulations 6, 7 and 8 of the Bachelor of Pharmacy (B. Pharm.) course regulations 2014 in 2016 and amended from 2017 onwards throughout India under same uniform syllabus. As per the norms, the book is compiled with semester wise syllabus in which Pharmaceutical microbiology is under Semester - III.

Semester - III, Pharmaceutical Microbiology subject is divided into five sub-units.

Unit–I, deals with the history of microbiology world, its general introduction and importance, description on Prokaryotes and Eukaryotes. Further detail study on bacteria world with their culture techniques for growth and their identification by various modern microscopic techniques.

Unit-II, deals with various staining techniques and biochemical tests for identification of various types of bacteria. Further most importantly, sterilization techniques are discussed in details with respect to equipments details, evaluation of efficiency and various sterility indicators.

Unit-III, describes about Fungi and Viruses in details. Disinfectants are also discussed in details for identification of bacteriostatic and bactericidal actions. Standard IP, BP and USP methods for sterility testing of products are also equally given importance in this sub-unit.

Unit IV deals with various conditions for culturing microbes under aseptic conditions. Principles and methods of different microbiological assay are highlighted with various methods for standardization of antibiotics, vitamins and amino acids. Assessment of new antibiotics is also added in this important sub-unit.

Unit V is most important where most care and precaution taken for storage of pharmaceutical products from the microbial spoilage and their stability. Microbial contaminants and their contamination processes are also revealed in this unit. Furthermore some methods are described on animal cell culture and their importance in pharmaceutical industry and research.

It is hoped that all the units will provide up to date knowledge to all the students with the detail information by systemic manners described in this book.

My attempt to publish this first edition of the pharmaceutical microbiology book is with better hope to gain the popularity by the students and readers throughout the country. Any criticism and suggestions from the readers are always welcome. In the future editions, such suggestions will be incorporated and other mistakes will be rectified.

It is my great privilege to acknowledge the help from all the published books and websites from the internet for completing this book. My sincere and heartiest gramercy to Dr. Raman Dang, Registrar of DPSRU New Delhi, for encouragement and positive motivation. My sincere thanks and respect to chairman sir, Prof Suresh Nagpal, vice chairperson madam, directors, present principal Dr. Amit Kumar Das, all my teaching, non-teaching staffs of my college for their active co-operation and encouragement.

I felt no word to express sense of indebtedness to my parents, Mrs. Kalyani Das and Dr. Dilip Kumar Das (Emeritus Fellow UGC), whose silent blessings, encouragement, and helping me to put my best foot forward in all my endeavors of chasing my dreams in life.

My special and sincere thanks to my wife Mrs. Sangita Das and son Master Niladri Das for their inspiration to pursue the work in all the situations.

Lastly but not the least, my sincere thank to M/S Nirali Prakashan publishers for kind publication of the book with much care.

Prof. Kuntal Das

M. Pharm, Ph.D

Syllabus

Unit I **10 Hours**

Introduction, History of microbiology, Its branches, Scope and its importance. Introduction to Prokaryotes and Eukaryotes. Study of ultra-structure and morphological classification of bacteria, Nutritional requirements, Raw materials used for culture media and physical parameters for growth, Growth curve, Isolation and preservation methods for pure cultures, Cultivation of anaerobes, Quantitative measurement of bacterial growth (total and viable count). Study of different types of phase constrast microscopy, dark field microscopy and electron microscopy.

Unit II **10 Hours**

Identification of bacteria using staining techniques (Simple, Gram's and Acid fast staining) and biochemical tests (IMViC).

Study of principle, Procedure, Merits, Demerits and applications of physical, Chemical, Gaseous, Radiation and mechanical method of sterilization. Evaluation of the efficiency of sterilization methods. Equipments employed in large scale sterilization. Sterility indicators.

Unit III **10 Hours**

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

Unit IV **08 Hours**

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

Unit V **07 Hours**

Types of spoilage, Factors affecting the microbial spoilage of pharmaceutical products, Sources and types of microbial contaminants, Assessment of microbial contamination and spoilage. Preservation of pharmaceutical products using antimicrobial agents, Evaluation of microbial stability of formulations. Growth of animal cells in culture, General procedure for cell culture, Primary, established and transformed cell cultures. Application of cell cultures in pharmaceutical industry and research.

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Contents

1. Basics of Microbiology 1.1 – 1.52

1.1 Introduction 1.1
1.2 History 1.2
1.2.1 Discovery Stage 1.2
1.2.2 Transition Stage 1.2
1.2.3 Modern Microbiology 1.3
1.3 Branches of Microbiology 1.7
1.4 Scope and Importance of Microbiology 1.9
1.4.1 Scope of Microbiology 1.9
1.4.2 Importance of Microbiology 1.11
1.5 Introduction to Prokaryotes and Eukaryotes 1.12
1.6 Study of Ultra-Structure and Morphological Classification of Bacteria 1.14
1.6.1 Ultra Structure of Bacteria 1.14
1.6.2 Morphological Classification of Bacteria 1.17
1.7 Culture Media 1.20
1.8 Raw Materials and Nutritional requirements for Culture Media 1.24
1.8.1 Nutrition required for Bacterial Culture Medium 1.25
1.9 Growth Factors 1.27
1.9.1 Physical Factors Affecting Bacterial Growth 1.28
1.9.2 Bacterial Growth Curve 1.31
1.10 Isolation Methods for Pure Cultures 1.32
1.11 Preservation Methods for Pure Cultures 1.38
1.12 Quantitative Measurement of Bacterial Growth (Total and Viable Count) 1.42
1.12.1 Total Count 1.43
1.12.2 Viable Count 1.45
1.13 Phase Contrast Microscopy, Dark Field Microscopy and Electron Microscopy 1.47
1.13.1 Phase Contrast Microscopy 1.47
1.13.2 Dark Field Microscopy 1.48
1.13.3 Electron Microscopy 1.49
• Questions 1.51
• Multiple Choice Questions 1.51

2. Sterilization Techniques and Identification of Bacteria 2.1 – 2.40

2.1 Identification of Bacteria using Staining Techniques 2.1
2.1.1 Simple Staining 2.2
2.1.2 Gram's Staining 2.3
2.1.3 Acid Fast Staining 2.5
2.2 Biochemical Test (IMViC) 2.6
2.3 Sterilization Process 2.9
2.3.1 Some Important Terminologies 2.10
2.3.2 Pharmaceutical Importance of Sterilization 2.10

2.4 Physical Methods of Sterilization 2.11
2.4.1 Heat 2.11
2.4.2 Dry Heat 2.11
2.4.3 Moist Heat 2.13
2.5 Mechanical Method of Sterilization 2.17
2.6 Chemical Methods of Sterilization 2.20
2.7 Gaseous Agents for Sterilization 2.25
2.8 Radiation Method of Sterilization 2.26
2.9 Evaluation of the Efficiency of Sterilization Methods 2.28
2.10 Equipments Employed in Large Scale Sterilization 2.31
2.11 Sterility Indicators 2.34
2.11.1 Dry Heat Sterilization 2.34
2.11.2 Moist Heat Sterilization 2.35
2.11.3 Gaseous Sterilization 2.35
2.11.4 Radiation Sterilization 2.36
• Questions 2.38
• Multiple Choice Questions 2.38

3. Study of Disinfectants, their Evaluation, Sterility Testing of Products and Study of Fungi and Viruses 3.1 – 3.54
3.1 Fungi 3.1
3.1.1 Morphology 3.2
3.1.2 Classification 3.4
3.1.3 Cultivation of Fungi 3.5
3.1.4 Reproduction of Fungi 3.6
3.2 Viruses 3.9
3.2.1 Morphology 3.9
3.2.2 Classification of Viruses 3.10
3.2.3 Cultivation of Viruses 3.13
3.2.4 Cultivation of Bacteriophages 3.16
3.2.5 Reproduction of Viruses 3.16
3.3 Classification and Mode of Action of Disinfectants 3.17
3.3.1 Classification 3.18
3.3.2 Mode of Action of Disinfectants 3.21
3.4 Antiseptics 3.23
3.5 Factors Influencing Disinfection and Antiseptics 3.25
3.6 Evaluation of Bactericidal and Bacteriostatic 3.27
3.7 Antibiotic Susceptibility Test 3.28
3.8 Sterility Testing of Products According to IP, BP and USP 3.46
• Questions 3.51
• Multiple Choice Questions 3.52

4. Aseptic Environment and Different Microbiological Assay 4.1 – 4.24
4.1 Designing of Aseptic Area 4.1
4.2 Laminar Flow Equipments 4.5
4.3 Study of Different Sources of Contamination in an Aseptic Area and Methods of Prevention 4.8

4.3.1 Atmosphere 4.8
4.3.2 Operator 4.9
4.3.3 Raw Materials 4.9
4.4.4 Building 4.10
4.4.5 Equipment 4.10
4.4.6 Packaging and Manufacturing Process 4.10
4.5 Clean Area Classification 4.11
4.5.1 Principle of Clean Room Air Flow 4.11
4.5.2 Classification 4.11
4.6 Principles and Methods of Different Microbiological Assay 4.14
4.7 Methods for Standardization of Antibiotics 4.17
4.8 Method for Standardization of Vitamins 4.18
4.9 Standardization of Amino Acids 4.22
• Questions 4.23
• Multiple Choice Questions 4.23

5. Microbial Spoilage, Stability of Products and Animal Cell Culture 5.1 – 5.26
5.1 Introduction 5.1
5.2 Types of Spoilage 5.2
5.2.1 Microbial spoilage 5.2
5.2.2 Non-Microbial spoilage 5.2
5.3 Factors Affecting the Microbial Spoilage of Pharmaceutical Products 5.5
5.3.1 Types and Size of Contaminant Inoculum 5.6
5.3.2 Nutritional Factors 5.6
5.3.3 Water 5.6
5.3.4 Storage Temperature 5.6
5.3.5 pH 5.7
5.3.6 Redox Potential 5.7
5.3.7 Packaging Design 5.7
5.4 Sources and Types of Microbial Contaminants 5.7
5.4.1 Sources of Contaminants 5.8
5.4.2 Types of Microbial Contaminant 5.9
5.5 Assessment of Microbial Contamination and Spoilage 5.10
5.6 Preservation of Pharmaceutical Products using Antimicrobial Agents 5.12
5.7 Evaluation of Microbial Stability of Formulations 5.15
5.8 Growth of Animal Cells in Culture 5.18
5.9 General Procedure for Cell Culture 5.21
5.9.1 Primary Culture 5.22
5.9.2 Established Cell Culture 5.23
5.9.3 Transformed Cell Culture 5.23
5.10 Application of Cell Cultures in Pharmaceutical Industry and Research 5.23
• Questions 5.25
• Multiple Choice Questions 5.25

• **References R.1 – R.2**

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Unit ... 1

BASICS OF MICROBIOLOGY

♦ LEARNING OBJECTIVES ♦

After completing this chapter, reader should be able to understand:

- About introduction, history, scope and importance of microbiology.
- About Introduction to Prokaryotes and Eukaryotes organisms.
- About detail study of Bacteria with respect to structures, their classification, growth requirements, their isolation techniques and quantitative measurements.
- About study of different types of microscopy for detection of microorganisms.

1.1 INTRODUCTION

Microbiology is the branch of science that deals with microorganisms. Microorganisms are the small living things that include unicellular, multicellular or acellular. Unicellular are single cells organisms like cocci, bacilli, virio and spirillae (Fig. 1.1). Multicellular are filaments and sheaths to form cell colonies like blue green algae (cyanobacteria), fungi, protozoans and bacteria (Fig. 1.2) whereas acellular are organism without cells, like viruses, prions (Fig. 1.3). These microorganisms are not visible by naked eyes, only observed under microscope. Like other organisms, microorganisms survive, grow and are also require a source of energy and nourishment to survive and to growth. Many microorganisms are beneficial to human and some are pathogenic in nature.

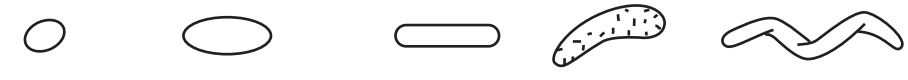


Fig. 1.1: Unicellular single cell organisms

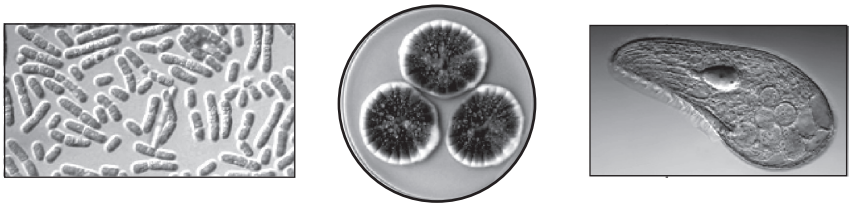
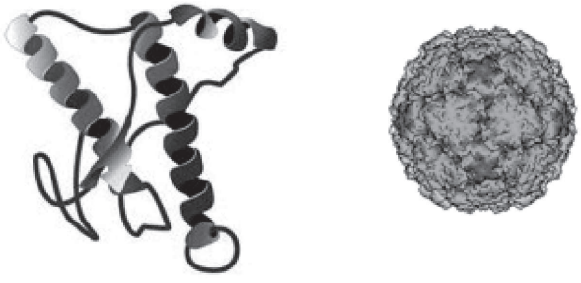


Fig. 1.2: Multicellular organisms



Prion Virus

Fig. 1.3: Acellular Organisms

1.2 HISTORY

History of microbiology is divided in to three stages namely **Discovery stage**, **Transition stage** and **Modern microbiology**.

1.2.1 Discovery Stage

Aristotle (384-322) described living and non-living organisms and their differentiation. **Roger Bacon** (13th Century) described that diseases are caused by living creatures (Fig. 1.4). Thereafter in 1546, **Fracastorius** described that communicable diseases were caused by living agents known as germs. In 1665, the first report on cell structure was described by **Robert Hooke**. Later, during mid of 1600's, **Antony van Leeuwenhoek (1632-1723)** was the first person who used a microscope of his own design to direct observations of microbes (Fig. 1.5). He discovered microorganisms in 1675 and named bacteria and protozoa as "Animalcules". He has provided full description of bacteria. From that time he was known as one of the founders of microbiology.



Fig. 1.4: Antonie van Leeuwenhoek (24 October 1632 – 26 August 1723)



Fig. 1.5: Roger Bacon (13th Century) (c. 1219/20 – c. 1292)

In 1659, **Kircher** was reported minute worms in the blood during plague attack to human.

1.2.2 Transition Stage

In this era, **Francesco Redi (1626-1697)** showed that maggots would not arise from decaying covered meat. Further **John Needham (1713-1781)** proposed that tiny organisms arise spontaneously on the mutton gravy and he supported the spontaneous generation theory. There after **Lazzaro Spallanzani (1729-1799)** demonstrated that air carried germs to

the culture medium and also revealed that boiled broth would not give growth of microorganisms (Fig. 1.6). In Next, **John Tyndall (1820-1893)** in 1877 proved the need for prolonged heating for elimination of microbial life from infusions, which are recently termed as tyndallization in which heat stable as well as heat sensitive bacteria both are killed (Fig. 1.7). In 1835, **Augustino Bassi** demonstrated that a silk worm disease called muscardine was due to fungal infection.



Fig. 1.6: Lazzaro Spallanzani (10 January 1729 – 12 February 1799)



Fig. 1.7: John Tyndall (2 August 1820 – 4 December 1893)

1.2.3 Modern Microbiology

The actual development of microbiology came with Louis Pasteur, Robert Koch, Lord Lister, Alexander Flemming and Paul Ehrlich.

Louis Pasteur is known as the father of medical microbiology because he has coined the terms microbiology, aerobic and anaerobic. In 1897, he suggested that mild heating at 62.8°C for 30 minutes was more effective than boiling to destroy the pathogenic organisms without change of taste of the product. This method was known as **Pasteurization**. Hence, he was known as the inventor of the Pasteurization. Thereafter he also invented fermentation process and developed effective live attenuated vaccines against rabies and anthrax. He also demonstrated disease of silkworm was due to protozoan parasite (Fig. 1.8).

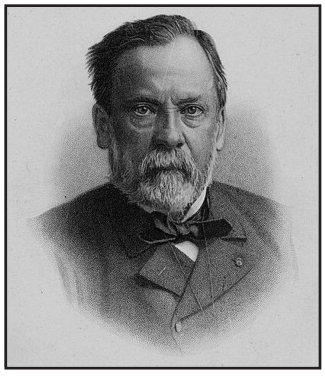


Fig. 1.8: Louis Pasteur (December 27, 1822 – September 28, 1895)

Lord Joseph Lister: He is known as father of antiseptic surgery. He also revealed that wound infections were due to microorganisms and discovered the method of destroying microorganisms in the operation theatre by spraying a fine mist of carbolic acid in the air (Fig. 1.9).

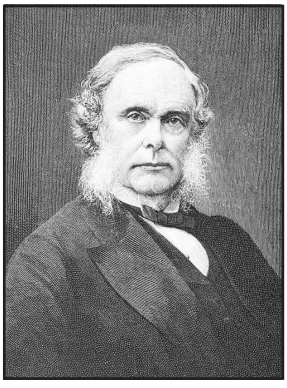


Fig. 1.9: Lord Joseph Lister
(5 April 1827 – 10 February 1912)

Robert Koch (Fig. 1.10): He demonstrated the role of bacteria in causing diseases and also invented technique for bacteria isolated from pure culture. He only explained first the germ theory of diseases in 1876. He prepared gelatin for solid media but was not ideal because gelatin is a protein which is digested by the bacteria and produce a proteolytic exo-enzyme gelatinase that hydrolyses protein into amino acids; thereafter gelatin also melted in temperature more than 25°C.

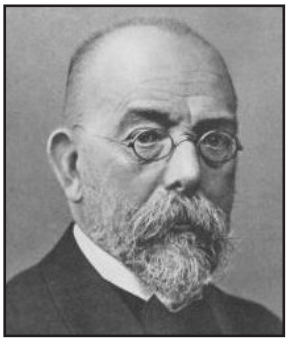


Fig. 1.10: Robert Koch
(11 December 1843 – 27 May 1910)

There are **four Koch's Postulates** viz.**:** (a) The causative agents are surely present in every individual with the disease. (b) They must be isolated and grown in pure culture, (c) When the pure culture inoculated into an experimental animal it surely cause the disease and (d) The causative agent must be re-isolated and re-identified from the experimental animal as well as in pure culture respectively.

Further, **Fanne Eilshemius Hesse (1850-1934)** used agar as solid culture media and proved the media was not attacked by most of the bacteria. Thereafter it can sustain at

higher temperature (96°C) and solidify at 40-45°C. **Richard Petri (1887)** has developed petri dishes, the glass containers which are used for solid culture media. **Edward Jenner (1749-1823)** was the first scientist who prevents small pox by the technique discovered of vaccination. **Martinus Willem Beijerinck (1851–1931)** was one of the founders of virology and environmental microbiology.

Paul Erlich: He discovered the treatment of syphilis by using arsenic (Arsphenamine). He was the founder of biological standardization with his work on toxins and antitoxins in quantitative manner. He also invented the precursor technique to Gram staining bacteria (Fig. 1.11).

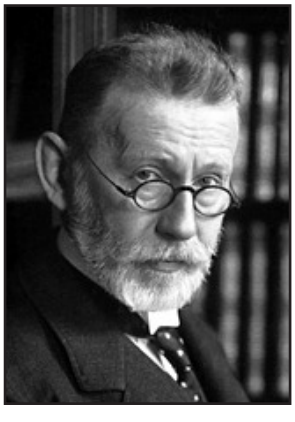


Fig. 1.11: Paul Erlich

(14 March 1854 – 20 August 1915)

Alexander Flemming (1881-1955) was the first scientist who discovered World's first antibiotic substance benzylpenicillin (Penicillin G) from the mould *Penicillium notatum* in 1928 that destroy many pathogenic bacteria and received Nobel Prize in 1945 (Fig. 1.12).

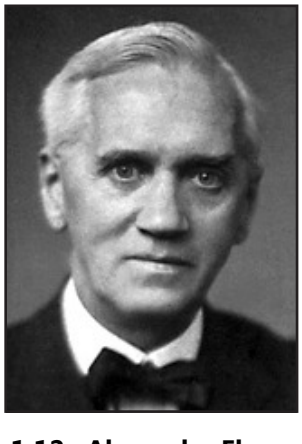


Fig. 1.12: Alexander Flemming

(6 August 1881 – 11 March 1955)

Table 1.1: Some important discoveries by the biological scientists

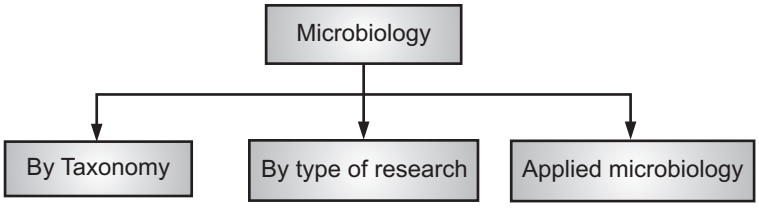
Name of the Scientists	Discoveries
Emile Roux (1853-1933) and Alexandre Yersin (1863-1943)	Discovered Diphtheria toxin
Joseph Hansen (1874)	Leprosy bacillus
Albert Ludwig Sigesmund Neisser (1879)	Causative agent of gonorrhea (*Neisseria gonorrhoeae*)
Alexander Ogston (1881)	*Staphylococcus*
Friedrich August Johannes Loeffler (1884)	Diphtheria causing bacteria (*Corynebacterium diphtheriae*) and the cause of foot and mouth disease (Aphthovirus)
Louis Pasteur (1885)	Developed Rabies vaccine
Frederick Twort (1915) and Felix d'Herelle (1917)	*Bacteriophages*
Good Pasteur (1981)	Cultivation of viruses in chick embryos

Table 1.2: Nobel laureates in modern era with their discoveries

Scientists	Discoveries
Emil von Behring (1901)	Diphtheria antitoxin
Sir Ronald Ross (1902)	Transmission of Malaria
Robert Koch (1905)	Research on tuberculosis
Ilya Ilyich Mechnikov (1908)	Work on immunity and Phagocytosis
Sir Alexander Fleming (1945)	Penicillin
James Watson and Francis Crick (1962)	Structure of DNA
Har Gobind Khorana, Marshall W Nirenberg and Robert W Holley (1968)	Genetic code
B. S. Blumberg and D. C. Gajdusek (1976)	Antigen important in diagnosing serum hepatitis
Stanley Benjamin Prusiner (1997)	Prion research
Malcolm K. Brenner (2002)	Genetic regulation of organ development and cell death
Harald zur Hausen (2008)	Human papilloma viruses can cause cervical cancer, discovered HIV virus
Bruce A. Beutler, Jules A. Hoffmann and Ralph M. Steinman (2011)	Role of dendritic cell in adaptive immunity
Jacques Dubochet, Joachim Frank and Richard Henderson (2017)	Developed cryo-electron microscopy for the high-resolution structure determination of biomolecules in solution

1.3 BRANCHES OF MICROBIOLOGY

General microbiology is classified into main three branches which are tabulated below.



Flowchart 1

By Taxonomy

- **Bacteriology:** It is the study of bacteria.
- **Immunology:** It is the study of the immune system. It looks at the relationships between pathogens such as bacteria and viruses and their hosts.
- **Mycology:** It is the study of fungi, such as yeasts and molds.
- **Nematology:** It is the study of nematodes (roundworms).
- **Parasitology:** It is the study of parasites. Not all parasites are microorganisms, but many are. Protozoa and bacteria can be parasitic; the study of bacterial parasites is usually categorized as part of bacteriology.
- **Phycology:** It is the study of algae.
- **Protozoology:** It is the study of protozoa, single-celled organisms like amoebae.
- **Virology:** It is the study of viruses.

By Type of Research

- **Astromicrobiology:** It is the study of the origin of life on Earth, and the search for extra-terrestrial life.
- **Evolutionary microbiology:** It is the evolution of microorganisms.
- **Microbial cytology:** It is the study of microscopic and submicroscopic details of microorganisms.
- **Microbial physiology:** It is the study of biochemical functioning of the microbial cells with respect to the study of microbial growth, microbial metabolism and microbial cell structure.
- **Microbial ecology:** It is the relationship between microorganisms and their environment.
- **Microbial genetics:** It is the study of how genes are organized and regulated in microbes in relation to their cellular functions which is closely related to the field of molecular biology.
- **Cellular microbiology:** It is a discipline bridging microbiology and cell biology.
- **Evolutionary microbiology:** It is the study of the evolution of microbes. This field can be subdivided into**:**
  - **Microbial taxonomy:** It is the study of naming and classification of microorganisms.
  - **Microbial systematics:** It is the study of the diversity and genetic relationship of microorganisms.
- **Generation microbiology:** It is the study of those microorganisms that have the same characters as their parents.

- **Molecular microbiology:** It is the study of the molecular principles of the physiological processes in microorganisms.
- **Nano microbiology:** It is the study of those microorganisms on Nano level.
- **Biological agent:** It is the study of those microorganisms which are being used in weapon industries.

Applied Microbiology

- **Medical microbiology:** It is the study of the pathogenic microbes that are responsible for human illness. It includes the study of microbial pathogenesis and epidemiology and is related to the study of disease pathology and immunology.
- **Pharmaceutical microbiology:** It is the study of microorganisms that are related to the production of antibiotics, enzymes, vitamins, vaccines, and other pharmaceutical products and that cause pharmaceutical contamination and spoil.
- **Industrial microbiology:** It is the exploitation of microbes for use in industrial processes like industrial fermentation and waste water treatment which is linked to the biotechnology industry.
- **Microbial biotechnology:** It is the study of genetic and molecular level manipulation of microorganisms to generate useful products.
- **Food microbiology and Dairy microbiology:** It is the study of microorganisms causing food spoilage and food borne illness.
- **Agricultural microbiology:** It is the study of agriculturally relevant microorganisms. This field can be further classified into the following**:** Plant microbiology and Plant pathology, where the interactions between microorganisms and plants and plant pathogens are studied. Secondly soil microbiology**:** It is the study of those microorganisms that are found in soil.
- **Veterinary microbiology:** It is the study of the role of microbes in veterinary medicine or animal taxonomy.
- **Environmental microbiology:** It is the study of the function and diversity of microbes in their natural environments. This involves the characterization of key bacterial habitats such as the rhizosphere and phyllosphere, soil and groundwater ecosystems, open oceans or extreme environments (extremophiles). This field includes other branches of microbiology such as:
  - **Microbial ecology:** It is the study of the relationship of microorganisms with one another with their environment.
  - **Microbially mediated nutrient cycling:** It is the study of the movement and exchange of organic and inorganic matter back into the production of matter through microorganisms.
  - **Geomicrobiology:** It is the interdisciplinary study of the interactions of microorganisms and earth materials such as soil, sediment, atmosphere, minerals and rocks.
  - **Microbial diversity:** It is the study of number of species and abundance of each species of microorganisms that are distributed in an ecological community.

- **Bioremediation:** It is a process used to treat contaminated media, including water, soil and subsurface material, by altering environmental conditions to stimulate growth of microorganisms and degrade the target pollutants.
- **Aquatic microbiology:** It is the study of those microorganisms that are found in water.
- **Aero microbiology:** It is the study of airborne microorganisms.

1.4 SCOPE AND IMPORTANCE OF MICROBIOLOGY

1.4.1 Scope of Microbiology

In the previous headings, various branches of microbiology were discussed and with the view of that it is well understood that microbiology has wide range of scope. It has impact on medicine, agriculture, food science, ecology, genetics, biochemistry, immunology and many other fields. Based on the specific groups of the microorganisms, the specialists are known as bacteriologist (Specialist in Bacteria study), virologists (specialist in virus study), mycologist (specialist in fungi study) and so on. Hence, different branches of microbiology has different role in the advancement pharmaceutical sciences where microbiologists are actively involved in research study (immunology, virology, chemotherapy, genetics etc.), helps to perform diagnostic tests, develops antibiotics and vaccines, protects the environment and plays an important role in food and beverages industries (Fig. 1.13).

Some of the recent scopes are highlighted below**:**

1. **Ecology and environment:**

- Bacteria are primary decomposers as they recycle nutrients back into the environment.
- Winogradsky and M. Beijerinck studied soil microbes and their roles in the sulfur, carbon, nitrogen biochemical cycles.
- Some bacteria are used as bio-pesticides to control Insects pest. For example, *Bacillus thuringiensis*.
- Microbes are also used to clean up pollutants and toxic wastes through bioremediation. For example, *Pseudomonas putida* is used to remove petroleum spill.

2. **Food microbiology:**

- Microbes are used in various food and dairy industries to produce various food products like cheese, pickles, sauerkraut, green olives, yogurt, soy sauce, vinegar, bread etc. Pasteur (1856) described fermentation technology which helped in production of Beer, Wine, Alcohol etc.

3. **Medicines: clinical and pharmaceutical microbiology:**

- **Disease Treatment:** Microbes are used to produce Antibiotics like *Penicillium notatum (*Penicillin); discovered by **Alexander Fleming (1928)**. Bacteria also synthesize vitamins which are needed for our body like *E. coli*. Vitamin-B and K are needed for metabolism and blood clotting respectively. Recently, gene therapy for treatment of genetic diseases is advanced scope of microbiology in medical field.

4. **Vaccine and immunology:**

- Vaccine activates immune response. Sir Edward Jenner inoculated people with cowpox to protect against smallpox. Thereafter Pasteur developed the rabies vaccine (1885), Von Behring and Kitasato (1890) produced toxoid vaccine against diphtheria and tetanus. Furthermore Metchnikoff (1884) described role of phagocytic cell in defense.

5. Genetic engineering:

- Microorganisms are used in Recombinant DNA Technology to manipulate their gene for the production of enzymes, hormones, interferons, etc. Microorganisms are used as model organism in molecular biology.

6. Biochemistry and physiology:

- Microorganisms are used as a model for study of many biochemical and physiological processes.

7. Industrial microbiology:

- Microbes are used in economic and industrial purposes. Like Biotechnology, fermentation technology, food and beverages etc. are now established on large industrial scale for income and there is a scope of Patients right for procedure used. Proteases, amylase from bacillus are used in textile and detergent industries. Thiobacillus is used in metal processing from mines. Methanococcus is used for the production of methane in fuel industry.

8. Agriculture microbiology:

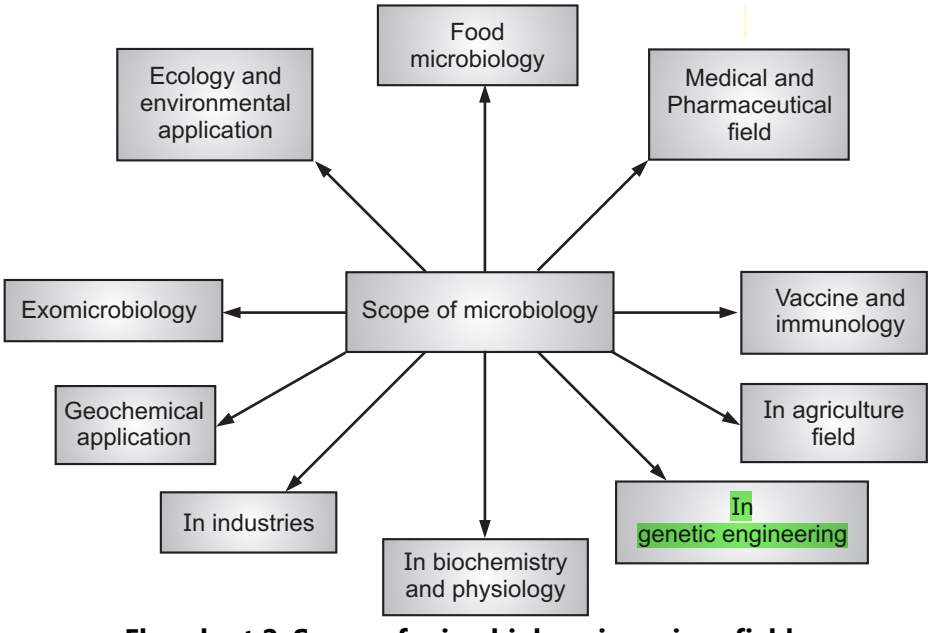
- Genetic engineering is used for the production of transgenic plants and animals for better production, resistant to environmental fluctuation. Transgenic animals or plants are used as bioreactor for mass production through molecular farming.

9. Geochemical microbiology:

- **Bioleaching:** Recovery of minerals from low grade ores also plays a role in geochemical cycle.

10. Exomicrobiology:

- It is also exploring for life in outer space.



Flowchart 2: Scope of microbiology in various fields

1.4.2 Importance of Microbiology

Microbiology is a part of our daily life and plays vital role to health care system. The importance of microbiology began with the discovery of antibiotics which prevent serious infections. Thereafter vaccination and better sanitation practices reduced the incidence of infectious diseases and most infectious diseases were under control but some are reappearing because pathogens are showing increasing resistance to antibiotics. Hence, many new diseases are emerging and many harmless organisms are discovered as causative agents for new diseases. Furthermore interest in bioterrorism has progressed from past few eras. Bioterrorism is terrorism involving the intentional release or dissemination of any pathogenic microorganisms like bacteria, viruses, or toxins that may be in a natural or a human modified form. The common examples of bioterrorism are anthrax, small pox, botulinum toxin, bubonic plague, H1N1, AIDS/HIV, zika and ebola viruses etc. Recently, microbiologists have also invented detection of bioterrorism event at the earliest possible moment through RODS (Real time Outbreak Disease Surveillance). This process is known as **biosurveillance**. Biosurveillance is the science of real-time disease outbreak detection. Its principles are applied to both natural and man-made epidemics. A tiny electronic chip has been invented to identify broad range of toxins. It contains living nerve cells that warn of the presence of bacterial toxins. Another fibre optic tube that is lined with antibodies coupled to light emitting molecules identifies specific pathogens like anthrax, botulinum etc. Recent research invented ultraviolet avalanche photodiodes which detect anthrax and other bioterrorism agents in the air in advance. Vaccines are in research stage to control and prevent many endemic diseases. Recently, vaccine has been discovered for treatment of dengue virus. India is currently working on vaccines to prevent dengue and by the year 2020 it will be in Indian market. Recently in 2017, a vaccine has discovered against malaria which is 100% effective. The importance of microbiology in various fields is such as:

Pharmaceutical industry: Pharmacists are discovering various antimicrobial drugs to prevent an escalating number of communicable diseases by understanding the human cell mechanism. They work with microbiologists to ensure that drug therapies target the opportunistic microbes without harming its human host. Another important role in pharmaceuticals is the use of medically important studies on microbes, such as Bacteriorhodopsin, a protein from the plasma membrane of *Halobacterium salinarum*.

Medical devices: Microbiology plays a significant role in medical devices like fluorescent fusion. It is mainly used for fast and precise detection of pathogens in tissue samples. It is a technology for carrying out immunofluorescence studies that may be applied to find specific cells in complex biological systems. Thereafter ultraviolet avalanche photodiodes are devices which detect anthrax and other bioterrorism agents in the air.

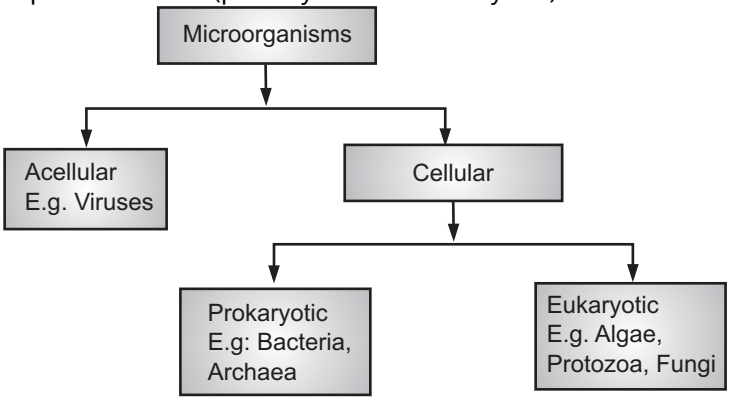
Cosmetic microbiology: Microbial contamination in cosmetic products is a major concern to the industry. Contamination with microorganisms is become a major cause of both product and economic losses. Not only that, they are also converted into products hazardous for consumers. The water and nutrients present in cosmetics make them susceptible to microbial growth. More often, microorganisms are the cause of offensive odours, and changes in viscosity and colour.

Microbiological processes are used in waste treatment, particularly dumping, waste incineration, composting and biomethanization.

1.5 INTRODUCTION TO PROKARYOTES AND EUKARYOTES

Classification of Microorganisms:

Microbes are widely spread in environment in which some are responsible for serious harm and some are beneficial to life. They are classified into six major groups namely bacteria, archaea, fungi, protozoa, algae, and viruses. All these microorganisms are broadly belong to two groups viz. Cellular (prokaryotic and eukaryotic) and acellular.



Flowchart 3

(a) Acellular organisms: These are the microorganisms that have neither cells nor cell membrane. They are composed of few genes which are protected by protein coat. Outside of the host they acts as dead but inside host they are alive and reproduce. Example: Viruses.

(b) Cellular organisms: These are the microorganisms which have definite cell structures. Based on the cellular structure they are further divided into prokaryotic and eukaryotic. Prokaryotics are cells without nucleus and have no membrane bond organelles. They are less complex in structures. Examples: Bacteria and Archaeac.

A. Bacteria: (singular: Bacterium) (study of bacteria: **bacteriology**)

1. They are prokaryotic and unicellular
2. The size is 1/1000 the volume of a typical eukaryotic cell.
3. Mainly two groups of bacteria are:
   (a) **Archaeobacteria:** Ancient bacteria.
   (b) **Eubacteria:** True bacteria.
4. They are in various shapes likely bacillus (rod), coccus (spherical), spirillum (spiral), vibrio (curved rod).
5. They are motile or non-motile.
6. They obtain their energy from sun as well as from organic and inorganic molecules, and as per that they are classified as: **Photosynthetic autotrophs** (use energy from the sun to produce their own carbohydrates for energy), **Chemosynthetic autotrophs** (process inorganic molecules for energy (example: sulfur or iron) and **heterotrophs** (depend on outside sources of organic molecules (example: carbohydrates or sugars) for energy.
7. **Optimum temperature:** $-20^{o}C$ to $110^{o}C$ (freezing is $0^{o}C$ and boiling is $100^{o}C$).

B. Algae:

1. They are eukaryotic, unicellular or multicellular.
2. They are microscopic and macroscopic in size.
3. They are motile or non-motile.
4. They obtain their energy from photosynthetic autotrophs.

C. Fungi:

1. They are eukaryotic.
2. They are unicellular or multicellular (yeasts are unicellular, molds are multicellular).
3. They are non-motile.
4. They obtain their energy from outside source of organic molecules.
5. They acts as scavengers, they live off dead matter and thus, decompose it.

D. Protozoa:

1. They are eukaryotic.
2. They are unicellular.
3. They are motile or non-motile.
4. They obtain energy from outside source of organic molecules.

E. Viruses:

1. They are acellular and obligate intracellular parasites.
2. **Structure of a virus:** A piece of nucleic acid (RNA or DNA) enclosed by a protein coat (capsid); possess no nucleus, organelles, cell membrane, or cytoplasm.
3. Size - 1/10 to 1/1000 the size of an ordinary bacterial cell.
4. They are non-motile.

Thereafter the differences between the eukaryotic and prokaryotic cells are depicted in Table 1.3.

Table 1.3: Differences between Eukaryotic and Prokaryotic cells

Organelles	Eukaryotic Cell	Prokaryotic Cell
Nucleus	Present	Absent
Chromosome	More than one	One (Plasmid)
Cell type	Usually multicellular	Usually unicellular
Lysosomes	Present	Absent
Microtubules	Present	Absent
Genetic recombination	Meiosis and fusion of gamates	Undirectional transfer of DNA
Endoplasmic reticulum	Present	Absent
Mitochondria	Present	Absent
Ribosome	Larger	Smaller
Golgi apparatus	Present	Absent
Flagella	Microscopic in size; membrane bound	Submicroscopic in size, composed of only one fibre
Cell size	10-100 μm	1-10 μm
Vacuoles	Present in large size	Present in small size
Nuclear membrane	Selective	Not present
Examples	Animal and Plants	Bacteria and Archaea

General classification of Bacteria:

A. Based on the wall structure: Gram Positive (Staphylococcus, Streptococcus, Clostridium, Bacillus), Gram negative (*E. coli*), Acid-fast (Mycobacterium), Wall-less (Mycoplasma).

B. Unusual: Obligate intracellular (Rickettsia, Chlamydia).

C. Cell morphology:

1. Shapes (Rod, Cocci, Spiral)
2. Associations (Individual, Diplococci, Staphylococci, Streptococci).

D. Growth characteristics:

1. Oxygen requirement (Aerobic, Anaerobic, Microaerophilic, aerotolerant, Facultative),
2. Spore formation
3. Intracellular/extracellular
4. Fastidious/non-fastidious

1.6 STUDY OF ULTRA-STRUCTURE AND MORPHOLOGICAL CLASSIFICATION OF BACTERIA

1.6.1 Ultra Structure of Bacteria

Under electron microscope, the structure of bacterial cell is look like a capsule. It has following components like outside the cell membrane and internal to cell membrane (Fig. 1.13).

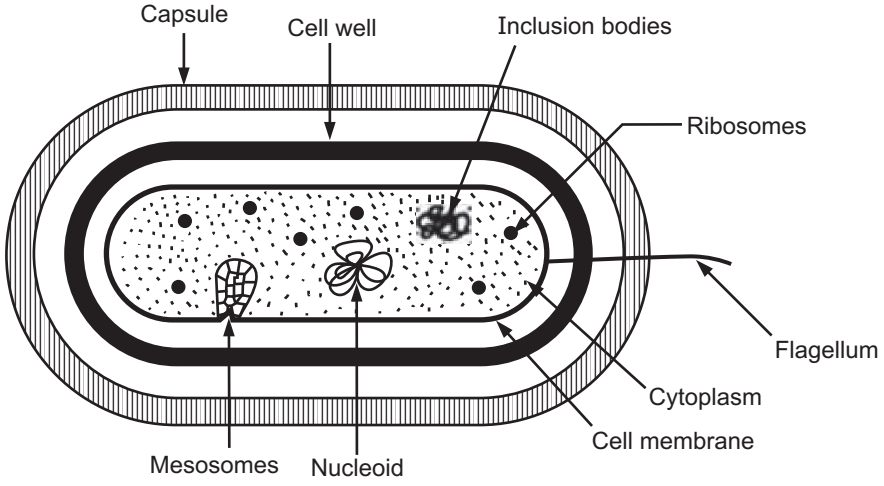


Fig. 1.13: Ultra structure of Bacterial cell

Capsule: It is the outer layer of the bacteria cell. Depending on the chemical nature capsules are thick or thin, and rigid or flexible. It is most important virulence factor of bacteria which is visualized by negative staining technique. Generally, it composed of 98% water and 2% polysaccharide or glycoprotein/polypeptide or both. Only some bacterial species possess capsule. Capsule is usually made of polysaccharide (e.g. pneumococcus), occasionally polypeptide (e.g. anthrax bacilli) and hyaluronic acid (e.g. streptococcus). Most bacterial capsules are composed of polysaccharides especially with homopolysaccharides which are usually synthesized outside the cell from disaccharides by exocellular enzyme. Capsule of acetic acid bacteria cell is composed of homopolysaccharide (hemicellulose).

Glucan is synthesized by *Streptococcus mutans* which are composed of several types of sugar and are termed as heteropolysaccharides. A few capsules are polypeptides like *Bacillus anthracis*, is composed entirely of a polymer of glutamic acids.

There are two types of capsules,

1. **Macrocapsule:** Thickness of 0.2 µm or more, visible under light microscope.
2. **Microcapsule:** Thickness is less than 0.2 µm, visible under Electron microscope.

Functions:

1. **Preventive nature:** Capsular polysaccharide bind significant amount of water making cell resistant to drying and also prevent attachment of bacteriophage on cell surface.
2. **Protection:** It protect from mechanical injury, temperature, drying etc.
3. **Attachment:** Capsule helps in attachment on the surface. For example: *Streptococcus mutants* that cause dental carries attach on teeth surface by its capsule.
4. **Anti-phagocytic:** Capsule resist phagocytosis by WBCs.
5. **Source of nutrition:** It acts as a resource organ of nutrition during insufficient nutrient supply.
6. **Repulsion:** Same charge capsulated bacteria repel each other.

Cell wall:

Cell wall is the rigid wall situated outside of the plasma membrane. It provides shape of the bacteria as well as provides protection from osmotic lysis. Cell wall is made up of peptidoglycan. The gram positive bacteria contains thick homogeneous peptidoglycan or murein layer (20 to 80 nm thickness) outside the plasma membrane whereas gram negative bacteria has 2-7 nm peptidoglycan layer surrounded by 7-8 nm thick outer membrane. Peptidoglycan is a polymer consists of two sugar molecules viz. N-acetylglucosamine and N-acetylmuramic acid. Apart from that it also contains several amino acids.

Flagella:

Flagella are slender and rigid hair like structures. They are about 20 nm cross and up-to 15 to 20 µm long. The eukaryotic flagellum is a long, rod-like structure, surrounded by an extension of the cell membrane like a sheath. The bulk of the structure is a filament called an **axoneme**. Monotrichous bacteria have one polar flagellum whereas amphitrichous bacteria have either singe or duster of flagellum at both pole. Lophotrichous bacteria have a cluster of flagella at one ends. Peritrichou bacteria have surrounded lateral flagella.

Flagellar Ultra-Structure:

Transmission electron microscope studies revealed three regions of flagella which are:

(1) The outermost region is filament which is extended from the cell surface to tips.
(2) Basal bodies consist of small central rods inserted into cells
(3) A short curved segment, the flagellar hook which links the filament to basal bodies and acts as flexible coupling.

The filament is a hollow, rigid cylinder made up of protein subunits flagellin. The molecular weight is varies from 30,000 dalton to 6,000 dalton depends on the bacterial species.

Gram negative bacteria have four rings connected to a central rod. Two rings (M and S rings) are embedded in cell membrane and other two rings (L and P rings) are associated with cell wall which is made up of lipopolysaccharide and peptidoglycan respectively.

Gram positive bacteria have only two rings in their basal body. M-ring which is inner ring, connected to the plasma membrane and S-ring which is outer ring, connected to the peptidoglycan.

Fimbriae and Pili:

Many Gram negative bacilli contain short, fine, hair like, non-flagellar appendages called fimbriae. Pili are similar appendages which are about 1-10 cells. The filament of pilus is straight and diameter is 7 nm to 10 nm, larger than diameter of fimbriae. It is made up of pilin protein. Molecular weight is 17,000. Pili are non-motile but adhesive structure. They enable the bacteria to stick firmly to other bacteria, to a surface or to some eukaryotic such as mould plants, plants and animal cells. They are genetically determined by sex factor or conjugative plasmid and hence they help in conjugation of male bacteria, in the attachment of pathogenic bacteria to their host cell.

Spinae:

Some Gram-positive bacteria have tubular unicellular and rigid appendage of single protein moiety, known as spinae. They help the bacterial cell to tolerate environmental stress such as salinity, pH and temperature etc.

Cell Membrane:

Cell or plasma membrane is a thin structure that completely surrounds the cell. It is about 8 nm thick. This structure is critical barrier separating the inside of cell from environment. The cell membrane is also highly selective barrier enabling the cell to concentrate a specific metabolite and excrete waste material. Mostly biological membrane is composed primarily of Phospholipids (about 20 to 30 per cent) and proteins (about 60 to 70 per cent).

One major difference in chemical composition of membrane between eukaryotic cells and prokaryotic cells is that eukaryotes have sterol in their membrane depending on cell type, sterol can make up from 5-25% of total lipid of eukaryotic membrane. Sterols are absent from membranes of all prokaryotic cells (methanotropns are major exception). Sterols are rigid, planner molecules, whereas fatty acids are flexible.

The main function of cell membrane is selective permeability and transport of solute, electron transport and oxidative phosphorylation in aerobic species, excretion of hydrolytic exoenzymes.

Inclusion bodies:

Various types of inclusion bodies organic and inorganic granules are present in the cytoplasmic matrix. They are mainly used for storage of energy and reduce osmotic pressure by tying up molecules in particulate forms like polyphosphate granules, glycogen granule etc. Some of the membrane enclosed inclusion bodies are like glycogen-sulfur granules, carboxysomes etc.

In many cyanobacteria, green photosynthesis bacteria gas vacuole is present as organic inclusion body. Gas vacuoles are aggregates of huge number of small, hollow cylindrical structures and provide them buoyancy.

Ribosomes:

Ribisomes are loosely attached to the plasma membrane (70S ribosomes). They are made up of both protein and ribonucleic acid. They are the site of protein synthesis. Plasma membrane ribosomes make proteins for transport to the outside.

Nucleoid:

Prokaryotic chromosome is located in an irregularly shaped region called nucleoid. Nucleoid is composed of 60% DNA, 30% RNA and 10% protein by weight. Prokaryotes contain single circle of double stranded DNA but some are have linear DNA chrosome.

Mesosome:

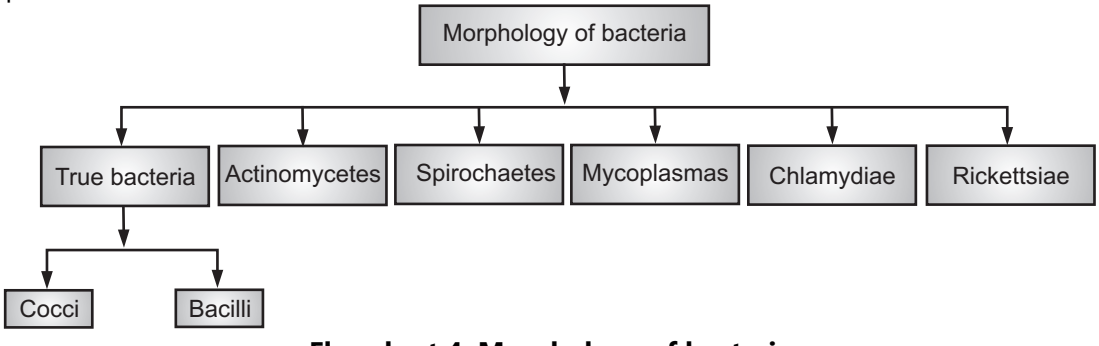
Mesosomes are present in the cell membrane of bacterial cells that fold inward. They play a major role in cellular respiration. They appear as a pocket form by the cytoplasmic membrane, filled with vesicles, tubules or lamellae.

Some of the differences of gram positive and gram negative bacteria are given in Table 1.4.

Gram Positive	Gram Negative
1. The cell wall is made up of peptide-glycan layer which is thick	1. They have a thin lipopolysaccharide exterior cell wall.
2. **Effect on dyes:** Retain the crystal violet dye, and change into purple during staining identification.	2. **Effect on dyes:** Do not retain the crystal violet dye, and react only with a counter-stain, generally stain pink.
3. **Effect on antibiotics:** Susceptible to the enzyme lysozyme and to penicillin	3. **Effect on antibiotics:** Resistant to penicillin and contain an endotoxin called Lipopolysaccharide.
4. The flagellum has two supporting rings, in the peptidoglycan layer, and in the plasma membrane.	4. If present, the flagellum has four supporting rings, namely 'L' ring, 'P' ring, 'M' ring, and 'S' ring.
5. Teichoic acids is present.	5. Teichoic acids is absent.
6. Liproproteins are absent.	6. Liproproteins are attached to the polysaccharide backbone.
7. Periplasmic space is absent.	7. Periplasmic space is present.

1.6.2 Morphological Classification of Bacteria

As per the morphological basis, bacteria are classified into six major groups which are depicted as follows:



Flowchart 4: Morphology of bacteria

1. **True bacteria:** They are major group of classification which is further divided in to two sub-groups namely Cocci and Bacilli. Cocci are various types. Like Mono cocci (single cocci): *Monococcus species*, Diplococci (Cocci in pairs): *Streptococcus pneumonia*, Staphylococci (Cocci in a bunch form): *Staphylococcus aureus*, Streptococci (Cocci in chain form): *Streptococcus pyogenes*, Tetrad (Cocci in group in four): *Micrococcus species*, Sarcina (Cocci in group in eight, cube like): *Sarcina ventriculi* (Fig. 1.14).

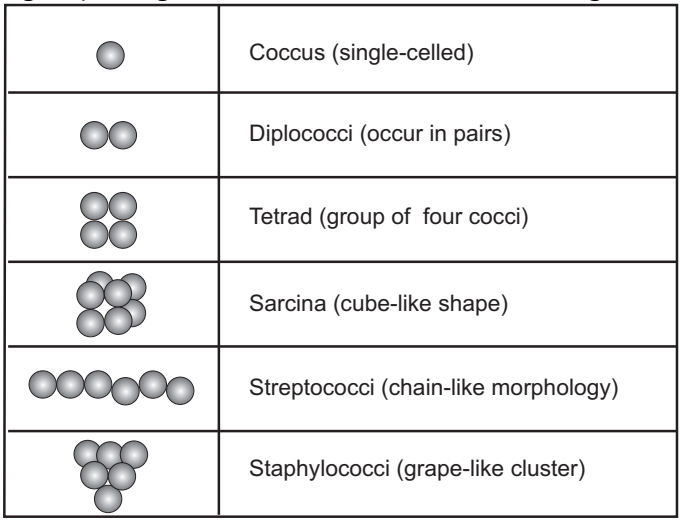


Fig. 1.14: Various cocci

Thereafter, Bacilli, which are rod like structure on the basis of arrangement of organisms they are classified in to: Diplobacilli (Pairs), streptobacilli (Chais), palisades, coccobacilli (Oval), comma shaped bacilli (Vibrio), club rods (Corynebacteriaceae), enlarged rods (Fusobacterium) (Fig. 1.15).

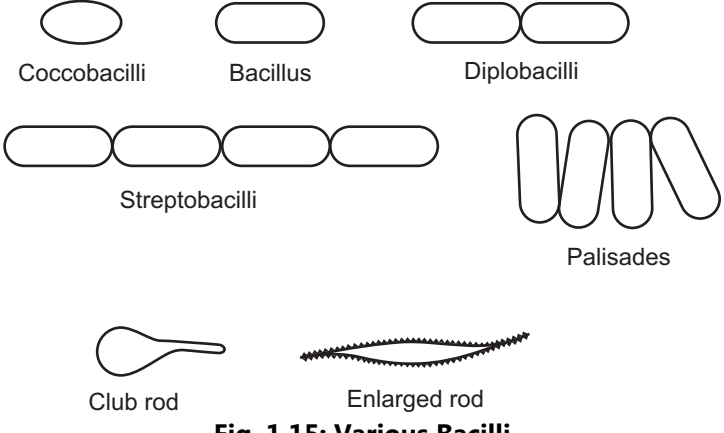


Fig. 1.15: Various Bacilli

2. **Actinomycetes:** They are rigid organism but look like fungi. They exhibit branching and tend to form filaments. Basically they are Gram positive, but several species have complex cell wall structures that make the Gram staining unsuitable (e.g. Mycobacteriaceae). They are widely distributed in soil, compost etc. They are heterotrophic, aerobic, and mesophilic organisms. Some organisms are thermophilic growing at more than 55°C like thermoactinomycetes, streptomyces etc. (Fig. 1.16).

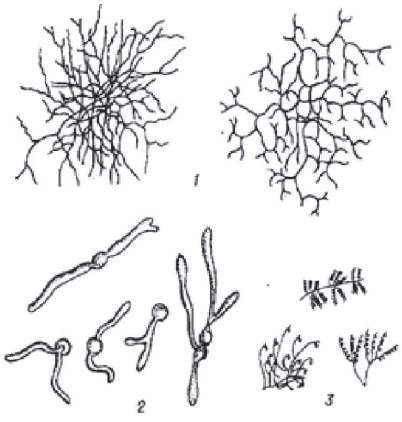


Fig. 1.16: Various Actinomycetes

3. **Spirochaetes:** They are double-membrane bacteria, most of which have long, non-branched, helically coiled cells. They are Gram negative and are chemoheterotrophic in nature. The length varies between 3 and 500 µm and diameters around 0.09 to 3 µm. Most spirochaetes are free-living and anaerobic in nature. Example: *Leptospira interrogans* (Fig. 1.17).

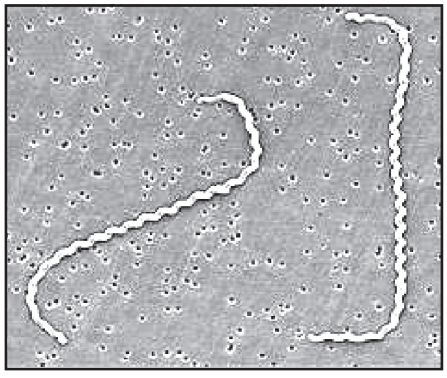


Fig. 1.17: Spirochaete

4. **Mycoplasmas:** They are the smallest bacterial cell and not having rigid cell wall. They are indefinite shape and highly pleomorphic (alter their shape or size in response to environmental conditions) in nature. They can be parasitic or saprotrophic. Several species of mycoplasmas are pathogenic in humans. Like *Mycoplasma pneumoniae* which causes pneumonia and other respiratory disorders. *M. genitalium* which involves in pelvic inflammatory diseases (Fig. 1.18).

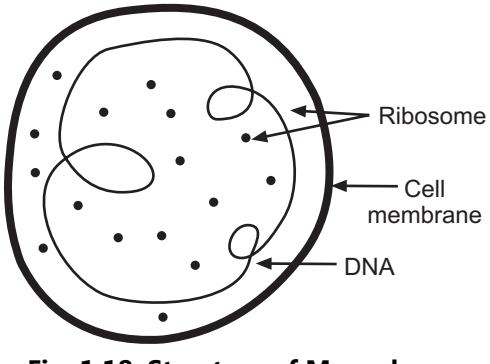


Fig. 1.18: Structure of Mycoplasma

5. **Rickettsia:** They are non-motile, Gram negative, non-spore forming, small intracellular and pleomorphic bacteria. They are also present as cocci (0.1 µm in diameter), rods (1-4 µm long), or thread-like (10 µm long) forms. *Rickettsia* species are the pathogens responsible for typhus, rickettsial pox, Boutonneuse fever and are susceptible to antibiotics of the tetracycline group. Examples: *Rickettsia conorii, Rickettsia typhi* etc. (Fig. 1.19).



Fig. 1.19: *Rickettsia typhi*

6. **Chlamydiae:** They are ovoid in shape and Gram negative bacteria. Their cell wall is consisting of peptidoglycan and other proteins. They are very small, obligate intracellular parasites. They multiply in the cytoplasm of their host cell by a distinctive developmental cycle. They commonly infect humans are eye disease, pneumonia, psittacosis etc. Examples: *Chlamydia trachomatis* (causes trachoma eye disease), *Chlamydophila pneumonia* (causes pneumonia), *Chlamydophila psittaci* (causes psittacosis) etc. (Fig. 1.20).

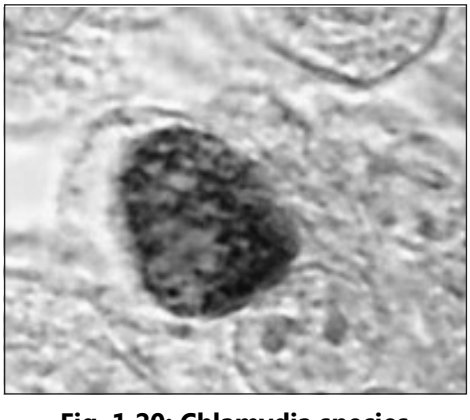


Fig. 1.20: Chlamydia species

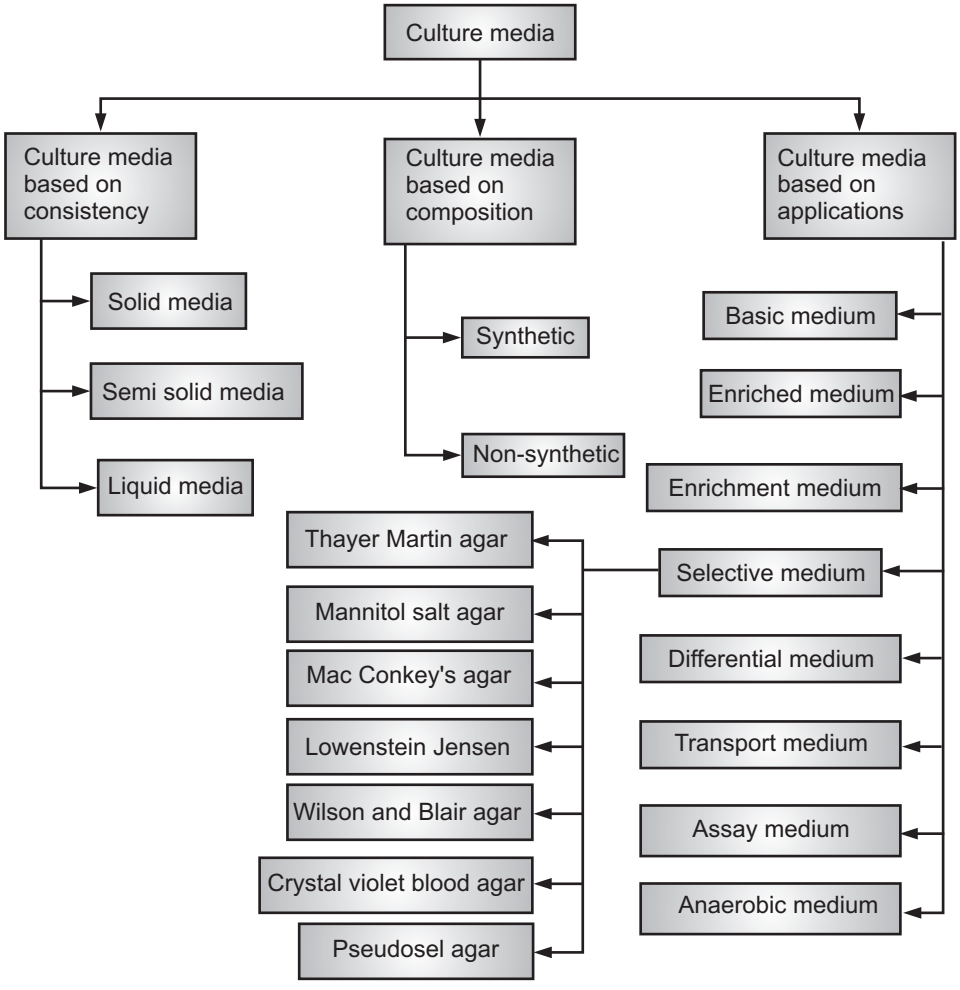
1.7 CULTURE MEDIA

Culture media are the base where microorganisms can grow with the help of nutrients and under the influence of physical growth parameters. All microorganisms cannot grow in a single culture medium and in fact many cannot grow in any known culture medium. Some

organisms cannot grow in artificial culture medium are known as **obligate parasites**. Examples: *Mycobacterium leprae, Rickettsias, Chlamydias, Treponema pallidum*.

Based on the consistency nature of the media, they are classified into solid, semis-solid and liquid. Solid media contains agar (1.5-2.0%) as solidifying agent and suitable for isolation of bacteria and determination of colony characteristics. Examples**:** Nutrient agar, BAP etc. Semi-solid media also contains agar but the percentage content is less (0.5%). This media is useful for determination of motility of bacteria and cultivation of microaerophilic bacteria. Liquid media that do not contains agar except for required amount of nutrients. This media is useful for propagation of large number of organisms, fermentation studies and various tests (Sugar fermentation test).

There are various types of culture media based on the applications as well as media compositions.



Flowchart 5: Various types of culture media

The importance of culture medium is to grow microorganisms outside the body for some important applications such as:

- To identify the cause of infection from the clinical samples.
- To study the characteristics and properties of microorganisms.
- To prepare the biological products like vaccines, antigens, toxoides etc.

Composition of Culture Medium:

- Energy source
- Carbon source
- Nitrogen source
- Water
- Specific growth factors
- Mineral salts

Synthetic medium: This medium is prepared from purified ingredients so that the whole composition of the medium is known. They are simple or complex, based on the supplements incorporated in the medium. A simple non-synthetic medium has all the nutrient requirements of organisms and some growth factors whereas complex non-synthetic medium does not contain purified or completely characterized or constant consistency between their batches and this medium supports the growth of more fastidious microorganisms (has a complex nutritional requirement).

Example: Nutrient broth is derived from cultures of yeasts.

1. **General purpose media/Basic media:** Basal media are simple media that supports most non-fastidious bacteria and are used for the isolation of primary microorganisms. Peptone water, nutrient broth and nutrient agar are considered as basal medium.
2. **Enriched medium:** Addition of extra nutrients in the basal medium in form of blood, serum, egg yolk etc. to make the medium enriched. They are used to grow nutritionally exacting bacteria. Examples: Blood agar, chocolate agar etc.
3. **Selective and enrichment media:** They are designed to inhibit unwanted contaminating bacteria and help to recover pathogen from a mixture of bacteria. Selective media are agar based and enrichment media are liquid in consistency. Addition of antibiotics, dyes, chemicals, alteration of pH etc. and makes a medium selective.

They are of various types like:

1. **Thayer Martin Agar:** It is used to recover *N. gonorrhoeae*; contains antibiotics; vancomycin, colistin and nystatin.
2. **Mannitol salt agar and salt milk agar:** They are used to recover *S. aureus*; contains 10% NaCl.
3. **Potassium tellurite:** This medium is used to recover *C. diphtheriae*; contains 0.04% potassium tellurite.
4. **Mac Conkey's Agar:** It is used for Enterobacteriaceae members contains bile salt that inhibits commonly gram positive bacteria.

5. **Pseudosel Agar or Cetrimide Agar:** It is used to recover *P. aeruginosa* contains cetrimide (antiseptic agent).
6. **Crystal Violet Blood Agar:** It is used to recover *S. pyogenes* contains 0.0002% crystal violet.
7. **Lowenstein Jensen:** This medium is used to recover *M. tuberculosis* is made selective by incorporating malachite green.
8. **Wilson and Blair's Agar:** It is used for recovering *S. typhi* is rendered selective by the addition of dye brilliant green.

Enrichment Culture Medium:

Enrichment medium, is liquid media, used to increase the relative concentration of certain microorganisms in the culture prior to plating on solid selective medium. It is mainly used as broth medium. Example**:** Selenite F broth, tetrathionate broth, alkaline peptone water are used to recover pathogens from fecal specimens.

Differential/Indicator Medium:

In this medium dyes, metabolic substrates etc. are added so that the bacteria that utilize them appear as differently colored colonies. Such media are called differential media or indicator media. Differential media allow the growth of more than one microorganism but with morphologically distinguishable colonies.

Examples:

1. **Mannitol salts agar** (mannitol fermentation = Gram positive bacteria *Staphylococcus aureus* produces yellow colonies with yellow zones.
2. **Blood agar** (various kinds of hemolysis i.e. α, β and γ hemolysis).
3. **Mac Conkey Agar** (lactose fermenters, pink colonies whereas non-lactose fermenter produces pale or colourless colonies.
4. **TCBS** (*Vibrio cholerae* produces yellow colonies due to fermentation of sucrose).

Transport media:

These media are used when specimen cannot be cultured soon after collection. This media prevent drying (desiccation) of specimen, maintain the pathogen to commensal ratio and inhibit overgrowth of unwanted bacteria. Example: Cary Blair medium, Amie's medium, Stuart's medium etc. Stuart's and Amie's media are semi-solid media in consistency. Addition of charcoal serves to neutralize inhibitory factors. Cary Blair transport medium is used to transport feces from suspected cholera patients. Pike's medium is used to transport streptococci from throat specimens.

Anaerobic media:

Anaerobic media is mainly supplemented with hemin and vitamin K where bacteria need low oxygen content, reduced oxidation–reduction potential and extra nutrients for their growth. The media is also contains 1% glucose, 0.1% thioglycollate, 0.1% ascorbic acid, 0.05% cysteine or red hot iron filings. Before use the medium is to be boiled in water bath to expel any dissolved oxygen and then sealed with sterile liquid paraffin. Example: Robertson Cooked Meat (RCM) medium is commonly used to grow *Clostridium* spps. contains a 2.5 cm column

of bullock heart meat and 15 ml of nutrient broth. Thioglycollate broth contains sodium thioglycollate, glucose, cystine, yeast extract and casein hydrolysate.

Assay Media:

These media is used for assay of amino acids, vitamins and other antibiotics. Antibiotic assay media are used for determination of antibiotic potency (by microbial assay).

1.8 RAW MATERIALS AND NUTRITIONAL REQUIREMENTS FOR CULTURE MEDIA

Raw materials play an important role in media preparation. Quality of media depends on the quality of raw materials. The most important raw materials are used for preparations of media are water, agar, peptone, casein hydrolysate, meat extract, yeast extract and malt extract.

1. **Water as solvent:** Water plays a vital role in preparation of media. Water should be free from copper ion because copper ion inhibits the growth of the microorganisms. Conductivity of water should be less than 15 µS (microsiemens) and the pH of the water should be slightly acidic but should not be less than 5.5.
2. **Petri dishes:** Petri dishes are generally sterilized with ethylene oxide (EtO) or gamma irradiated. If EtO sterilized they should be then checked for residual EtO toxicity which affect the growth of the microorganisms. The maximum permissible limit for residual EtO is 1 µg/g. Only borosilicate glassware should be used because soda glass can leach alkali into the media.
3. **Energy source:** The most common substance added to culture media is glucose that acts as source of energy and also increases the rate of growth of organisms. Other carbohydrates are also added to media at 5-10 g/lt as biochemical substrates to detect the production of specific enzymes in the identification of organisms.
4. **Nutrients:** The nutrients of culture media are selected to recover the required spectrum of organisms in the sample e.g. coliforms or anaerobes. Proper nutrients help for bacterial growth.
5. **Essential Metals and Minerals:** The inorganic essential components of culture media are divided on a semi-quantitative basis**:** Macro-components (gm/litre): Na, K, Cl, P, S, Ca, Mg, Fe. Micro-components (mgm-microgm/litre): Zn, Mn, Br, B, Cu, Co, Mo, V, Sr, etc.
6. **Buffering Agents:** The pH of a culture medium is poised around the optimum necessary for growth of the desired micro-organisms. The use of buffer compounds at specific pK values is especially necessary when fermentable carbohydrates are added as energy sources. Such examples of buffering agents are phosphates, acetates, citrates, zwitterion compounds and specific amino-acids that added to culture media.
7. **Indicator Substances:** The addition of coloured indicator substances in the medium is very effective of detecting fermentation of specific carbohydrates. They change colour distinctly and rapidly at critical pH values. Examples: phenol red, bromo-cresol

purple, fuchsin, etc., are toxic and it is essential to use in low concentrations. Known sensitive strains of micro-organisms are also used in the screening tests.

8. **Selective Agents:** Chemicals or antimicrobials are added to culture media to make them selective for certain micro-organisms. They are added at specific concentrations to suppress the growth of unwanted organisms in a polymicrobial sample. Examples: bile salts, dye-stuffs, selenite, tetrathionate, tellurite and azide. Thereafter antimicrobial agents are used in mixtures when suppressing polymicrobial contaminating flora. Antimicrobials are more specific in their selective action.
9. **Gelling Agents:** Gelatin is still used for a few specific media and carrageenans, alginates, silica gel and polyacrylamides are sometimes used as gelling agents but the most important gel-forming substance used in culture media is agar. It is inert to microbial action and melting temperatures (38°C and 84°C respectively) the high gel strength which allows low concentrations of agar to be used. It has low toxicity and also not hydrolyzed by agarase enzyme in high temperature. Microbiological agar is specially processed to yield a low toxicity, high clarity, low mineral and high diffusion gel.
10. **Other Components:** Many other substances are added to culture media for specific purposes like growth factors for fastidious organisms, pH-reducing compounds for anaerobic organisms (thioglycollate and cysteine), whole blood to detect haemolytic enzymes etc.
11. **Meat extract:** It is obtained by hot water extraction of lean beef and then concentrated by evaporation. It contains gelatin, albumoses, peptrones, proteoses, amino acids, creatinine, purines, and accessory growth factors.
12. **Yeast extract:** It is prepared from washed cells of bakers' yeast and contains wide range of amino acids, growth factors and inorganic salts.
13. **Malt extract:** It is prepared by extracting soluble materials from sprouted barley in water at 55^{o}C and concentrated by evaporation. It contains maltose, starch, dextrin, glucose and small amounts of protein and protein breakdown products and growth factors.

1.8.1 Nutrition required for Bacterial Culture Medium

Most of the microorganisms are grown in specific culture media in laboratory conditions and for proper growth nutrition plays an important role for structural and functional properties of the cells. Nutrition are generally composed of:

- Major macronutrients: C, H, O, N, S, P, K, Mg, Fe, Ca and Mn.
- Major micronutrients: Zn, Co, Cu, and Mo.
- Carbon and energy sources.
- Growth factors.
- Vitamins.

Major Macronutrients:

- **Carbon:** The main source is organic compounds and CO_2. It is the main component of cellular materials.

- **Hydrogen:** The main source is organic compounds. It is the main component of cell water.
- **Oxygen:** The main source is organic compounds, CO_2 and O_2. It is the main constituent of cell material and cell water. It is electron acceptor in aerobic respiration.
- **Nitrogen:** The main source is organic compounds, NH_3, NO_3, N_2 etc. It is the main constituent of amino acids, nucleic acids nucleotides and coenzymes.
- **Sulfur:** The main source is organic sulfur compounds. It is the main constituent of some amino acids like cysteine, methionine, glutathione and several coenzymes.
- **Phosphorus:** The main source is inorganic phosphates. It is the main component of nucleic acids, nucleotides, phospholipids etc.
- **Potassium:** The main source is potassium salt. It is the main component of cellular inorganic cation and cofactor for certain enzymes.
- **Magnesium:** The main source is magnesium salt. It is the main component of inorganic cellular cation, cofactor for certain enzymatic reactions.
- **Iron:** The main source is iron salt. It is the main component of cytochromes and certain non-heme iron-proteins and a cofactor for some enzymatic reactions.
- **Calcium:** The main source is calcium salt. It is the main component of inorganic cellular cation, cofactor for certain enzymes and a component of endospores.
- **Manganese:** The main source is manganese salt. It is the main component of inorganic cellular cation, cofactor for certain enzymes.

Major Micronutrients:

They are also known as trace elements because they require very small amount. They usually act as cofactors for essential enzymatic reactions in the cell. They are cations requires for bacterial nutrition. Examples: Mn, Co, Zn, Cu, and Mo.

Zn^{2+} is present at the active site of some enzymes but is also involved in the association of regulatory and catalytic subunits in *E. coli aspartate* carbomoyl transferase. Mn^{2+} aids many enzymes catalyzing the transfer of phosphate groups. Mo^{2+} is required for nitrogen fixation and Co^{2+} is a component of Vitamin B_{12}. Cu^{2+} is an important micronutrient required as a redox co-factor of multiple proteins.

Carbon and Energy Sources for Bacterial Growth:

A source of carbon as energy source and other nutrients as growth parameters are required in a permissive range of physical conditions viz. O_2 concentration, temperature, and pH. Sometimes bacteria are referred to as individuals or groups based on their patterns of growth under various chemical or physical conditions. For example, phototrophs are organisms that use light as an energy source; anaerobes are organisms that grow without oxygen; thermophiles are organisms that grow at high temperatures.

All living organisms require energy source. Organisms that use light energy is called phototrophs, when organisms use an organic form of carbon are called heterotrophs and when organisms that oxidize inorganic compounds are called lithotrophs. The source of carbon is either organic carbon or CO_2. Organisms that use organic carbon are heterotrophs and organisms that use CO_2 as a sole source of carbon are known as autotrophs (Table 1.5).

Table 1.5: Prokaryotes as per nutritional requirement

Nutritional Type	Energy Source	Carbon Source	Examples
Photoautotrophs	Light	CO_2	Cyanobacteria, some Purple and Green Bacteria
Photoheterotrophs	Light	Organic compounds	Some Purple and Green Bacteria
Chemoautotrophs or Lithotrophs	Inorganic compounds, e.g. H_2, NH_3, NO_2, H_2S	CO_2	A few Bacteria and many Archaea
Chemoheterotrophs or Heterotrophs	Organic compounds	Organic compounds	Most Bacteria, some Archaea

1.9 GROWTH FACTORS

All the bacteria require small amounts of organic compounds for growth because they are essential substances that the organism is unable to synthesize from available nutrients. Such compounds are called growth factors. These compounds are added in prior to preparation of culture media. They are required in small quantities by cells to fulfill specific roles in biosynthesis and metabolism. They are organized into three categories.

1. **Purines and pyrimidines:** They are required for synthesis of nucleic acids (DNA and RNA).
2. **Amino acids:** They are required for the synthesis of proteins.
3. **Vitamins:** They are needed as coenzymes and functional groups of certain enzymes.

Some bacteria (e.g. *E. coli*) do not require any growth factors they can synthesize all essential purines, pyrimidines, amino acids and vitamins, starting with their carbon source, as part of their own intermediary metabolism. Certain other bacteria (e.g. *Lactobacillus*) require purines, pyrimidines, vitamins and several amino acids for their growth. Mutant strains of bacteria that require some growth factor not needed by the wild type (parent) strain are referred to as **auxotrophs**. Some vitamins that are frequently required by certain bacteria as growth factors are tabulated in Table 1.6.

Table 1.6: Vitamins required in the nutrition

Vitamin	Coenzyme form	Function
p-Aminobenzoic acid (PABA)	-	Precursor for the biosynthesis of folic acid
Folic acid	Tetrahydrofolate	Transfer of one-carbon units and required for synthesis of thymine, purine bases, serine, methionine and pantothenate
Biotin	Biotin	Biosynthetic reactions that require CO_2 fixation

Vitamin	Coenzyme form	Function
Lipoic acid	Lipoamide	Transfer of acyl groups in oxidation of keto acids
Mercaptoethane-sulfonic acid	Coenzyme M	CH_4 production by methanogens
Nicotinic acid	NAD (nicotinamide adenine dinucleotide) and NADP	Electron carrier in dehydrogenation reactions
Pantothenic acid	Coenzyme A and the Acyl Carrier Protein (ACP)	Oxidation of keto acids and acyl group carriers in metabolism
Pyridoxine (B_6)	Pyridoxal phosphate	Transamination, deamination, decarboxylation and racemation of amino acids
Riboflavin (B_2)	FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide)	Oxidoreduction reactions
Thiamine (B_1)	Thiamine pyrophosphate (TPP)	Decarboxylation of keto acids and transaminase reactions
Vitamin B_{12}	Cobalamine coupled to adenine nucleoside	Transfer of methyl groups
Vitamin K	Quinones and napthoquinones	Electron transport processes

1.9.1 Physical Factors Affecting Bacterial Growth

Some of the important factors affecting bacterial growth are:

1. Nutrition concentration
2. Temperature
3. Gaseous concentration
4. pH
5. Ions and salt concentration
6. Water

1. Nutrient concentration:

- Bacterial growth is directly proportional with the available nutrients. If culture media is rich in nutrients, growth of bacteria occurs faster and vice versa.
- Nutritional requirement varies with the bacterial species.

The relationship between substrate concentration (nutrition) and growth rate is shown in Fig. 1.21.

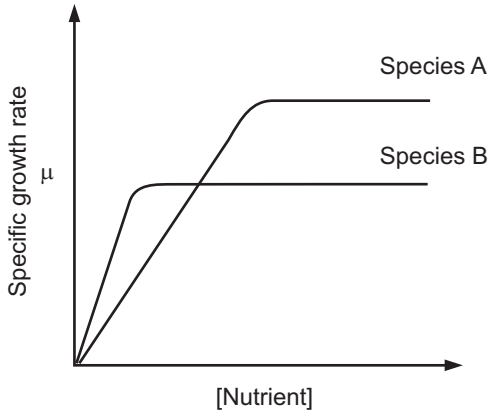


Fig. 1.21: Nutrient vs growth rate

- With increase in concentration of nutrients, growth rate of bacteria increases up to certain level and then growth rate remains constant irrespective of nutrition addition.

2. Temperature:

- The lowest temperature that allows the growth is called minimum temperature and the highest temperature that allows growth is called maximum temperature.
- There is no growth below minimum and above maximum temperature.
- Below minimum temperature cell membrane solidifies and become stiff to transport nutrients in to the cell, hence no growth occurs.
- Cellular proteins and enzymes denatures at maximum temperature, so the bacterial growth ceases.

The relationship between temperature and growth rate is shown in Fig. 1.22.

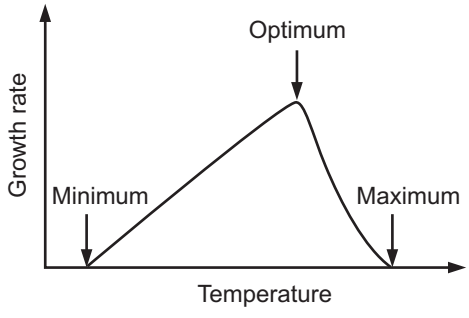


Fig. 1.22: Temperature vs growth rate

- Growth rate of bacteria increases because the rate of metabolic reaction increases with increase in temperature.
- At certain temperature the growth rate become maximum, this temperature is known as optimal temperature.
- On further increasing the temperature above optimal, growth rate decreases drastically and completely ceases with reaching maximum temperature.

Thermophile organisms are heat-loving bacteria. Their optimum growth temperature is between 45°C and 70°C and are commonly found in hot springs and in compost heaps.

Hyperthermophile bacteria grow at very high temperatures. Their optimum growth temperature is between 70°C and 110°C. They are usually members of the Archaea. Psychrophiles are grown between −5°C to 20°C. Mesophiles are grown between 20°C to 45°C.

3. pH:

- pH affects the ionic properties of bacterial cell so it affects the growth of bacteria.
- Most of the bacteria grow at neutral pH (6.5-7.5). However, there are certain bacteria that grow best at acidic or basic pH.

The relationship between pH and bacterial growth is given in Fig. 1.24.

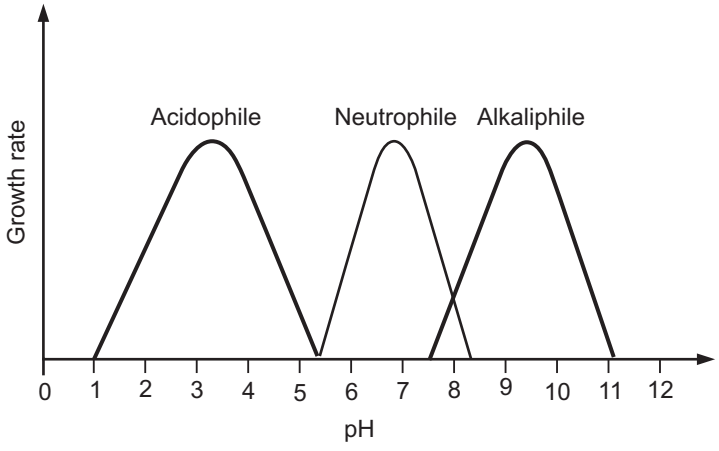


Fig. 1.23: pH vs growth rate

Microorganisms can be placed in one of the following groups based on their optimum pH requirements:

1. Neutrophiles grow best at a pH range of 5 to 8.
2. Acidophiles grow best at a pH below 5.5.
3. Alkaliphiles grow best at a pH above 8.5.

4. Ions and salt:

- All bacteria requires metal ions such as K^{+}, Ca^{++}, Mg^{++}, Fe^{++}, Zn^{++}, Cu^{++}, Mn^{++} etc. to synthesize enzymes and proteins.
- Most bacteria do not require NaCl in media and they can tolerate very low concentration of salt.
- Some halophilic bacteria such as *Archeobacteria* require high concentration of salt in media.
- It is balanced with Osmosis process.

Osmosis is the diffusion of water across a membrane from an area of higher water concentration (lower solute concentration) to lower water concentration (higher solute concentration) without requirement of metabolic energy. While water molecules are small enough to pass between the phospholipids in the cytoplasmic membrane, their transport can be enhanced by water transporting transport proteins known as aquaporins. Based on the

conditions, a cell has three environments such as isotonic, hypertonic and hypotonic. In an isotonic environment both the water and solute concentration are the same inside and outside the cell and water goes into and out of the cell at an equal rate.

With hypertonic environment, the water concentration is greater inside the cell while the solute concentration is higher outside whereas with hypotonic condition, the water concentration is greater outside the cell and the solute concentration is higher inside hence water goes into the cell.

Most bacteria require an isotonic environment or a hypotonic environment for optimum growth.

5. Gaseous requirement:

- Oxygen and carbon-dioxide are important gases that affect the growth of bacteria.
- Oxygen is required for aerobic respiration and obligate aerobic bacteria must require O_2 for growth. For example: *Mycobacterium, Bacillus*.
- For obligate anaerobes oxygen is harmful or sometime lethal but facultative anaerobes can tolerate low concentration of O_2.
- Carbon-dioxide is needed for capnophilic bacteria. Such as *Campylobacter, Helicobacter pylori*.

6. Water:

- Water is the most essential factor for bacterial growth.
- Available water in the culture media determines the rate of metabolic and physiological activities of bacteria.
- Sugar, salts and other substances are dissolved in water and are made available for bacteria.

Preparation of Nutrient Agar:

1. 28 g of nutrient agar powder is suspended in 1 litre of distilled water.
2. Heated the mixture while stirring to fully dissolve all components.
3. Autoclaved the dissolved mixture at 121°C for 15 minutes.
4. Allowed it to cool but not solidify.
5. Poured nutrient agar into each plate and left the plates on the sterile surface until the agar has solidified.
6. Stored the plates in a refrigerator.

Uses of Nutrients Agar

1. It is frequently used for isolation and purification of cultures.
2. It is also used for producing the bacterial lawns needed for antibiotic sensitivity tests.

1.9.2 Bacterial Growth Curve

Bacterial growth curve has four distinct phases viz. Lag phase, Log phase, Stationary phase and Decline phase (Fig. 1.25).

(a) Lag Phase: The bacteria first undergoes a period of acclimatization when they are inoculated into the sterile nutrient medium. During that time, necessary enzymes and intermediate metabolites are synthesized and before multiplication the bacterium

reaches a critical stage and finally multiplication takes place at this stage. Hence, this stage is also known as initial stage or dormant stage. The duration of this phase depends on the type of bacteria, quality of culture medium, size of inoculum and some environmental factors like CO_2, temperature, pH, etc. The average time of lag phase is 2 hours but it varies from species to species and ranges between 1-4 hours.

(b) Log Phase: After the Lag phase the next phase is Log phase. In this phase, the bacteria undergo cell division and their population increase exponentially at a logarithmic rate. Hence this phase is also known as exponential phase. The number of viable count, when plotted against time it gives a straight line of inclined fashion. The average time of log phase is 8 hours but also varies with the bacterial species.

(c) Stationary Phase: In this phase, the cell division of bacteria is almost ceases due to exhaustion of nutrients and also the accumulation of toxic products. Secondary metabolites like antibiotics are also produced in this phase. The cell death starts at a slow rate and is compensated by the formation of new cell through cell division. The total cell number increases at a slow rate, but the viable count remains almost constant. The duration of this phase is varies from few days to few hours.

(d) Decline Phase: This is the last stage of bacterial growth cycle. In the phase of decline, the total number of cells remains constant, but the number of viable cells gradually decreases due to exhaustion of nutrients and also the accumulation of toxic products. In some cases, a few cells remain viable for long time. These viable cells probably grow by utilizing nutrients released from dead cells. The cells attain maximum size at the end of lag phase and become smaller in log phase.

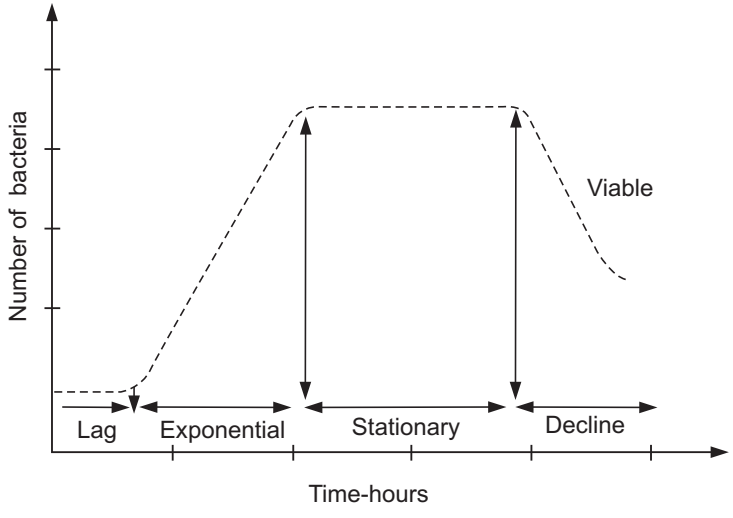


Fig. 1.24: Growth curve of Bacteria

1.10 ISOLATION METHODS FOR PURE CULTURES

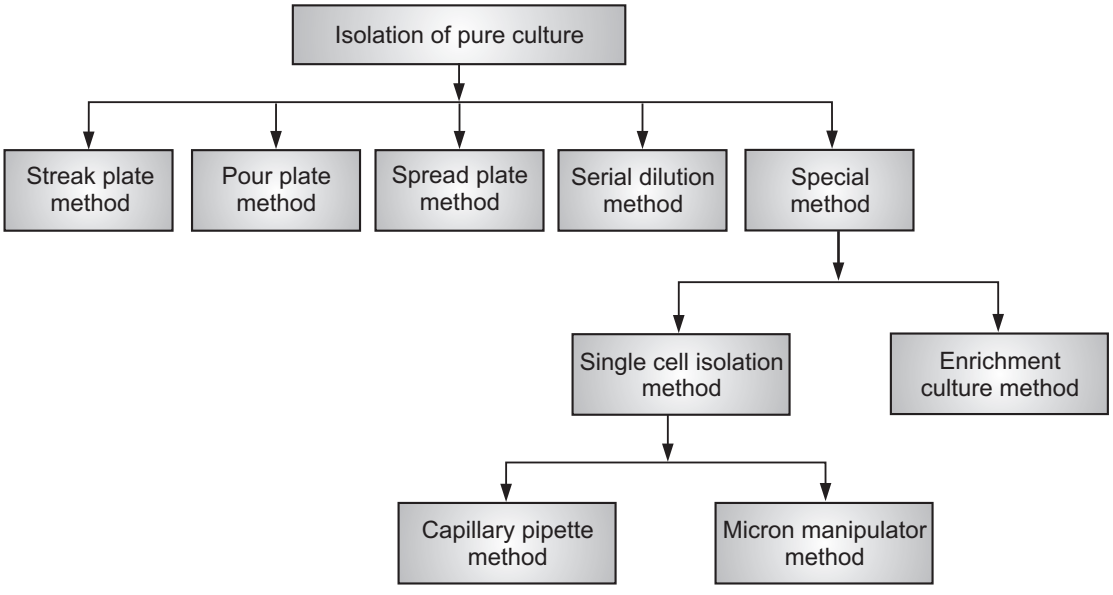
A pure culture is usually derived from a mixed culture by transferring a small sample into new, sterile growth medium in such a manner as to disperse the individual cells across the medium surface or by thinning the sample several times before inoculating the new medium. Hence, pure culture is defined as a laboratory culture containing a single species of organism.

Importance of Pure Culture:

- Desired known species of microorganisms can grow once pure culture is isolated.
- Pure culture can be correctly identified for accurate study and testing of microorganism in a clinical environment.
- Experimenting with pure culture ensures the same results after repetition of experiment many times.
- In pure culture, the spontaneous mutation rate is low.

Hence, pure culture is required to isolate which is carried out by:

1. Streak plate method
2. Pour plate method
3. Spread plate method
4. Serial dilution method
5. Special methods:
   (a) Single Cell Isolation Methods
   (b) Enrichment Culture Method.



Flowchart 6: Types of isolation of pure culture

1. Streak Plate Method: The principle of this method is that, by streaking, a dilution gradient is established across the face of the Petri plate where bacterial cells are deposited on the agar surface. This method is used most commonly to isolate pure cultures of bacteria where a small amount of mixed culture is placed on the tip of an inoculation loop and is streaked across the surface of the agar medium by different fashions (Fig. 1.25) and the plates are incubated to allow the growth of colonies and each colony is the progeny of a single microbial cell thus representing a clone of pure culture. Finally, the micro-organisms

are separated from each other. The isolated colonies are picked up separately using sterile inoculating loop and re-streaked onto fresh media to ensure purity.

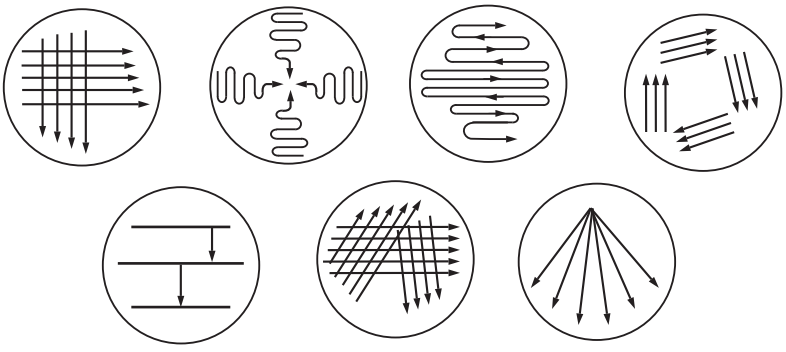


Fig. 1.25: Different types of streak plates

2. Pour Plate Method: The main principle of this method is dilution of the inoculum in successive tubes containing liquefied agar medium so as to permit a thorough distribution of bacterial cells within the medium.

In this method, the mixed culture of bacteria is diluted directly in the liquid agar medium tube (42-45°C) and mixed well. In this method, fixed amount of inoculum (generally 1 ml) from a broth/sample is placed in the center of sterile petri dish using a sterile pipette. The contents of each tube are poured into separate petri plates and then allowed to solidify. These plates are the incubated to develop bacterial colonies in both within the agar medium (subsurface colonies) and on the medium (surface colonies). Finally, isolated the colonies and are picked up by inoculation loop and streaked onto another petri plate to insure purity (Fig. 1.26). This method is also used for determining the number of viable bacterial cells present in a culture.

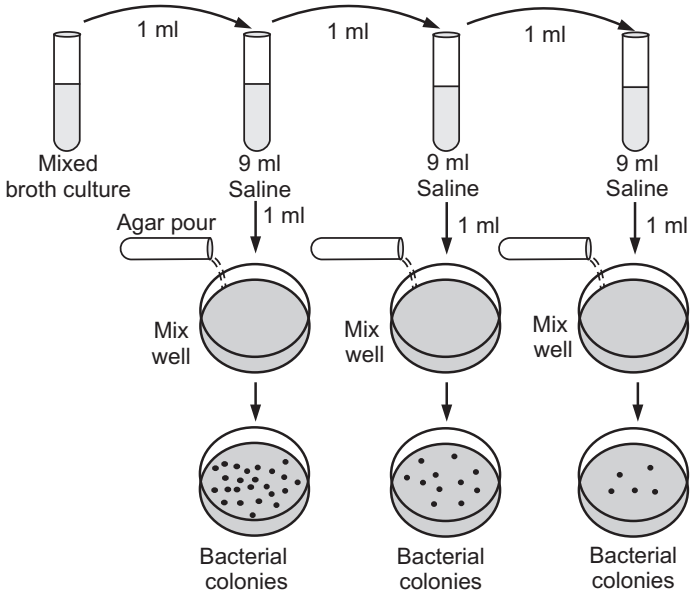


Fig. 1.26: Pour plate method

3. Spread Plate Method: The principle of this method involves using a sterilized spreader with a smooth surface made of metal or glass to apply a small amount of bacteria suspended in a solution over a plate that needs to be dry and at room temperature so that the agar can absorb the bacteria more readily. A successful spread plate will have a countable number of isolated bacterial colonies evenly distributed on the plate.

The technique is easier to quantify bacteria in a solution. A drop of diluted liquid from each tube is placed on the center of an agar plate and spread evenly over the surface by means of a sterilized bent-glass-rod. The medium is incubated for the colonies development on the agar medium plates. The isolated colonies are picked up and transferred onto fresh medium to ensure purity (Fig. 1.27).

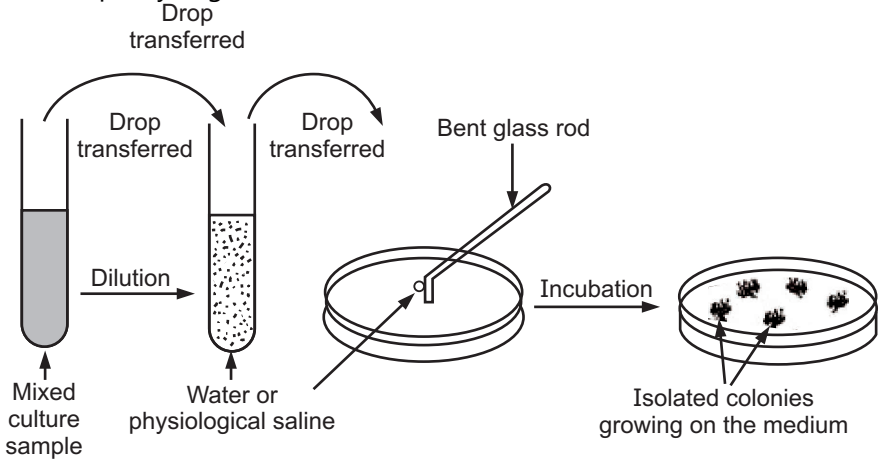


Fig. 1.27: Spread plate technique

Some useful differences between pour plate and spread plate methods

Parameters	Pour plate method	Spread plate method
Procedure	Inoculum from a sample is placed in the center of sterile petri dish using a sterile pipette. Molten cooled agar (approx. 15 ml) is then poured into the petri dish containing the inoculum.	Inoculum from a sample is pipette out and spread evenly on sterile nutrient agar by spreader.
Amount	Inoculum is 1 ml.	Inoculum is 0.1 ml.
Medium	Colonies are grown in solid culture medium.	Colonies are grown in solid culture medium.
Sample Application	More surface area is covered as the sample is spread throughout the media.	Sample is spread only to a limited area i.e. only on the surface of agar.
Uses	It is used to determine CFU/ml or PFU/ml.	It is used to isolate specific clonal colonies.
Advantages	1. It allows the growth of microaerophiles. 2. It helps in identification of various bacteria, like an aerobe, anaerobe or facultative aerobe bacteria.	1. Picking surface colony will not interrupt other colonies by digging out of agar. 2. Get even distribution of colonies.

Parameters	Pour plate method	Spread plate method
Disadvantages	1. Picking subsurface colonies can interrupt other colonies by digging out of agar. 2. Do not get even distribution of colonies.	1. It does not allow growth of microaerophiles. 2. It does not allow growth of obligate anaerobes.
Benefit	• It is beneficial to isolate certain bacteria which are motile and do not grow as colony.	• It is beneficial for isolation of bacteria from soil or water.
Application	• This technique is used in Bacteriologic examination of milk, bacterial number of urine to signify urinary tract infection.	• This technique is used in enrichment, selection and screening experiment.

4. Serial Dilution Method: A microorganism that predominates in a mixed culture can be isolated in pure form by a series of dilutions. The inoculum is subjected to serial dilution in a sterile liquid medium, and a large number of tubes of sterile liquid medium are inoculated with aliquots of each successive dilution.

The aim of this dilution is to inoculate a series of tubes with a microbial suspension so dilute that there are some tubes showing growth of only one individual microbe. Example: A culture containing 10 ml of liquid medium, containing 1,000 microorganisms i.e., 100 microorganisms/ml of the liquid medium. 1 ml of this medium is taken out and mixed with 9 ml of fresh sterile liquid medium, then resulted medium have 100 microorganisms in 10 ml or 10 microorganisms/ml. Further 1 ml of this suspension is added to another 9 ml of fresh sterile liquid medium, each ml is now contained a single microorganism. If this tube showed any microbial growth, there is a very high probability that this growth has resulted from the introduction of a single microorganism in the medium and represents the pure culture of that microorganism (Fig. 1.28).

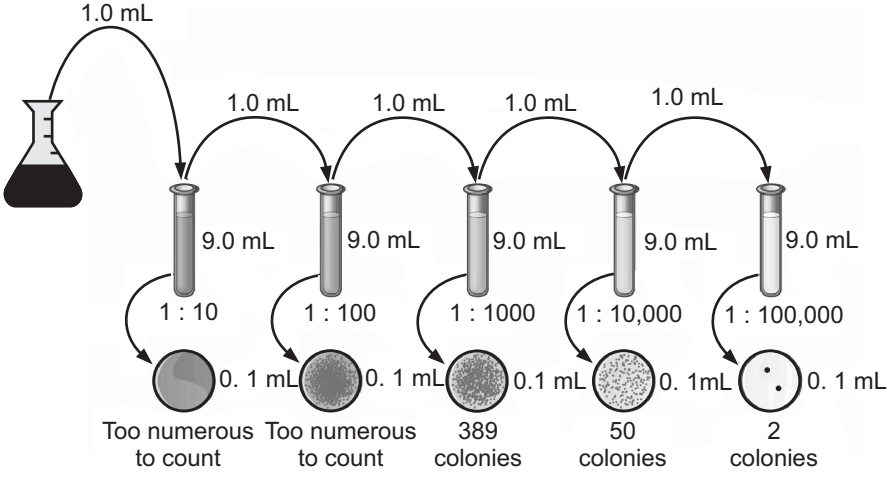


Fig. 1.28: Serial dilution method

5. Special Methods

1. Single Cell Isolation methods

An individual cell is picked out by this method from the mixed culture and is allowed to grow to procure pure culture. There are two methods used for the isolation of single cell:

(i) Capillary pipette method: Several small drops of a suitably diluted culture medium are placed on a sterile glass-coverslip by a sterile pipette. Examine then each drop under the microscope until find one microorganism in one drop. This drop is then removed with a sterile capillary pipette to fresh medium for multiplication to yield a pure culture (Fig. 1.29).

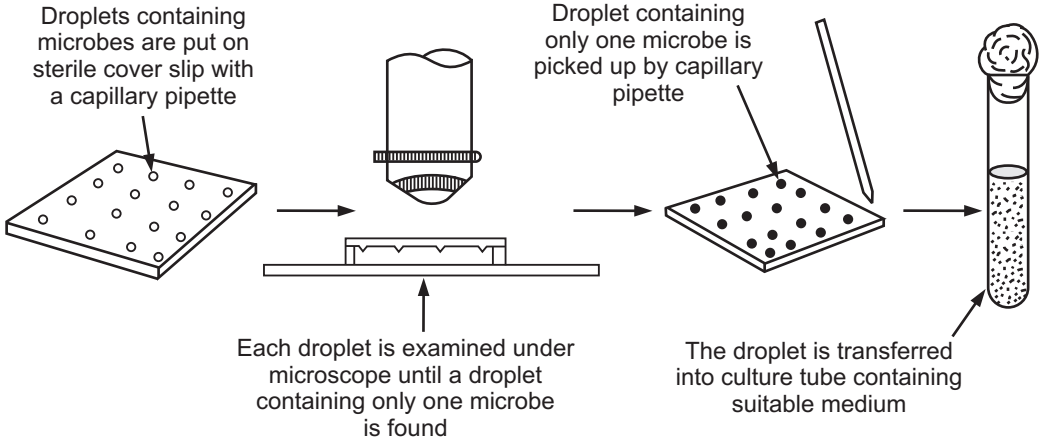


Fig. 1.29: Capillary pipette method

(ii) Micromanipulator method: Micromanipulators permit one to pick out a single cell from a mixed culture. This instrument is used in conjunction with a microscope to pick a single cell (particularly bacterial cell) from a hanging drop preparation. The micro-manipulator has micrometer adjustments with micropipette that can move in all directions. A series of hanging drops of a diluted culture are placed on a special sterile coverslip by a micropipette. Hanging drop is searched for a single microorganism cell. This cell is drawn into the micropipette by gentle suction and then transferred to a large drop of sterile medium on another sterile coverslip. When the number of cells increases in that drop, the drop is transferred to a culture tube having suitable medium which yields a pure culture of the required microorganism.

Advantage: It can be reasonably sure that the cultures come from a single cell. It can obtain strains within the species.

Disadvantage: The equipment is expensive and it requires a skilled operator.

2. Enrichment Culture Method:

This method is used to isolate those microorganisms that are present in relatively small numbers or have slow growth rates in the mixed culture. The enrichment culture strategy provides a specially designed cultural environment by incorporating a specific nutrient in the medium and by modifying the physical conditions of the incubation. The medium of known composition and specific condition of incubation favors the growth of desired microorganisms. This method is unsuitable for the growth of other types of microorganisms.

1.11 PRESERVATION METHODS FOR PURE CULTURES

Preservation is a process to maintain pure culture for extended periods in a viable condition without any genetic changes. Once a pure culture has been obtained from the mixture, the microbes may be maintained in the laboratory over long periods. The most important factor of preservation is to stop microbial growth or at least suppress the growth rate.

Objectives of Preservation:

- To maintain isolated pure cultures for longer periods in a viable conditions.
- To avoid contamination.
- To restrict genetic changes.

Several techniques are used to preserve cultures viz. period transfer in fresh medium or Subculturing, Storage in sterile soil, Saline suspension, Refrigeration, Paraffin Method, Cryopreservation, Lyophilization.

1. **Subculturing:** Strains are maintained by periodically preparing a fresh culture from the previous stock culture. Culture medium, storage temperature and the time interval at which transfers are made vary with the species. Nutrient agar medium is commonly used where most of the heterotrophs remain viable for several months.
2. **Storage in sterile soil:** The soil is prepared by mixing enough sand with a rich garden soil that makes friable and easy to handle. A small amount of calcium carbonate may be added and the mixture is transferred to screw capped tubes or tubes plugged with cotton. The tubes are autoclaved until complete sterility. This soil is widely used in preservation cultures that form "spores". To this thick suspension of spores is added to the sterile soil. Moisture is removed by placing the cap and maintaining reduced pressure over a drying agent.
3. **Saline suspension:** Sodium chloride in high concentration is frequently an inhibitor of bacterial growth. Bacteria are suspended in 1% salt solution. The tubes are stored at room temperature. Whenever needed the transfer is made on agar slant.
4. **Refrigeration:** Pure cultures are also stored at 0-4°C either in refrigerators or in cold-rooms. This method is applied for short duration (2-3 weeks for bacteria and 3-4 months for fungi) because the metabolic activities of the microorganisms are greatly slowed down but not stopped.
5. **Paraffin method:** This is a simple and most economical method of maintaining pure cultures of bacteria and fungi. In this method, sterile liquid paraffin in poured over the slant (slope) of culture and stored above at room temperature. The layer of paraffin ensures anaerobic conditions and prevents dehydration of the medium. This condition helps microorganisms or pure culture to remain in a dormant state. Hence, the culture is preserved for several years. This method is also known as **Agar Slant Culture Covered with Oil**.
6. **Cryopreservation:** Cryopreservation (i.e., freezing in liquid nitrogen at −196°C) helps in survival of pure cultures for long storage times. In this method, the

microorganisms of culture are rapidly frozen in liquid nitrogen at −196°C in the presence of stabilizing agents (glycerol). It prevents the formation of ice crystals and promotes cell survival.

7. **Lyophilization:** It is also known as Freeze-Drying. It is the method of removal of frozen solvent by sublimation under vacuum and unfrozen solvent by desorption. In this method, the culture is rapidly frozen at a very low temperature (−70°C) and then dehydrated by vacuum. Under these conditions, the microbial cells are dehydrated and their metabolic activities are stopped; as a result, the microbes go into dormant state and retain viability for years. Lyophilized or freeze-dried pure cultures and then sealed and stored in the dark at 4°C in refrigerators.

Cultivation of Anaerobes:

Anaerobic microorganisms are widely spread in environment. They do not require oxygen for their growth. Sometimes these organisms are very toxic. There are two types of anaerobes namely, facultative anaerobes, obligate anaerobes and aerotolerant anaerobes.

- **Facultative anaerobes:** They are the organisms that can grow in presence or absence of oxygen. They obtain energy by both respiration as well as fermentation.

  Some examples of facultative anaerobic bacteria are *Staphylococcus spp*. *Streptococcus spp.*, *Escherichia coli*, *Salmonella*, *Listeria spp.* and *Shewanella oneidensis*.
- **Obligate anaerobes:** They are the organisms that can grow totally in absence of oxygen. Oxygen is toxic for their normal growth. Examples: Actinomyces, Bacteroides, Clostridium, Fusobacterium, Peptostreptococcus, Porphyromonas, Prevotella, Propionibacterium, Veillonella etc.
- **Aerotolerant anaerobes:** They are the organisms that cannot use oxygen for growth but are able to tolerate its presence. They have superoxide dismutase and peroxidase but do not have catalase. Examples: *Streptococcus*, *Clostridium*, and *Actinomyces*.

Principle:

Reduce the O_2 content of culture medium and remove any oxygen already present inside the system or in the medium. Oxygen is ubiquitous in the air so special methods are used to culture anaerobes. Four types of culture media are used likely:

1. Special Anaerobic Culture Media
2. Anaerobic Chamber
3. Anaerobic Bags or Pouches
4. Anaerobic Jars.

1. Special Anaerobic Culture Media: This media is also known as pre-reduced media. During preparation of this media the liquid culture medium is boiled by holding in boiling water bath for 10 minutes to remove the dissolved oxygen. Liquid media soon become aerobic so that to keep media anaerobic, reducing agents such as cysteine 0.1%, ascorbic acid 0.1%, sodium thioglycollate 0.1% are added to lower the oxygen content.

Oxygen-free N_2 is bubbled through the medium to maintain anaerobic condition. The medium is then dispensed into tight stoppered tubes and autoclaved for sterilization. Such

tubes are stored for many months before being used. During inoculation, the tubes are continuously flushed with oxygen free CO_2 with gas cannula, re-stoppered and incubated (Fig. 1.30).

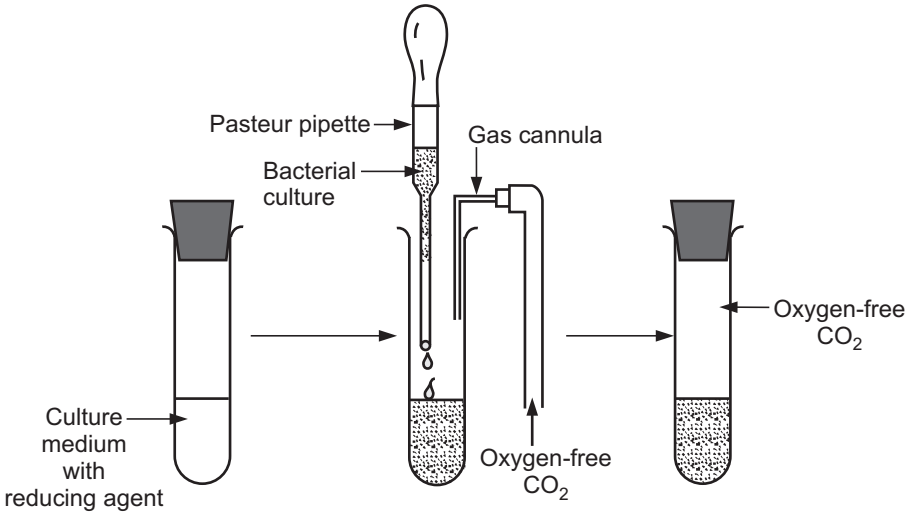


Fig. 1.30: Pre-reduced culture media

The inoculum of the bacterium is inserted deep in the medium in contact with the meat. Meat particles are placed in 30 ml bottles to a depth of about 2.5 cm and covered with about 15 ml broth.

Cooked meat broth is suitable for growing anaerobic bacteria in air and also for the preservation of their stock cultures which is also known as **'Robertson's bullock-heart medium'**. Thioglycollate broth is also useful media for anaerobes. Some other media such as Brucella blood agar, bacteroides, bile, aesculin agar, phenylethyl alcohol agar, kanamycin blood agar, etc. are used for recovering anaerobes. Vitamin K, haemin and yeast extract are used as essential nutrition for all the anaerobes media.

2. Anaerobic Chamber: Anaerobic chamber is an ideal anaerobic incubation system, which provides anaerobic environment for inoculation of media and incubation of cultures. The chamber is made up of a plastic anaerobic glove box that contains an atmosphere of H_2, CO_2, and N_2. Glove ports and rubber gloves are used during experimentation to perform manipulations within the chamber. There is an air-lock with inner and outer doors. Culture media are placed within the air-lock with the inner door. Air of the chamber is removed by a vacuum pump connection and replaced with N_2 through outer doors. The culture media are then transferred from air-lock to the main chamber containing H_2, CO_2, and N_2. A circulator fitted in the main chamber circulates the gas atmosphere through pellets of palladium catalyst causing any residual O_2 present in the culture media to be used up by reaction with H_2. The existence function of CO_2 in the chamber is because of many anaerobic bacteria for their optimum growth. Hence, when the culture media becomes completely anaerobic, they are inoculated with bacterial culture and are placed in an incubator in the chamber (Fig. 1.31).

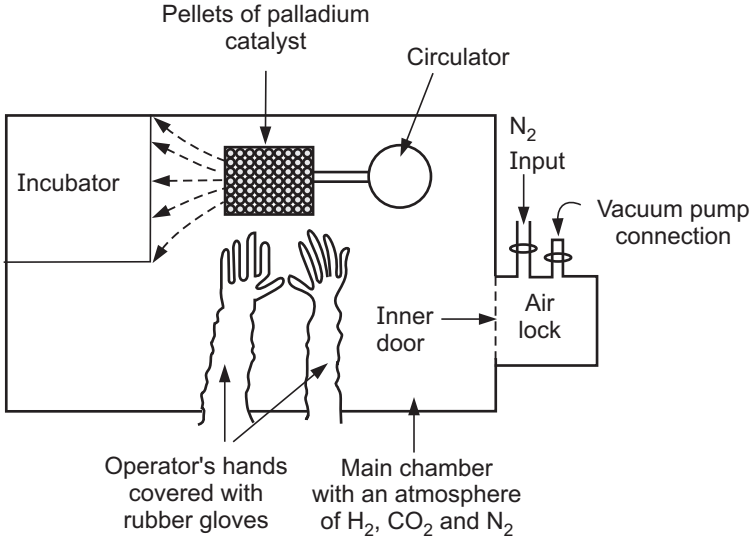


Fig. 1.31: Anaerobic chamber

3. Anaerobic Bags or Pouches: Anaerobic bags or pouches make convenient containers when only a few samples are to be incubated anaerobically. They are available commercially. Bags or pouches have an oxygen removal system consisting of a catalyst and calcium carbonate to produce CO_2-rich atmosphere.

One or two inoculated plates are placed into the bag and the oxygen removal system is activated and the bag is sealed and incubated. Plates are examined for growth without removing the plates from bag. These bags are also useful in transport of biopsy specimen for anaerobic cultures.

4. Anaerobic Jars (or GasPak Anaerobic System):

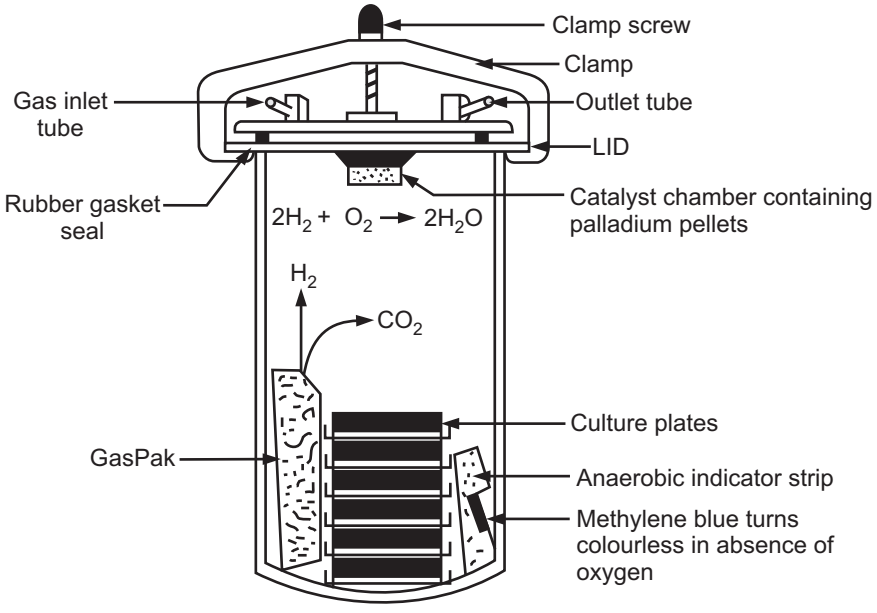


Fig. 1.32: Anaerobic Jar

These anaerobic jars are required for obtaining surface growth of anaerobic bacteria in anaerobic condition. The most reliable and widely used anaerobic jar is the Melntosh-Fildes' anaerobic jar. It is a cylindrical vessel made of glass or metal with a metal lid, which is fitted by a clamp. The lid possesses two tubes with taps, one acting as gas inlet and the other as the outlet. On it's under-surface it carries a gauze sachet carrying palladium pellets, which acts as a room temperature catalyst for the conversion of hydrogen and oxygen into water. Palladium pellets acts as catalyst (Fig. 1.32).

Inoculated culture plates are placed inside the jar and the lid clamped tightly. The outlet tube is connected to a vacuum pump and the air inside is evacuated. The outlet tap is then closed and the gas inlet tube connected to a hydrogen supply from where hydrogen is drawn rapidly. After 5 minutes inlet tube is further opened and the catalyst creates a reduced pressure within the jar due to the conversion of hydrogen and leftover oxygen into water. The jar is left connected to the hydrogen supply for about 5 minutes and then the inlet tube is closed and the jar is placed in the incubator.

The gasPak is now the method of choice for preparing anaerobic jar. The gasPak is commercially available as a disposable envelope containing chemicals that generate hydrogen and carbon dioxide when water is added. After the inoculated plates are kept in the jar, the gasPak envelope with water is added and is placed inside and the lid screwed tight. The culture plates should be removed from the jar and placed in the oxygen-free holding system. From there culture plates should be removed one by one for rapid microscopic examination of colonies, and then quickly returned to the holding system.

1.12 QUANTITATIVE MEASUREMENT OF BACTERIAL GROWTH (TOTAL AND VIABLE COUNT)

After incubation, the growth of micro-organisms is detected followed by counting. There are several techniques involved in counting microbes. Direct counting techniques are used. The oldest of these is microscopy which involves magnification of individual cells to become visible to the naked eye. Direct counting techniques do not rely on cell population growth. A recent direct counting technique is the use of immunofluorescence and epifluorescence adaptations of cell labeling used in conjunction with cytometry.

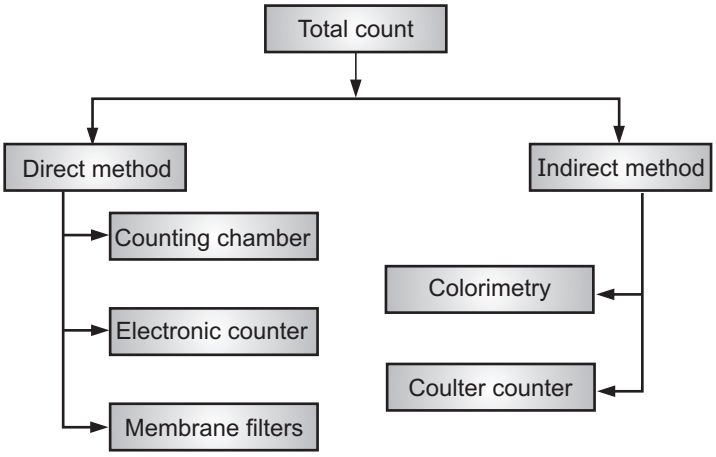
Total and viable counts of microorganisms are important practices in microbiology. The main difference between the two is that total count determines the count of all cells both dead and alive while viable count estimate the number of viable or live cells only capable of growing into distinct colonies.

Importance:

- Knowing how to count organisms and understanding their growth cycles is often important in treating infections.
- By counting individual organisms and experimenting, we can determine how many of them are needed to cause disease.
- Counting bacteria is also important in environmental microbiology; to control environmental conditions or enhance growth to obtain desired results.

1.12.1 Total Count

Total count is also termed as standard plate count or colony count. It gives the total number of microbes both viable and non-viable. All cells are counted. These include bacteria, yeasts and moulds. It is usually done by pour plate method. Total count generally requires a microscope.



Flowchart 7: Types of Total Count

Counting chamber: This method is easy, quick and inexpensive. It is used for both prokaryotes and eukaryotes. A haemocytometer is used for cell counting. It is a microscope slide. The central part of this slide has etched grids with precisely spaced lines to enable accurate counting. In order to get an accurate count in this method, the cell number should range between 40 to 70 cells in a one mm square (Fig. 1.33). There are 1000 mm cubed per ml, so cell can calculate by unit of cells/ml. Sometimes need to dilute a cell suspension to get the cell density low enough for counting. In that case, the final count is multiplied by the dilution factor.

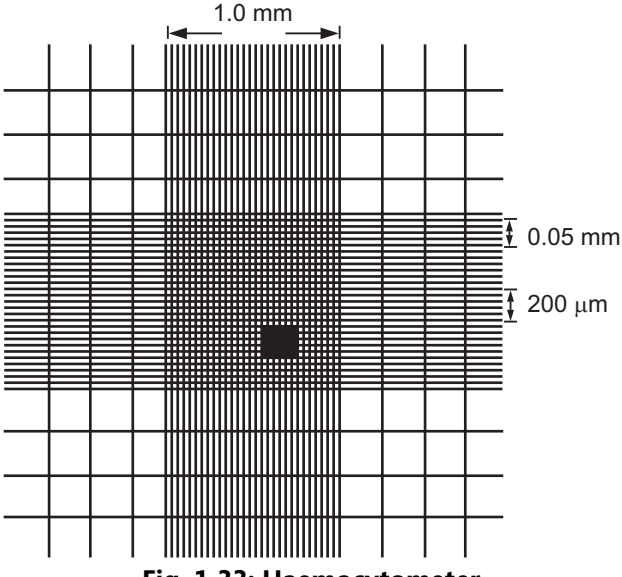


Fig. 1.33: Haemocytometer

Electronic counter: This method is known as flow cytometry method. This method is easy, fast but expensive. This machine detects the difference in current as individual microorganisms pass through a small orifice. This orifice connects two compartments of the counter which contain conductive solution. In this instrument two electrodes passing constant current are placed on either side of a small hole and due to that the major resistance in the circuit is at the aperture and when a non-conducting particle is passing through the resistance is changed to electrical pulse (Fig. 1.34). When each bacterium passes through the orifice, the electrical resistance between two compartments increases. The number of pulses is equal to the number of cells counted and the strength of the signal is directly proportional to the cell volume.

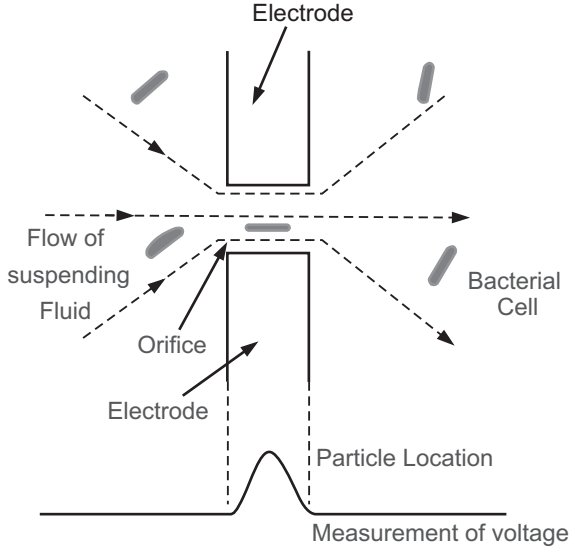


Fig. 1.34: Coulter counter

Coulter counter: It is a probe which measures variation in conductivity of a solution as a bacteria passes through a narrow gap.

Advantage: Direct and indirect counts processes are automated.

Disadvantage: They cannot differentiate dead cells from living ones.

Membrane filters method: This method is useful for counting bacteria. Cells are stained with fluorescent dyes then cells are filtered through special membrane that provides dark background for observing cells. These filters have known uniform porosity of predetermined size to trap microorganisms. The membrane with trapped bacteria is then placed in a special plate contained a pad saturated with specific medium. During incubation the bacterial colonies grow which appear on the surface of the membrane. This technique is mainly useful in analyzing aquatic samples.

Indirect count method: These depend on the effects of the organisms to estimate their numbers.

Colorimetry: This method is also known as turbidity method. When the organisms are grown in the medium the nutrient broth became turbid. The turbidity in the form of optical

density is measured by colorimetry method. It consists of light source, a filter which allows only a single wavelength of light to pass through, the sample tube containing the bacterial suspension and a photocell that compares the amount of light coming through the tube with the total light entering the tube. The ability of the culture to block the light can be expressed as either percent of light transmitted through the tube. The percent of light transmitted is inversely proportional to the bacterial concentration. Optical density of the solution is high with the maximum growth of the microorganism in the medium i.e. the absorbance is directly proportional to the cell concentration.

McFarland standards are used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range (Fig. 1.35).

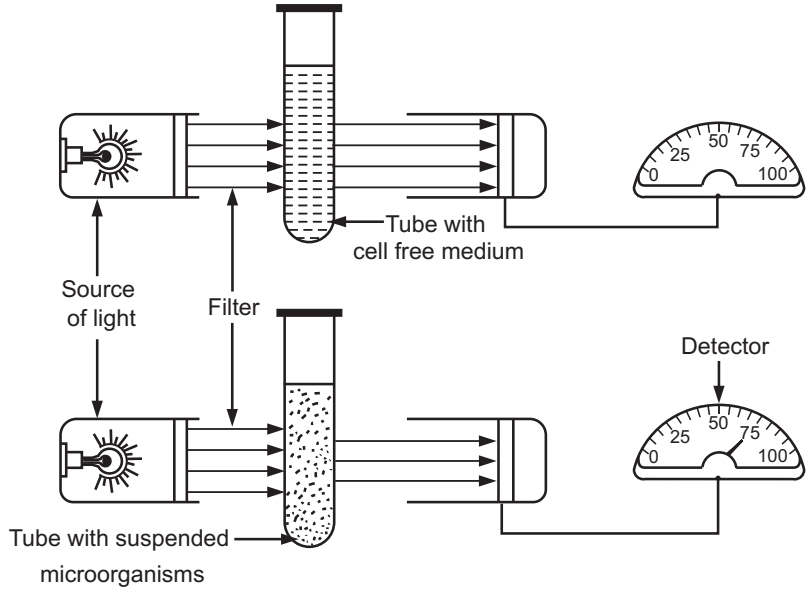
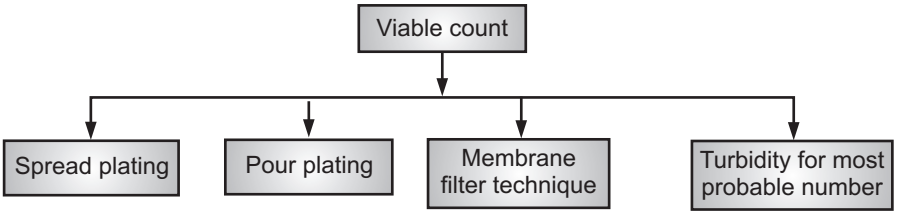


Fig. 1.35: Turbidity method

1.12.2 Viable Count

Viable count involves counting of colonies produced by only viable cells under favorable growth conditions. Number of living or dead bacterial cells (viable count) in the culture is estimated and is expressed as a Colony Forming Unit (CFU). This can be accomplished by techniques like pour plating, spread plating and most probable number with an assumption that each and every viable cell gives rise to a pure colony. This method is useful in the dairy industry and the food industry for quantitative analysis of milk and spoilage of food products. It helps in estimation of shelf life of processed food products as well as evaluation of sanitary conditions under which the products were manufactured. The efficiency of certain treatment processes like pasteurization, sterilization and cold storage done during production is also evaluated by viable microbial count.

The advantage of viable count is that the method is very sensitive and that can able to count subsets of population. The disadvantage is that the method is time consuming (counts require at least few hours or overnight for incubation) and sometimes the colony forming units may underestimate the number of cells because of clumping or chains of cells.



Flowchart 8: Types of viable count

Pour plating: In this method, the liquid media and the diluted sample are poured together in Petri dishes while still in liquid form and left to solidify. After solidifying, the Petri dishes are incubated at appropriate temperature for the required period of time during which the growth is realized. The plates are then removed and distinct colonies counted and expressed in colony forming units per ml (Fig. 1.36).

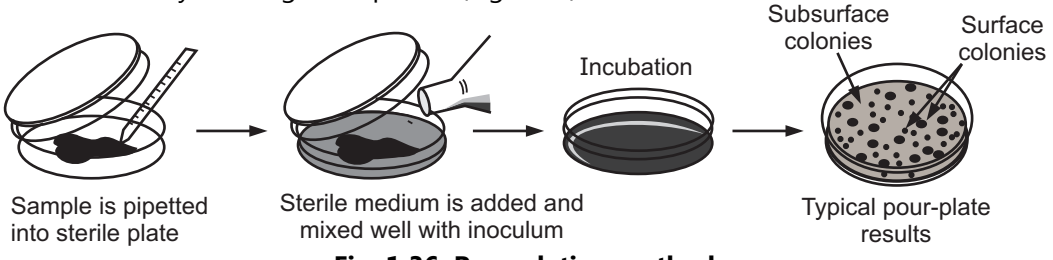


Fig. 1.36: Pour plating method

Spread plating: In this method, the media is prepared separately and poured in Petri dishes while still in liquid form. It is then left to solidify. After solidifying, a small known volume from the diluted sample is put to each Petri dish and with the help of a sterilized spreading rod, the sample is evenly spread over the media. The plates are then incubated at an appropriate temperature for a given period during which growth is realized. The plates are then taken for counting of colonies using magnifying lens (Fig. 1.37).

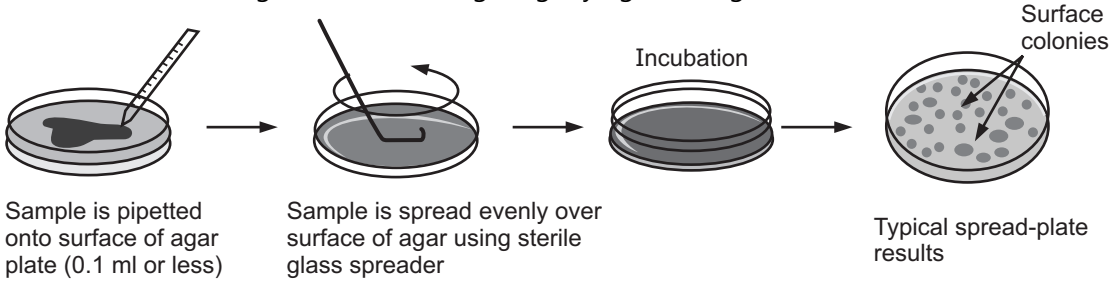


Fig. 1.37: Spread plating method

Membrane filter technique: This method is used in laboratory during water testing. Bacteria from aquatic samples are trapped on membranes by placed on culture media. Bacterial colonies are grown on membrane and then colony count determines the number of bacteria present in the sample.

Turbidity for Most Probable Number: If microbe cannot be cultured on plate media then dilutions are made and added to suitable media. After growth of microorganisms the turbidity determined to yield the most probable number. This method is useful where it is beneficial to use broth other than solid media especially for highly motile organisms which

are poor in forming colonies. It is mostly used when the organism is not able to grow on solid agar or in situations where the microorganisms are too few to give reliable measure of population size by the standard plate count method.

Serial dilution: In this method, increments are made in 1000, 100, or 10. The number of dilutions to be done depends on the concentration of the original solution and the required concentration. If small quantities of solutions are needed then more numbers of dilutions are required. Serial dilution allows small aliquots to be diluted instead of unnecessary big volumes of materials. During dilution, a small amount of original sample is removed to another container and its volume adjusted to original volume using a suitable buffer or distilled water e.g. if 1 ml of the original solution is taken and 10 µL removed and added in 990 µL of media or water then the dilution is 1 : 100. If the original solution contained 5×10^6 cells/ml then the concentration is of 5×10^4 cells/m*l* because that divided the concentration by 100.

1.13 PHASE CONTRAST MICROSCOPY, DARK FIELD MICROSCOPY AND ELECTRON MICROSCOPY

1.13.1 Phase Contrast Microscopy

Frits Zernike in 1953 has discovered this microscope. It is mainly used to visualize unstained living cells. It is an optical microscopy technique, converts phase shifts in light passing through a transparent specimen to brightness changes in the image. Phase shifts themselves are invisible, but based on brightness variations, it become visible. This microscope made it possible for biologists to study living cells and their multiplication through cell division.

Principle:

The main principle is based on the small phase changes in the light rays, induced by differences in the thickness and refractive index of the different parts of an object, can be transformed into differences in light intensity.

In simple term, it is the translation of invisible phase shifts into visible differences of intensities (Fig. 1.38). In a phase-contrast microscope, image contrast is increased in two ways: by generating constructive interference between scattered and background light rays in regions of the field, and by reducing the amount of background light that reaches the image plane.

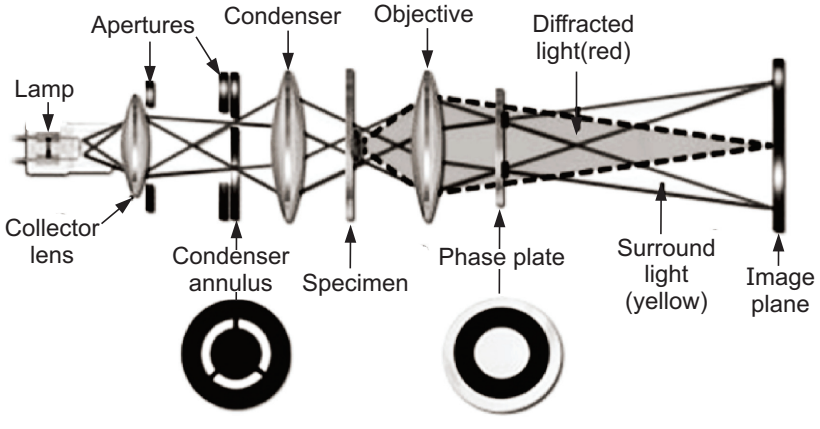


Fig. 1.38: Phase contrast microscope

Components:

It has light source, condenser, objective lens system, ocular lens system, annular diaphragm and phase plate.

Applications:

1. It enables the visualization of living and unstained cells.
2. It is used to visualization of various cell organelles like mitochondria, nucleus, vacuoles etc.
3. It helps to study cellular events like cell division, phagocytosis etc.
4. It helps to visualize cellular movements like chromosomal and flagellar movements.
5. It is used to observe growth of the living cells in the plant tissue culture techniques.
6. It is used to study membrane permeability of the cells.

Advantages:

1. It provides the clear images of unstained cells.
2. It provides high contrast images of the cells.
3. It restricts the damages of the cells due to chemical preparation and staining.
4. It enhances prolong observation of living cells.
5. Its cost is affordable.
6. It is widely applied in biological and medical research, especially throughout the fields of cytology and histology.

Disadvantages:

1. It produces bright holo surrounding the image because of partial formation of direct and deviated rays.
2. It is only effective to observe individual cells.

1.13.2 Dark Field Microscopy

Dark-field describes an illumination technique used to enhance the contrast in unstained organisms or any samples. It works by illuminating the sample with light that will not be collected by the objective lens and thus will not form part of the image. This produces the appearance of a dark, almost black, background with bright objects on it.

Principle:

It creates a contrast between the object and the surrounding field so that the back ground is dark and the object is bright. The objective and the ocular lenses are used in this microscope are same like ordinary microscope but a special condenser is used that prevent the transmitted light from directly illuminating the specimen. Only oblique scattered light reaches the specimen and passes on to the lens and causing the bright objects against dark background (Fig. 1.39).

Components: It has dark ground condenser that focuses only the oblique rays of light on to the specimens, high intensity light lamp, a funnel stop that reduces the apparatus of the objective to less than 1.

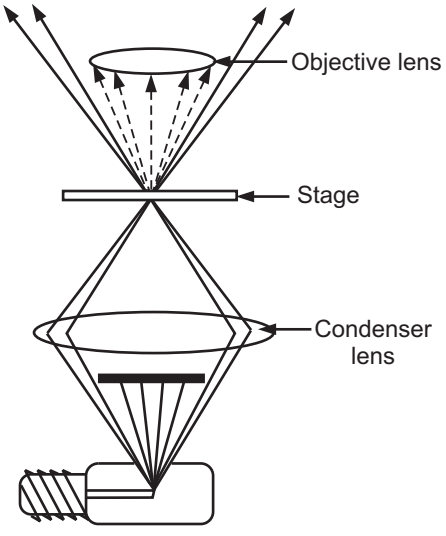


Fig. 1.39: Dark field microscopy

Applications:

1. It is used for diagnosis of Syphilis.
2. Viewing bacteria, algae and blood cells.
3. Viewing hair line metal fracture.

Advantages:

1. It is ideal for viewing unstained, transparent and little absorbed objects.
2. It is ideal to study marine organisms such as diatoms, algae, plankton etc.
3. It is used for research on live bacterium, mounted cells and tissues.
4. It is used to examine external details like out lines, edges, grain boundaries etc.

Disadvantages:

1. The images are prone to degradation and distortion.
2. It needs an intense amount of light to work.
3. It is not reliable tool to obtain accurate measurement of specimens.
4. If oil or water is used on the condenser then it is impossible to avoid air bubbles on slides.

1.13.3 Electron Microscopy

It is a microscope that uses a beam of accelerated electron as a source of illumination. The wavelength is 100,000 times shorter than visible photon light. It has higher resolving power than light microscope. Based on the working, it is four different types like analytical electron microscopy (AEM), scanning transmission electron microscope (STEM), Scanning electron microscope (SEM) and transmission electron microscope (TEM).

Principle:

AEM is a type of microscopy for capturing information on the interaction between the incident electrons and the atoms of the specimen. It is a tool for observing nano-scale structures also. It is a transmission electron microscope equipped with analytical functions such as energy dispersive X-ray spectrometry and electron energy loss spectrometry, to enable qualitative and quantitative measurements, elemental distribution mapping, and chemical state analyses in the micro-size observation areas.

TEM is a microscopy technique in which a beam of electrons is transmitted through an ultra thin specimen, interacting with the specimen as it passed through it. It is mainly used for cancer research, virology, to examine small column of atom, crystal orientation etc.

STEM is a modified type of TEM. It uses the magnetic lenses to focus a beam of electron. The image is formed by the primary electrons coming through the specimen.

SEM is a microscopy technique that produces images of a sample by scanning it with a focused beam of electrons. The electrons interact with the atoms in the sample and produce various signals that contain information about the sample's surface topography and composition. It is mainly use in ultra high vacuum, air and various liquid states. It is also used for the examination of live specimen (Fig. 1.40).

Applications:

1. The ability to view the microscopic structure of a specimen at a higher resolution.

2. It is used for particle analysis or material characterization in a research laboratory.
3. It is used to explore the molecular nature and mechanisms of disease.
4. It is used to view 3D structure of biological tissues or cells, determine the structure of proteins and observe viruses in a biological context.

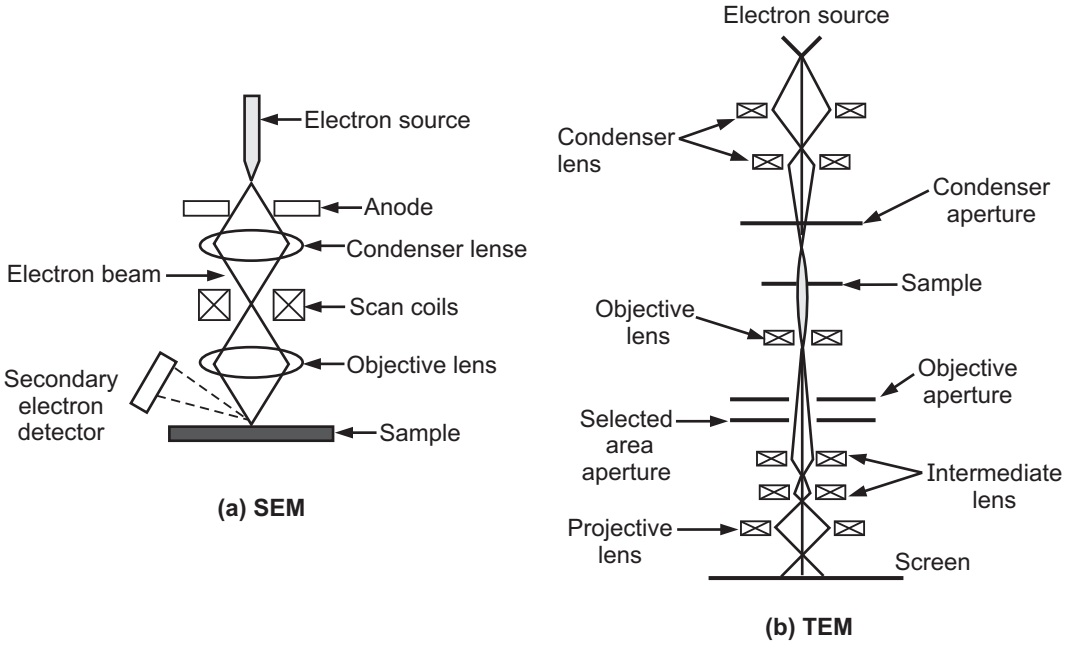


Fig. 1.40: Principles of electron microscopes

Advantages:

1. It is used to study the object of more than 0.2 micrometer.
2. It is used to analysis of subcellular structure.
3. It is used for study of intracellular pathogens and viruses.
4. It is used for cell metabolism.
5. It is used to study for micro structure of nature.

Disadvantages:

1. Instruments are highly expensive.
2. The electron microscope is dynamic, which means that the voltage needs to be highly stable.
3. The cooling system needs constant circulation pumping through the unit and the vacuum setup also requires consistent pressure and continuous pumping to keep the entire system in proper working condition.
4. An electron microscope requires that all samples be viewed in a vacuum. Otherwise, the molecules that occur naturally in the air would scatter and distort the electrons.

QUESTIONS

Long Essays:

1. Write the history, scope and importance of microbiology.
2. Explain ultra-structure and morphological classification of bacteria.
3. Explain various raw materials and nutritional requirement for culture media.
4. Explain various isolation and preservation method of pure culture.
5. Explain quantitative measurement of bacterial growth.

Short Essays:

1. Explain different branches of microbiology.
2. Explain the scope of microbiology.
3. Explain the importance of microbiology.
4. Explain the classification of microbiology.
5. Explain various culture media with examples.
6. Explain ultra-structure of bacteria.
7. Explain physical factors affecting bacterial growth.
8. Explain bacterial growth curve.
9. Explain isolation of pure culture.
10. Give the difference between pour plate and spread plate method.
11. Explain the cultivation method of anaerobes.
12. Explain viable count for bacterial growth measurement.
13. Explain principle and importance of electron microscope.
14. Explain phase contrast microscope.

MULTIPLE CHOICE QUESTIONS

1. Give the example of multicellular organisms.
   (a) Coccus (b) Spirillia
   (c) Fungi (d) Virus
2. Give one example of acellular organism.
   (a) Prion (b) Cyanobacteria
   (c) Protozoa (d) Coccus
3. Germs are discovered by ……
   (a) Robert Hooke (b) Roger Bacon
   (c) Aristotle (d) Fracastorius
4. Leprosy bacillus discovered by ……
   (a) Emile Roux (b) Joseph Hansen
   (c) Alexander (d) Good Pasteur
5. Mycology is the study of ……
   (a) Bacteria (b) Algae
   (c) Fungi (d) Virus

6. Phycology is the study of ……
   (a) Algae (b) Fungi
   (c) Molds (d) Protozoa
7. Axonema is also known as ……
   (a) Cell wall (b) Bulk filament
   (c) Pili (d) Spinae
8. Teichoic acid is absent in ……
   (a) Gram positive bacteria (b) Gram negative bacteria
   (c) Acid fast bacteria (d) Both gram positive and gram negative
9. Lithotrophs are ……
   (a) Organisms that use light energy
   (b) Organisms that use carbon energy
   (c) Organisms that use oxidize inorganic compounds
   (d) Organisms that use organic carbon
10. Psychrophiles are grown in temperature between ……
    (a) 20 to 45 °C (b) 70 to 110 °C
    (c) −5 to 10 °C (d) −5 to 20 °C
11. Exponential phase is also known as ……
    (a) Log phase (b) Lag phase
    (c) Stationary phase (d) None of these
12. Cryopreservation is ……
    (a) Freezing in liquid nitrogen at −196 °C
    (b) Freezing in liquid nitrogen at −19 °C
    (c) Freezing in liquid nitrogen at −27 °C
    (d) Freezing in liquid nitrogen at 196 °C
13. Obligate anaerobes can grow in ……
    (a) Presence or absence of oxygen (b) Absent of oxygen
    (c) Presence of oxygen (d) None of these
14. Facultative anaerobes can grow in ……
    (a) Presence or absence of oxygen (b) Absence of oxygen
    (c) Presence of oxygen (d) None of these
15. CFU stands for ……
    (a) cell forming unit (b) colony forming unit
    (c) cells per feet unit (d) colony per feet unit

Answers:

1. (c)	2. (a)	3. (d)	4. (b)	5. (c)	6. (a)	7. (b)	8. (b)	9. (c)	10. (d)
11. (a)	12. (a)	13. (b)	14. (a)	15. (b)					

***

Unit ... 2

STERILIZATION TECHNIQUES AND IDENTIFICATION OF BACTERIA

♦ LEARNING OBJECTIVES ♦

After completing this chapter, reader should be able to understand:

- To identify various bacteria through staining methods.
- To study the principle, procedure, merits and demerits of various sterilization methods.
- To evaluate the efficiency of sterilization.
- To know the various equipments used for large scale sterilization.
- To know about sterility indicators.

2.1 IDENTIFICATION OF BACTERIA USING STAINING TECHNIQUES

Stain: A stain is a substance that adheres to a cell which gives cell colour because bacteria are slightly negative charge at pH 7.0. The presence of colour gives the cells significant contrast so that they are prominently visible. Different stains have different affinities for different organisms. Hence, the staining technique enhances the contrast in the microscopic image. Generally, crystal violet, Gram's Iodine, acetone and safranin are used as stains. Table 2.1 indicates the colour changes for different bacteria.

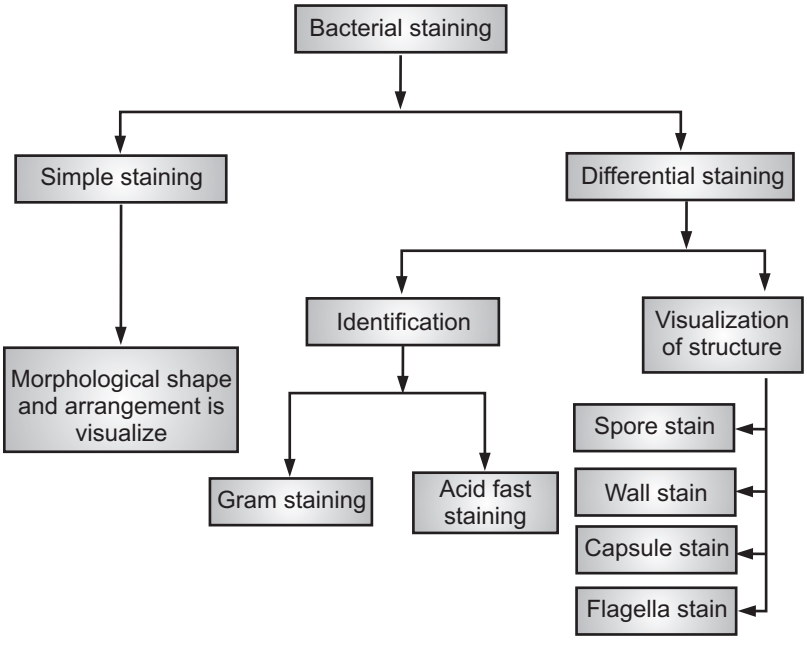
Table 2.1: Different stains for different bacteria

Reagent	Gram Positive	Gram Negative
Crystal violet	Purple or blue	Purple or blue
Gram's iodine	Purple or blue	Purple or blue
Acetone or Alcohol	Purple or blue	Colourless
Safranin	Purple or blue	Red or Pink

Importance:

- Bacteria have nearly same refractive index as water so when they are observed under microscope they are invisible to the naked eye. Different types of staining methods are used to make the cells and their internal structures more visible under the light microscope.
- Staining is the primary recognition of bacteria.
- It is of importance in diagnosis of infectious diseases in culture and directly from clinical samples.

Types of Staining Technique:



Flowchart 1: Classification of bacterial staining

2.1.1 Simple Staining

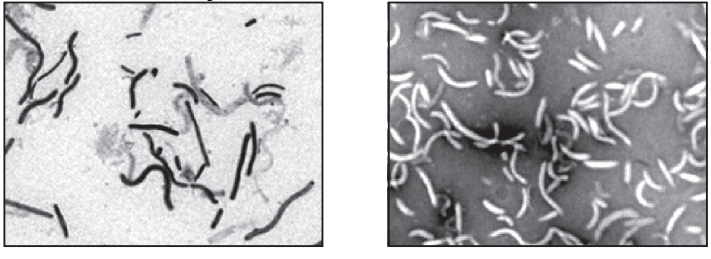
Simple staining is carried out to visualize bacteria and to compare morphological shapes and arrangements of bacterial cell. In this technique the bacterial smear is stained with single basic dye such as crystal violet, safranin, methylene blue etc.

Principle: These single basic dyes will give up a hydroxyl ion or accept a hydrogen ion that leaves the stain positively charged. Since, the surface of bacteria is negatively charged so these charges are strongly binds to the cell surface (cell wall). Then the cells are visible against a light background.

Steps (Fig. 2.1):

1. Clean and dry microscope slides thoroughly.
2. Flame the surface in which the smear is to be spread.
3. Flame the inoculating loop.

4. Transfer a loop full of tap water to the flamed slide surface.
5. Reflame the loop making sure the entire length of the wire that will enter the tube has been heated to redness.
6. Remove the tube cap with the fingers of the hand holding the loop.
7. Flame the tube mouth.
8. Disperse the bacteria on the loop in the drop of water on the slide and spread the drop over an area the size of a dime. It should be a thin, even smear.
9. Reflame the inoculating loop to redness including the entire length that entered the tube.
10. Allow the smear to dry thoroughly.
11. Fix the smear cautiously by passing the underside of the slide through the burner flame two or three times.
12. Stain the smear by flooding it with one of the staining solutions and allowing it to remain covered with the stain for the time designated below.
    Methylene blue - 1 minute
    Crystal violet - 30 seconds
    Carbol fuchsin - 20 seconds
    During the staining the slide may be placed on the rack or held in the fingers.
13. At the end of the designated time rinse off the excess stain with gently running tap water. Rinse thoroughly.
14. Wipe the back of the slide and blot the stained surface with a paper towel.
15. Place the stained smear on the microscope stage smear side up and focus the smear using the 10X objective.
16. Choose an area of the smear in which the cells are well spread in a monolayer. Center the area to be studied, apply oil directly to the smear, and focus the smear under oil with the 100X objective.



Positive staining **Negative staining**

Fig. 2.1: Simple staining

Observation: Spherical shaped bacteria coccus is turn blue colour whereas rod shaped bacteria, bacilli turn red colour.

Use: To study morphology and arrangement of bacteria cell.

2.1.2 Gram's Staining

It is a type of differential staining where two or more stains are used to differentiate between types of bacteria. This staining technique was discovered by Hans Christian Gram in the year 1884.

Importance:

- It is important test for rapid diagnosis of infectious agents.
- It differentiates types of bacteria whether Gram positive or Gram negative.
- It helps in study the morphology of bacteria.
- It helps in find the evidences of capsule, spores, pus cell, epithelial cells, Yeast cell etc.
- To understand how the Gram stain reaction affects Gram positive and Gram negative bacteria based on the biochemical and structural differences of their cell walls.

Steps:

1. **Crystal violet:** It is the primary stain which is used as a simple stain because it dyes the cell wall of bacteria cell.
2. **Gram's Iodine:** It is acts as mordant which helps to fix the primary dye to the cell wall.
3. **Decolourizer:** It is used to remove the stain of primary dye (crystal violet) from the Gram negative bacterial cell wall. Decolourizer is composed of an organic solvents like acetone, ethanol or in combinations.
4. **Safranin:** Finally, a counter stain is applied to stain those cells that have lost their primary stain (especially Gram negative bacteria) as a result of decolourization.

The procedure is based on the ability of microorganisms to retain colour of the stains used during the gram stain reaction. Gram-negative bacteria are decolourized by the alcohol, losing the colour of the primary stain, purple.

Gram-positive bacteria are not decolourized by alcohol and will remain as purple. After decolourization step, a counterstain is used to impart a pink colour to the decolourized gram-negative organisms.

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

Example: *Staphylococcus aureus*.

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

Example: *Escherichia coli*.

There are some differences between Gram positive and Gram negative bacteria which are listed in Table 2.2.

Table 2.2: Differences between Gram Positive and Gram negative bacteria

Gram Positive bacteria	Gram Negative bacteria
1. They retain crystal violet dye and stain dark violet or purple, they remain coloured blue or purple with gram stain when washed with absolute alcohol and water.	1. They are decolourized to accept counter stain such as Safranin or Fuchsine. They stain red or pink and they do not retain the Gram stain when washed with absolute alcohol and acetone.

Gram Positive bacteria	Gram Negative bacteria
2. Peptidoglycan is multilayered thick.	2. Peptidoglycan is single layered thin.
3. Outer membrane is absent.	3. Outer membrane is present.
4. Periplasmic space is absent.	4. Periplasmic space is present.
5. Teicholic acids are present.	5. Teicholic acids are absent.
6. Lipid and lipoprotein content is low.	6. Lipid and lipoprotein content is high.
7. They have high resistance to physical disruption and drying.	7. They have low resistance to physical disruption and drying.
8. Cell wall is 100-120 Å thick; single layered. Lipid content of the cell wall is low, whereas Murein content is 70-80%.	8. Cell wall is 70-120 Å thick; two layered. Lipid content is 20-30% (high), Murein content is 10-20%.
9. More susceptible to antibiotics.	9. More resistant to antibiotics.

2.1.3 Acid Fast Staining

Acid fast staining method is one of the differential staining techniques. This technique was first developed by Ziehl and later on modified by Neelsen and hence this method is also known as **Ziehl-Neelsen staining** techniques. In the year 1883, Neelsen used Ziehl's carbol-fuchsin and heated then decolourized with an acid alcohol followed by counter stained with methylene blue. Thus Ziehl-Neelsen staining technique was developed as acid fast staining (Table 2.3).

Objectives:

- To differentiate bacteria into acid fast group and non-acid fast groups.
- It is used for those microorganisms which are not staining by simple or Gram staining method, particularly the member of genus *Mycobacterium* which are resistant to other stains.

Principle:

The smear is prepared and stained with carbol fuchsin which solubilizes the lipoidal material present in the Mycobacterial cell wall and by heating, carbol fuchsin further penetrates through lipoidal wall and enters into cytoplasm, resulting in all cells appearing red. Then the smear is decolourized with 3% HCl in 95% alcohol (decolourizing agent) but the acid fast cells are resistant due to the presence of large amount of lipoidal material in their cell wall that prevents the penetration of decolourizing solution. Then the smear is stained with counterstain, methylene blue and only decolourized cells are absorbs and appears blue while acid-fast cells retain the red colour (Table 2.3).

Table 2.3: Cell colours with various dyes

Application of	Reagent	Cell colour	
		Acid fast	Non-acid fast
Primary dye	Carbol fuchsin	Red	Red
Decolourizer	Acid alcohol	Red	Colourless
Counter stain	Methylene blue	Red	Blue

Procedure (Fig. 2.2):

1. Prepare bacterial smear on clean and grease free slide, using sterile technique.
2. Allow smear to air dry and then heat fix.
3. Cover the smear with carbol fuchsin stain.

4. Heat the stain until vapor just begins to rise about 60°C. Allow the heated stain to remain on the slide for 5 minutes.
5. Wash off the stain with clean water.
6. Cover the smear with 3% v/v acid alcohol for 5 minutes or until the smear is sufficiently decolourized, i.e. pale pink.
7. Wash well with clean water.
8. Cover the smear with malachite green stain for 1–2 minutes, using the longer time when the smear is thin.
9. Wash off the stain with clean water.
10. Wipe the back of the slide clean, and place it in a draining rack for the smear to air-dry.
11. Examine the smear microscopically, using the 100 X oil immersion objective.

Acid-fast: *Mycobacterium tuberculosis, Mycobacterium smegmatis.*

Non-Mycobacterial bacteria: *Nocardia*; **Coccidian Parasites:** *Cryptosporidium*.

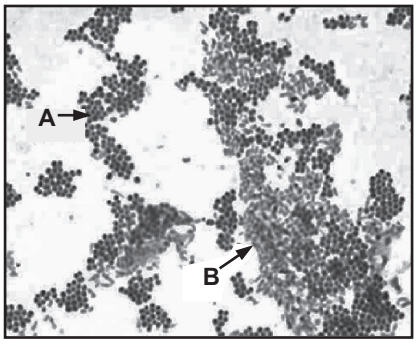


Fig. 2.2: A. Non acid fast: Blue colour; B. Bright red to intensive purple, Red, straight or slightly curved rods

2.2 BIOCHEMICAL TEST (IMViC)

It is a biochemical series of tests including the following tests: Indole, Methyl Red (MR), Voges-Proskauer (VP), and Citrate. All of the series were designed to differentiate among the Enterics (family Enterobacteriaceae).

I = Test for production of indole from tryptophan.
M = Methyl red test for acid production from glucose.
Vi = Voges-Proskauer test for production of acetoin from glucose.
C = Test for usage of citrate as a carbon source.

Significance:

- This test is useful for differentiating the Enterobacteriaceae, especially when used alongside the urease test.
- Particularly it is used for differentiate *E. coli, Enterobacter aerogens, Enterobacter cloacae and Klebsiella pneumonia.*
- Four different tests are used viz. indole test, methyl red test, Voges Proskauer test and citrate test.
- These four tests are combined with those carbohydrate tests to identify unknown bacterial species.
- This test detects the motility of bacteria and disulphane (H_2S_2) production.

Indole test:

Some microorganisms can metabolize tryptophan by the tryptophanase. It is based on the principle of formation of hydrolytic product from an amino acid tryptophan.

Media: Sulfide Indole Motility (SIM); Tryptophan is used as substrate.

Reagent: Kovac's reagent (Consist of isoamyl alcohol, para-dimethyl amino benzaldehyde and concentrated hydrochloric acid). It is used to determine the ability of the organism to split indole from the amino acid tryptophan.

Colour: Pink or red indicates positive test.

Tryptophan $\longrightarrow$ Tryptophanaseindole + Acid pyruvate + NH_3 $\longrightarrow$ Kovac's Reagent

Upper organic layer forms red colour (positive for indole); black (production of H_2S_2) and motility (turbidity forms) of bacteria.

Example: *Proteus mirabilis* can differentiate from *Proteus vulgaris* by this test. *P. vulgaris* shows positive test (red or pink).

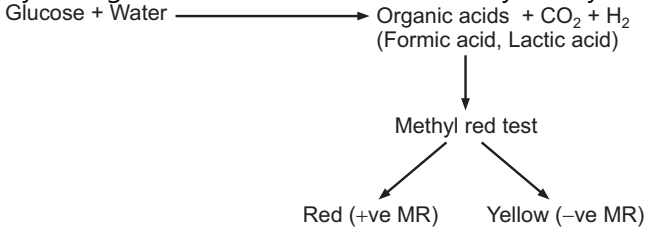
Methyl Red Test:

This test is a qualitative test for acid production. The bacteria are grown in MR-VP broth. It requires positive organisms to produce strong acids likely lactic acid, acetic acid, formic acid from glucose via mixed acid fermentation pathway. Only those organisms that are maintained low pH (4-5), give positive test for methyl red indicates for mixed acid fermentation. In alkaline medium, it gives yellow colour (Negative test) indicating negative test for fermentation.

Significance:

- It is used to identify bacteria to produce pyruvic acid from glucose metabolism.
- This test is used to differentiate *E. coli* and *E. aerogens*.

Medium: MR-VP medium. It contains glucose, peptone and buffer (buffer will neutralize weak acids so that only strong stable acids will be detected by methyl red).



Example:

E. coli:

Glucose + H_2O $\longrightarrow$ Lactic acid, Acetic acid + Methyl red, Formic acid $\longrightarrow$ CO_2 + H_2 + **Red colour** (Positive test) **pH 4**

E. aerogen

Glucose + H_2O $\longrightarrow$ Acetic acid $\longrightarrow$ 2,3 butanediol + Methyl red + CO_2 + H_2O $\longrightarrow$ Yellow color (Negative test) **pH 6**

Voges-Proskauer Test:

The test is mainly based on the principle of conversion of glucose to acetyl methyl carbinol. After break down of glucose it reacts with alpha naphthol and potassium hydroxide to form a red colour solution, known as acetoin.

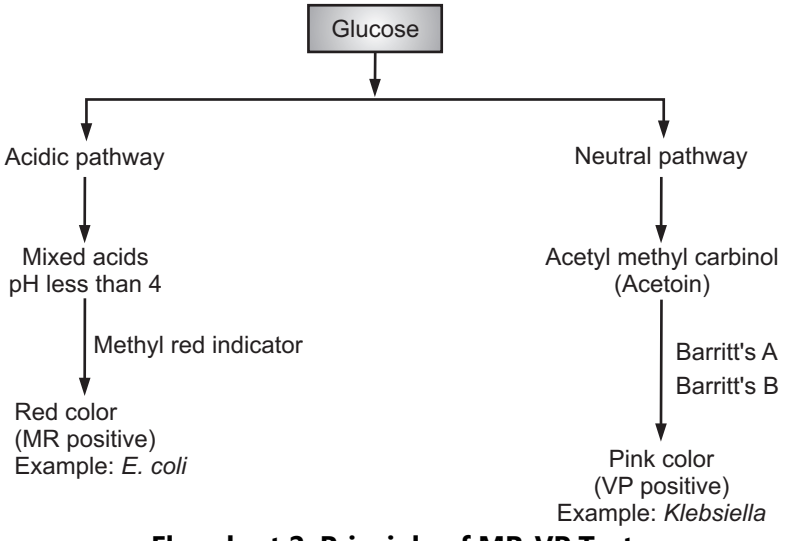
Reagents used: Barritt's A and Barritt's B which are mainly alpha naphthol and potassium hydroxide respectively. When these reagents are added to the broth containing acetyl methyl carbinol, it changes to pink colour after 20-30 minutes. This indicates the reaction is positive.

Procedure:

- Pipette out 1.0 ml of MRVP broth into empty sterile tube.
- Add 0.6 ml of alpha naphthol and 0.2 ml of potassium hydroxide respectively.
- Shake the tube well and stand for 20-30 minutes.
- Observe and record the colour.

Example: *E. coli* does not produce red colour (Negative test) whereas *Enterobacter* and *Klebsiella* give positive test.

Principle of MR-VP Test:



Flowchart 2: Principle of MR-VP Test

Citrate Test:

Objective:

- This test is used to determine the ability of an organism, using the enzyme citrase, to use citrate as its sole carbon source.
- Used occasionally to assist in the identification of enterobacteria.
- Inorganic ammonium salt is used as sole nitrogen source.
- It utilizes Simmon's citrate media to determine the growth of bacteria.

Principle:

Breakdown of ammonium salt gives ammonia and hydroxides. These hydroxyl ions raise the pH and that changes the colour of bromophenol blue indicator from green to royal blue.

When the bacteria metabolize citrate, the ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turns the bromothymol blue indicator in the medium from green to blue above pH 7.6.

Reagent: Bromothymol blue indicator.

Procedure: Inoculate the slant of Simmons citrate agar media (pH 6.9) with the bacterial culture by streaking the surface of media. Then incubate at 37°C for 1-2 days. Observe and record the colour changes.

Observation: If the organism did not grow, the slant remains green. This indicates test is negative. Example: *E. coli*.

If the organism grows, the slant became partially or completely Prussian blue in colour. Example: *Klebsiella pneumonia*.

Positive Reaction: Growth with colour change from green to intense blue along the slant.

Examples: *Salmonella, Edwardsiella, Citrobacter, Klebsiella, Enterobacter, Serratia, Providencia*, etc.

Negative Reaction: No growth and no colour change; Slant remains green. **Examples:** *Escherichia, Shigella, Morganella, Yersinia* etc.

Limitations:

1. Some organisms are capable of growth on citrate and do not produce a colour change. Growth is considered a positive citrate utilization test, even in the absence of a colour change.
2. Tests with equivocal results should be repeated.
3. The reactions of this medium alone are not sufficient for identification to the species level.

2.3 STERILIZATION PROCESS

Sterilization is the process that eliminates, removes, kills, or deactivates all forms of life and other biological agents such as fungi, bacteria, viruses, spore forms, prions, unicellular eukaryotic organisms such as plasmodium etc. Sterilization can be carried out through heat, chemicals, irradiation, high pressure and filtration. In an one sentence, sterilization is the process by which an article, surface or medium is freed from all living organisms either in the vegetative or spore state.

- **Autoclaves:** They are highly effective and inexpensive. Unsuitable for heat sensitive objects.
- **Hot air ovens:** They are inefficient compared to autoclaves.
- **Ethylene oxide:** It is suitable for heat sensitive items but leaves toxic residue on sterilized items.
- **Low-temperature steam and formaldehyde:** They are effective for instruments with cavities or tubular openings.
- **Sporicidal chemicals:** They are often used as disinfectants but can also sterilize instruments if used for prolonged periods.
- **Irradiation:** Gamma rays and accelerated electrons are excellent at sterilization.
- **Gas plasma:** Many types of gases are used as a source of plasma which are used for inactivation of microorganisms.

Principle:

- Thorough cleaning of instruments before sterilization.
- Contact of sterilizing agent with all surfaces of each item for specified period of time at specified temperature.
- Regular service and maintenance of sterilizing equipment.

Need for sterilization:

- Microorganisms are capable of causing infection.
- They are responsible for contamination and infection.

Mechanism:

- Denaturation of protein
- Oxidation
- Filtration
- Interference with protein synthesis
- Disruption of cell membranes
- Interruption of DNA synthesis/repair

2.3.1 Some Important Terminologies

Disinfectant: The process of destruction or removal of all pathogenic organisms. Examples: Alcohol, formaldehyde, chloramines etc.

Asepsis: It is the absence of disease producing organisms. It is two types viz. medical and surgical. Medical asepsis is the process that helps to reduce the number and spread of microorganisms. Surgical asepsis is the process by which complete removal of microorganism and their spores from the surface of an object.

Antisepsis: The procedure of antiseptic solution which inhibits growth of microorganism with their contact. Example: Betadiene.

Classification of Sterilization:

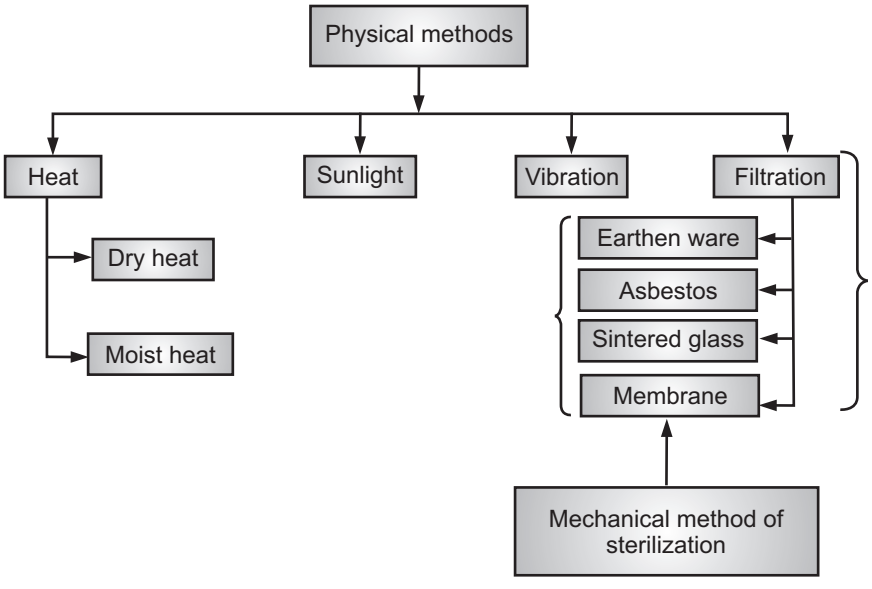
Broadly sterilization is classified as physical, chemical, gaseous, mechanical and radiation methods.

2.3.2 Pharmaceutical Importance of Sterilization

- Moist heat sterilization is the most efficient biocidal agent. In the pharmaceutical industry, it is used for Surgical dressings, Sheets, Surgical and diagnostic equipment, Containers, Closures, Aqueous injections, Ophthalmic preparations and Irrigation fluids etc.
- Dry heat sterilization is used for thermo-stable, moisture-sensitive or moisture impermeable pharmaceutical and medicinal. These include products like; Dry powdered drugs, Suspensions of drug in non-aqueous solvents, Oils, fats waxes, soft hard paraffin silicone, Oily injections, implants, ophthalmic ointments and ointment bases etc.
- Gaseous sterilization is used for sterilizing thermolabile substances like; hormones, proteins, various heat sensitive drugs etc.
- U.V. light is the most lethal component in ordinary sunlight used in sanitation of garments or utensils.

- Gamma-rays from Cobalt 60 are used to sterilize antibiotic, hormones, sutures, plastics and catheters etc.
- Filtration sterilizations are used in the treatment of heat sensitive injections and ophthalmic solutions, biological products, air and other gases for supply to aseptic areas. They are also used in industry as part of the venting systems on fermenter, centrifuges, autoclaves and freeze driers. Membrane filters are used for sterility testing.

2.4 PHYSICAL METHODS OF STERILIZATION



Flowchart 3: Types of physical method of sterilization

2.4.1 Heat

Heat is considered as most reliable method of sterilization of objects that can withstand heat. It is carried out by two ways viz. Moist and Dry heat. They are the most common sterilizing methods used in hospitals and are indicated for most materials. This method of sterilization is applied only to the thermostable products, but it can be used for moisture-sensitive materials for which dry heat (160-180°C) sterilization, and for moisture resistant materials for which moist heat (121-134°C) sterilization is used.

Advantages:

- Most common method for controlling microbial growth.
- Very effective in destroying unwanted microbes.
- Low cost.

Disadvantages:

- It is probabilistic i.e. not absolute sterilization.
- It can degrade thermally unstable media.

2.4.2 Dry Heat

Dry heat sterilization requires higher temperatures and longer exposure times than moist heat sterilization. It uses high temperatures to kill microorganisms and bacterial spores and is

used on items that cannot get wet and for glassware, oils, powders, metal instruments, and items wrapped in paper. There are three types of temperatures are recommended viz.

Temperature	Exposure time
150°C	150 minutes
160°C	60 minutes
170°C	30 minutes

Thermal death time (TDT): It is the minimum time required to kill a suspension of organism at a predetermined temperature at a specified environment. Thermal death time is inversely proportional to temperature. It is increased in presence of organic substance, proteins, nucleic acid, starch gelatin etc.

Principle: It causes denaturation of proteins and oxidative damage. There are several different types of dry heat sterilization such as:

- **Red Heat:** It is used for straight wires, bacterial loops and spatulas.
- **Flaming:** It is the process of heating over fire till they become red hot. It is used for bacterial loops, wires and spatulas.
- **Incineration:** It is the process that involves the combustion of organic substances contained in waste materials. It is used for soil dressing, pathological bedding.
- **Hot Air oven:** It is an electrical device. The oven uses dry heat to sterilize articles at 50 to 300°C. Thermostat is used to control the temperature. It is commonly used in dairy industry. Glasswares, forceps, scissors, scalpels etc. are sterilized by this method but surgical dressings, rubber items, or plastic materials are not sterilized by this method.

Temperature used in hot air oven with holding time of articles are described as 160°C for 45 minutes, 170°C for 18 minutes, 180°C for 7.5 minutes etc.

Operation:

(i) Articles to be sterilized are first wrapped or enclosed in containers of cardboard, paper or aluminium.

(ii) Then, the materials are arranged to ensure uninterrupted air flow.

(iii) Oven may be pre-heated for materials with poor heat conductivity.

(iv) The temperature is allowed to fall to 40°C, prior to removal of sterilized material (Fig. 3.3).

Types of Hot Air Oven:

There are two types of hot air ovens. (i) Forced air hot air oven and (ii) Static air hot air oven. The forced air hot air oven is more effective than the static air hot air oven. Forced hot air oven works by heating the oven and using a fan to move the hot air around whereas the static air hot air oven works by using a heating coil at the bottom of the oven. The heat rises throughout the oven and takes a longer time to reach the desired temperature.

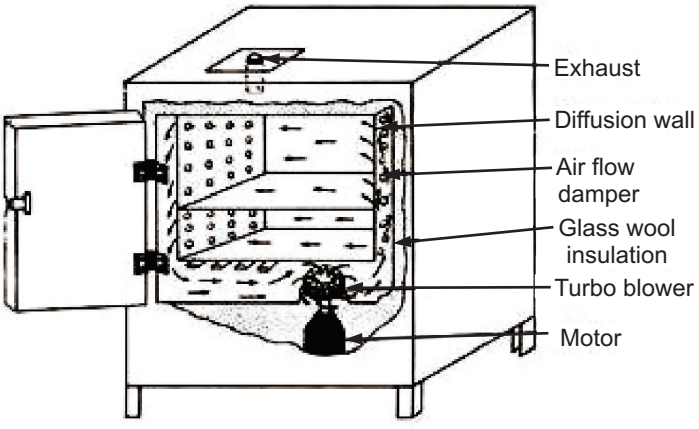


Fig. 2.3: Working of Hot air oven

Advantages:

There are several advantages that include:

- It does not cause metals to corrode or rust.
- It is relatively inexpensive.
- It does not release any harmful or hazardous fumes or pollutants.

Disadvantages:

- It is relatively slow (can take a couple hours).
- Many objects cannot withstand the very high temperatures required for dry heat sterilization (example: some plastics would melt).

Applications of Dry Heat Sterilization:

- The method is applicable for thermo stable, moisture-sensitive pharmaceutical and medical devices and materials such as dry powdered drugs, suspension of drugs in non-aqueous solvents, oils, fats, waxes, oily injections, implants, ophthalmic ointments etc.

2.4.3 Moist Heat

Principle: Moist heat is more efficient for sterilization in contrast to dry heat; it destroys microorganisms by the irreversible denaturation of enzymes and structural proteins. The temperature at which denaturation occurs varies inversely with the amount of water present.

Moist heat sterilization involves the use of steam in the range of 121-134°C. Steam under pressure is used to generate high temperature needed for sterilization. Saturated steam acts as an effective sterilizing agent. There are three types of temperatures are used in this technique.

At temperature below 100°C:

- **Pasteurization:** Food industry.
- **Vaccine bath:** Vaccine sterilization.
- **Serum bath:** Serum contaminants, does not kill spores survive.
- **Inspissation:** Egg and serum containing media, can kill spores.

At temperature 100°C:

- **Boiling:** Boiling water (100°C).
- Tyndallization (100°C).

At temperature above 100°C:

- Autoclave.

Pasteurization: This process was first discovered by Louis Pasteur. It is the process for heating liquids for the purpose of destroying viruses and harmful organisms such as bacteria, protozoa, molds etc. This method is not intended to kill all microorganisms in the food. Instead it aims to achieve a logarithmic reduction in the number of viable organisms, reducing their number so they are unlikely to cause disease.

Process:

- It involves milk.
- In Holder method, the sample is heated for 30 minutes at 60°C.
- In Flash method, the sample is heated for 15-20 sec at 72°C.
- Milk is not heated more than this temperature otherwise it forms aggregate resulting in spoiling of milk.
- By this method all non-sporing bacteria like mycobacteria, Brucellae and salmonellae are destroyed.
- *Coxiella burnetii* being heat resistant survives Holder's method.

Examples:

- Vaccines of non-sporing bacteria are pasteurized in special bath at 60°C for 1 hour.
- A temperature of 80°C for 5-10 minutes destroys all vegetative form of Bacteria, yeast and molds.
- Spores of *Clostridium botulinum* are destroyed at 120°C for 4 minutes.

Inspissator:

It is a system designed to produce large batches of uniform culture medium (Tuberculum bacteria culture) four to six times per day. Inspissation takes 50 minutes at 85°C. It is used for sterilization of media.

Example: Lowenstein-Jenson and Loeffler's rendered sterile at 80-85°C for 30 minutes on three successive days inspector.

Boiling:

It is the process of heating the sample to be sterilized material in a liquid at its boiling point to kill bacteria and other microorganism and also spores.

Example: Vegetative bacteria are killed immediately at 90-100°C.

Steam at 100°C:

It is an inexpensive method of utilizing free steam to sterilize culture medium. In this method, container and the medium are simultaneously sterilized.

Tyndallization: It is also known as intermittent sterilization. It helps first exposure to kills all bacteria and spores. It is used for media with sugar or gelatin which require 100°C for 20 minute on three successive days.

Autoclave: It is also known as steam under pressure.

Principle:

High-pressure steam sterilization is used to kill all microorganisms, including spores. It is the best and most widely used methods of sterilization. Example: Pressure cooker.

Vapour pressure within the autoclave increases that enables steam to reach higher temperatures. At 103.4 kPa vapor pressure, the temperature reaches to 121.3°C, for 15 ~ 20 minutes. This method is used for physiological saline, surgical instruments, containers, glass syringes, dressings and other article (Fig. 2.4).

Autoclaves have lower row pressure pre-vacuum steam sterilizers which are classified in to two types viz. portable and horizontal.

(i) Under the exhaust pressure steam sterilizer: With the upper part of the vessel vapor pressure forces the cold air from the bottom of the exhaust vent. When the pressure in the 102.97 ~ 137.30 kPa, the temperature can reach 121 ~ 126°C, 15 ~ 30 minutes to achieve sterilization purposes.

(ii) Pre-vacuum steam sterilizer: With vacuum before injecting steam into the first internal evacuated, a negative pressure is generated to facilitate steam penetration. When the pressure reaches 105.95 kPa, temperature of 132°C, 4 ~ 5 minute are enough for sterilization.

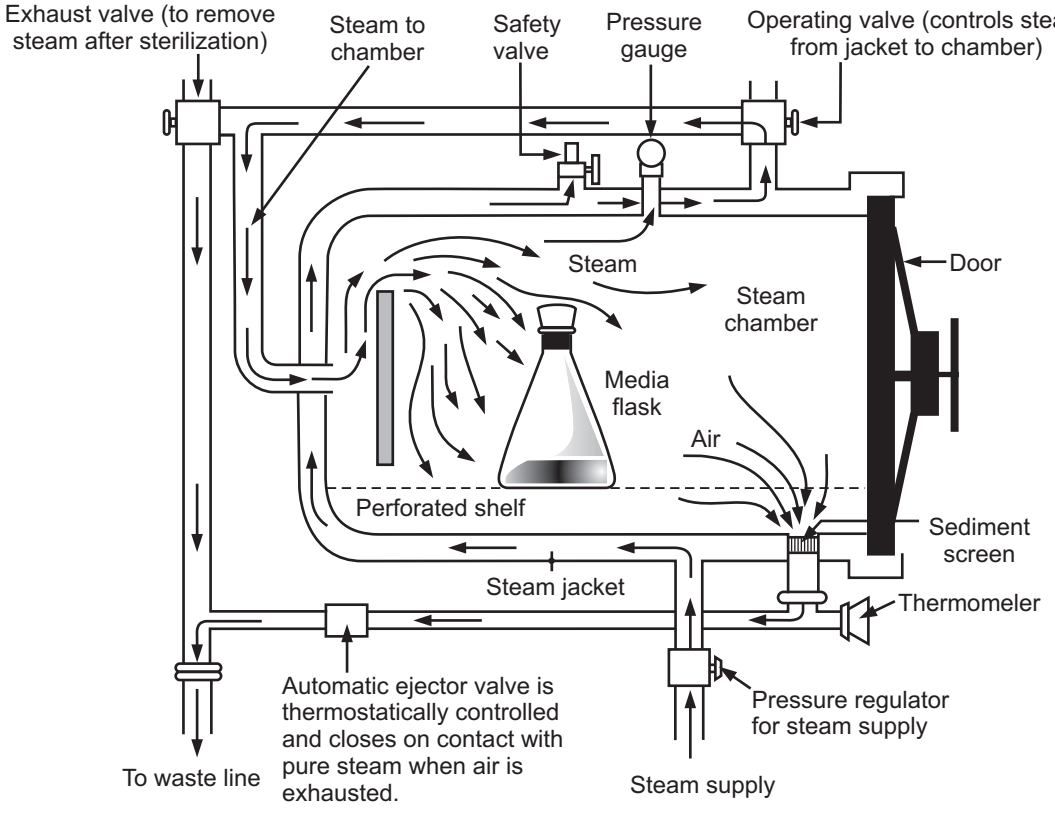


Fig. 2.4: Principle of Autoclave

Advantages of Autoclave:

- It is rapid and effective.
- It destroys microorganisms more efficiently than dry heat and therefore a shorter exposure at a lower temperature is possible.
- It can be used for a large proportion of the official injections.
- It is supplied with dry saturated steam porous materials and can be sterilized without damage.
- Equipment or components of rubber and certain plastics such as nylon and P.V.C will withstand the conditions.

Disadvantages of Autoclave:

- Items sensitive to heat cannot be sterilized.
- It is unsuitable for anhydrous materials such as powders and oils.
- It cannot be used for injections and articles such as plastics that deteriorate at 115°C.

Advantages of Moist Heat:

- It has good penetration.
- It maintains integrity of liquids due to the 100% humidity within the chamber.

Disadvantages of Moist Heat:

- Non-stainless steel metal items corrode in moist heat.
- It may damage rubber and plastic items.
- This method is not suitable for oils, fats, ointments, oily injections etc.

Applications of Moist Heat Sterilization:

- This method is most essential biocidal agent. It is used for surgical dressings, sheets, surgical and diagnostic equipments, containers, closures, aqueous injections, ophthalmic preparations etc.

There are some differences between dry heat and moist heat sterilization (Table 2.4).

Table 2.4: Differences between dry heat and moist heat sterilization

Moist heat	Dry heat
1. It has water and steam.	1. It do not require water and steam.
2. It is based on protein denaturation and coagulation.	2. It is based on protein denaturation.
3. Latent heat liberated when steam condenses on cooler surface.	3. It is based on oxidative damage.
4. Spores are killed by exposure to heat at 121°C for 10-30 minutes.	4. Spores are killed by exposure of temperature at 160°C for 1 hour.
5. This process is under pressure.	5. This process is on direct flame.
6. This process takes less time.	6. This process takes more time.
7. It is mainly dependent on boiling and autoclaving.	7. It is mainly dependent on flame and incineration.

Sunlight:

It possesses bacterial activity. The action is due to its content of UV rays. Direct sunlight has a powerful germicidal action. Exposure to direct sunlight for a sufficient time will kill spores as well as vegetative cells. Tuberculosis germs are killed in few hours. UV rays destroy bacteria in a few seconds or minutes; molds and yeasts are somewhat more resistant. Ultraviolet light is also used for purification of drinking water and swimming pool water.

Ultrasonic Vibration:

It is the sound waves above the frequencies. Vibrations have the property to disrupt the cells. Gram negative rods are more sensitive to ultra sonic vibration whereas Gram positive cocci, spores of fungi and resistant to the vibration. The current trend is to use ultrasonic as a cleaning agent to follow the process by sterilization in an autoclave.

2.5 MECHANICAL METHOD OF STERILIZATION

Filtration:

Filtration allows for the exclusion of organisms based upon size. There are many types of filtration techniques, but when sterilizing a system membrane filtration is used. To obtain a sterile filtrate it is necessary that the filter and all connecting parts likely to come into contact with the filtrate must be sterile. Contaminants are traps on the membrane filter due to larger size than the pore size of the membrane. It is useful for antibiotic solutions, sera, carbohydrate solution etc. This method does not destroy but removes the microorganisms. It is used for both the clarifications and sterilization of liquids and gases as it is capable of preventing the passage of both viable and non-viable particles.

The major mechanisms of filtration are sieving, adsorption and trapping within the matrix of the filter material. Based on the pores size of the membrane filter, various types of microbes are trapped. Like, 0.01 μM is used to trap smallest viruses, 0.025 μM is used to trap larger viruses, 0.45 μM is used to trap largest bacteria, 1.2 μM is used to trap protozoa and smallest unicellular algae etc.

Some observations are essential for successful sterilization by filtration:

1. The whole of the apparatus must be sterile.
2. An aseptic technique should be followed in order to minimize the risk of contamination.
3. The assembly of filtration unit should be so adjusted that there is minimum exposure of filtrate to the atmosphere.
4. The filter selected must be fine enough to obstruct the passage of all bacteria.

Four steps for sterilization by filtration:

1. Filtration of the solution through one of the bacteria-proof filters.
2. Aseptic distribution of the filtered solution into the previously sterilized final containers.
3. Aseptic closure of the containers.
4. Performing the sterility test.

There are various types of filtrations used for various purposes viz. HEPA filter, sintered glass filters, colloidal or membranous filtrates, earthenware candles etc. The various types of bacteria proof filters used are as follows.

1. **Ceramic filters:** These are also known as filter candles, made of porcelain or kieselguhr and are available in a range of pore size. Kieselguhr filters are usually softer than the porcelain type. The candle is placed in the solution to be sterilized and its opening is attached to the vacuum system. When vacuum is applied the pressure inside the candle is decrease. Due to difference in pressure between the outside and inside of the candle, the solution moves into the candle. The filtrate is collected in sterile container. The main disadvantage of ceramic filter is its tendency to absorb materials from aqueous solutions.
2. **Seitz filter:** It consists of two parts. The lower part holds a perforated disc and the upper part is compressed asbestos sheet. Two parts are joined together with the help of nuts. There is a valve on the upper part through which vacuum is applied. Due to the fibrous nature of asbestos pads, it may shed fibres into the filtrate and also absorb drugs from solution. Hence, few ml of filtrate should always be rejected and sintered glass disc may also be fixed in the filtration unit immediately after seitz filter.
3. **Sintered glass filters:** They are made from borosilicate glass. The glass is finely powdered and particles of the required sizes are separated and are then packed into disc moulds. These discs are fused to funnels of suitable shape and size. Sintered glass filters are available in different pore sizes and are numbered accordingly. For bacteria proof filtration number 5 or 3 is used. The filtration is carried out under a reduction pressure. Sintered glass filter do not absorb the medicaments from the solution. These filters are made from borosilicate glass, so change of pH of the solution not occurred.
4. **Sintered metal filters:** They are the metallic counter part of sintered glass filters. These are usually made from stainless steel. They have the advantage of having greater mechanical strength.
5. **Membrane filters:** These are made of cellulose acetate or cellulose nitrate. These are fixed in metallic holders similar to those used with asbestos pads. The pore size in the membranes lies in the range of 100-150 μ. They are also called millipores filters. They are suitable for sterilizing aqueous and oily solutions but are not suitable for organic solvents like alcohol, ketones, esters or chloroform.
6. **Air filter: (HEPA filter):** It is high efficiency particulate air or originally called High-Efficiency Particulate Absorber (HEPA). It is used to describe filters that are able to trap 99.97 per cent of particles that are 0.3 microns (Fig. 2.5). Air particles are circulated through HEPA filter by four directions viz.
   (a) Direct Impaction: Large contaminants, such as certain types of dust, mold, and pollen, travel in a straight path, collide with a fibre, and stick to it.

(b) Sieving: The air stream carries a particle between two fibres, but the particle is larger than the gap, so it becomes ensured.

(c) Interception: Airflow is nimble enough to reroute around fibres, but, thanks to inertia, particles continue on their path and stick to the sides of fibres.

(d) Diffusion: Small, ultrafine particles move more erratically than larger ones, so they are more likely to hit and stick to fibres (Fig. 2.5).

Advantages:

- The method is suitable for sterilization of thermolabile medicaments, such as, blood products, insulin and enzymes.
- All types of bacteria i.e., living as well as dead, are removed from the preparation.
- Both clarification and sterilization are done side by side.
- It is an excellent method for the rapid supply of a small volume of a parenteral solution in an emergency.

Disadvantages:

- The method is not a reliable one and therefore a sterility test is necessary.
- The suspension and oily preparations cannot be sterilized by this method.
- There are chances of absorption of medicaments from a solution by the filter.
- Defects in the media are not immediately detectable.
- Aseptic technique is necessary.
- Highly trained staff is required.
- The process is only suitable for medicaments which are in solution form.

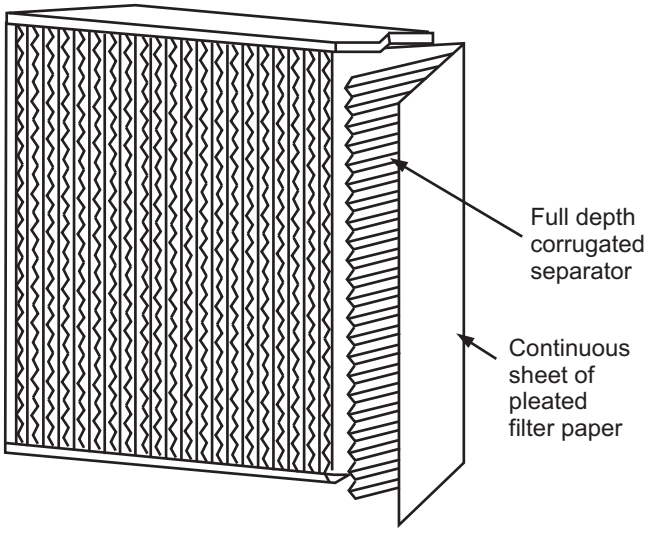


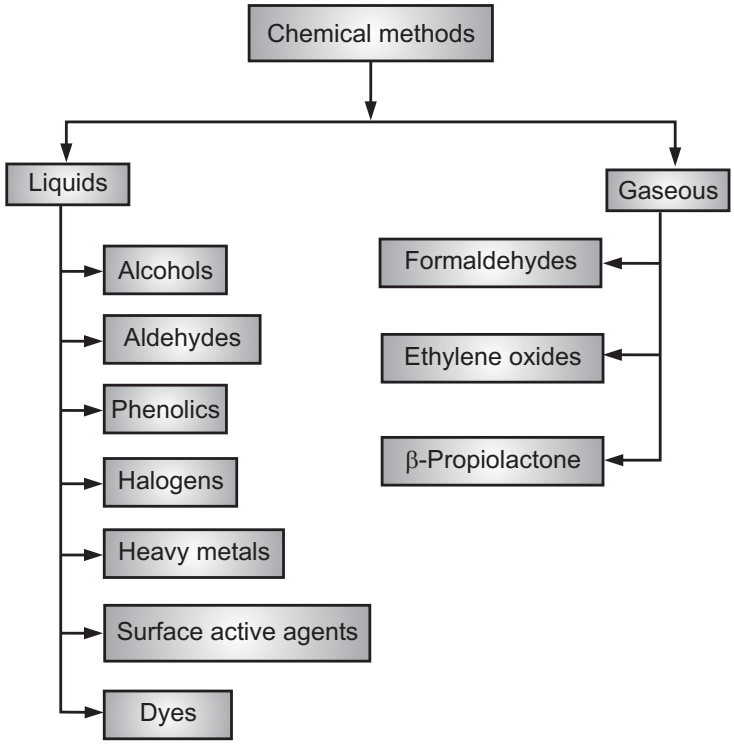
Fig. 2.5: HEPA filter

Applications:

This filtration method is useful for sterilization of parenteral solutions containing thermolabile medicaments without any decomposition e.g. insulin, blood serum and other products containing protein matters, heat sensitive injections, biological products etc.

2.6 CHEMICAL METHODS OF STERILIZATION

Disinfection is known as partial removal of pathogens whereas complete removal of microorganisms is called sterilization. Chemical sterilization is the process of elimination of all viable microorganisms and their spores. It is of two types viz. using liquids and gaseous compounds. Liquid sterilization involves submerging equipment in a chemical fluid for enough time to kill all viable microorganisms and their spores. Gas sterilization involves exposing equipment to chemical gases in an enclosed heated or pressurized chamber. Liquid sterilizing agents are impractical or ineffective for sterilizing items. Gaseous agents are more effective sterilants because they are able to permeate small openings and crevices easily. Gas chemicals also sterilize faster than liquids because they usually are combined with high heat. Gas residue is also easier to remove from sterilized articles but requires more expensive equipment.



Flowchart 4: Types of chemical methods of sterilization

Ideal Properties of Chemical Sterilization:

- Wide spectrum of activity.
- Active in presence of organic matter.
- Effective in acid as well as alkaline media.
- High penetration power.
- Stable.
- Speedy action.

- Compatible with other antiseptics and disinfectants.
- Safe and easy to use.
- Cheap and easily available.
- Not corrode metals.

Mode of Action of Chemical Disinfectants:

- It acts by protein coagulation.
- Disruption of cell membrane resulting in exposure, damage or loss of the contents.
- Removal of free sulfhydryl group essential for the functioning of enzymes.
- Substrate competition: A compound resembling the essential substrate of the enzyme diverts the enzymes necessary for the metabolism of the cell and causes cell death.

Alcohol:

Mainly ethanol (80% v/v ethanol) or isopropyl alcohol (60-70% v/v) solutions are used to disinfect skin and decontaminate clean surfaces. Methanol is also used as disinfectant but less bactericidal than ethanol and highly poisonous. Other alcohols such as propyl, butyl, amyl alcohols are more powerful germicidal than ethanol. Alcohols are effective against fungi, vegetative bacteria, mycobacterium species and some lipid containing viruses. They are more effective at concentration 70% in water. In tinctures, alcohols enhance the effectiveness of other antimicrobial chemicals.

Principle: It is based on denaturing coagulating proteins and dissolving membrane lipid of microorganisms.

Advantages:

- It is inexpensive and non-toxic.
- Widely available.
- Rapidly effective.
- Active against bacteria, viruses, mycobacterium.

Disadvantages:

- Not effective against bacterial spores.
- Not effective with organic materials.

Aldehydes:

They are low molecular weight compounds and act as antimicrobial. The most important two aldehydes are formaldehyde and glutaraldehyde. 2% solution of glutaraldehyde is known as Cidex which is used for bactericidal and viricidal in 10 minutes and sporicidal in 3-10 hours. Both these compounds are highly microbicidal and also kill the spores. Formaldehyde is also a common aldehyde which is a gas in high concentration but at room temperature it polymerizes and form solid substance. Formaldehyde solution as well as in gaseous form is used for sterilization and disinfection of enclosed area respectively where vegetative cells are killed more quickly than spores. Formalin and paraformaldehyde are two important sources of formaldehyde when it is used for gaseous disinfection. Formalin is the aqueous solution of 40% formaldehyde. Formalin is extensively used for preservation of specimens, inactive viruses and bacteria in vaccines.

Principle:

Aldehyde combines with important proteins and nucleic acids of the bacterial cells. These interactions of aldehyde with these cellular substances produce antimicrobial action. Formaldehyde inactivates microorganisms by alkylating the amino acids and sulfhydryl groups of proteins and ring nitrogen atoms of purine bases.

Advantage:

- They have good activity against spores, viruses and fungi.

Disadvantages:

- They are toxic.
- They need long exposure time for the action, minimum 3 hours.
- Freshness and pH are critical.
- They are irritant and carcinogenic.
- Glutaraldehyde is moderately active against TB.

Phenolics and Phenolic Agents:

Phenol or carbolic acid was first used by Lister as a disinfectant. Phenolics are the chemical derivatives of phenols. They are very effective disinfectants. Examples: cresols, biphenols etc. Cresols are derived from coal tar. A 5% aqueous solution of phenol rapidly kills the vegetative cells of microorganisms but spores are resistant. Phenols are used as standard chemical to determine antimicrobial activity of other similar chemical compounds by Phenol coefficient method. This method is used for evaluating the effectiveness of disinfectants. Especially *Staphylococcus aureus* or *Salmonella typhi* are used for testing.

Hexylresorcinol is a phenolic derivative which is marketed in a solution of glycerin and water, acts as strong surface tension reductant as well as high bactericidal activity. Pure crystalline phenol is colourless, its 5% aqueous solution is used as disinfectant of sputum, urine, feces etc. Quaternary ammonium compounds e.g. cetrimide is used in combination with other agents and acts as good detergent properties. Some other examples of phenolic compounds are amyl phenol, benzyl-4-chlorophenol etc.

Principle:

They are having various modes of action. They act by destroying plasma membranes and denature proteins. They also affect plasma membrane, inactivate enzymes, and denature proteins. Some phenolics are mild enough for use as antiseptics.

Advantages:

- They are stable, persist for long times after application, and remain active in the presence of organic compounds.
- They are active against wide range of organisms.
- They have good antimicrobial activity and are rapid bactericidal.
- They are more active in acid pH.
- They remain active on surfaces long after application.

Disadvantages:

- They are having caustic effects on skin.
- They are having systemic toxicity.
- They are not effective against spores.

- They are not effective at low temperature.
- They are incompatible with non-ionic and cation surfactants.
- They have a disagreeable odor

Halogens:

Halogens, mainly, iodine and chlorine are used as antimicrobial agents. Iodine is used as antiseptic against all microbes, fungi and viruses. It inhibits protein synthesis and oxidizes sulphydryl group of amino acids. There are various preparations of iodine such as 2% iodine plus 2% sodium iodide diluted in alcohol, 7% iodine plus 5% potassium iodide in 83% alcohol, 5% iodine plus 10% potassium iodide in aqueous solution. Iodine is also used in the form of iodophors. Iodophors are mixtures of iodine with surface active agents, used for germicidal activity. Chlorine is used as disinfectant. Hypochlorous acid (HOCl) is an aqueous solution of chlorine which is used as disinfectant. Some other compounds like calcium hypochlorite [$Ca(OCl)_2$], sodium hypochlorite (NaOCl) etc. are the preparation of chlorine. Chloramines are also chlorinated compound (chlorine and ammonia) which acts as antiseptics.

Principle:

Iodine is an oxidizing agent. These agents can irreversibly oxidize and inactivate essential metabolic compounds like proteins with sulphhydryl groups. It also has halogenations action of tyrosine unit of enzymes and other cellular proteins require tyrosine for activity.

Chlorinated compounds show antimicrobial activity due to formation of hypochlorous acid in water. This hypochlorous acid is further decomposed in to nascent oxygen which shows strong oxidation reaction.

Advantages of Iodine compounds:

- Iodine compounds are effective against gram positive bacteria.
- They produce residual activity.
- They retain microbial action in the presence of organic debris.
- They are available in solutions, sprays and in gel preparations.

Disadvantages of iodine compounds:

- Iodine compounds when used alone are major irritants.
- They are weak against myco bacteria, fungi, viruses.
- They are absorbed into skin and can be toxic.
- They can cause 1^{st} and 2^{nd} degree burns.

Advantages of Chlorine Compounds:

- They are cheap and readily available.
- Chlorine is highly soluble in water.
- They leave a residue in solution.
- They are toxic to most microorganisms.
- They remove iron and manganese and ammonia nitrogen during oxidation.
- They destroy taste and other odour compounds.

Disadvantages of Chlorine Compounds:

- Chlorine is a poisonous and toxic gas.
- They are corrosive, requires special non-metal conduits.

- They require careful handling, operation and storage.
- The vapours of chlorine are irritant.
- They are strong oxidizing agents, react with most elements and compounds.

Heavy Metals:

Most of the heavy metals have detrimental effect on microorganisms. The compounds such as mercury, silver, copper are more effective against microorganisms. Mercury compounds such as mercuric chloride, mercurous chloride, mercuric oxide are in dilution form act as bactericidal. They are highly toxic to animals but used in ointments as antiseptics. Some of the organic compounds of mercury are Mercurochrome, metaphen, mercresin are used as antiseptics. They are less irritants and less toxic.

Silver compounds such as silver nitrate, silver lactate, silver picrate act as bacteriostatic as well as bactericidal when these metals are in contact with proteins of microorganisms. Silver nitrate is mainly used as antiseptics and other compounds are act as germicidal.

Copper compound such as copper sulfate is more effective against algae and molds than bacteria. 2 ppm in water prevents algal growth. It is used in the form of Bordeaux mixture as a fungicide. Zinc salt controls infection caused by anaerobic bacteria.

Principle:

Heavy metals and their compounds have antimicrobial activity due to combination with cellular proteins and inactivating their function. Example: mercuric chloride inhibits the action of enzyme by acting on sulfhydryl group of enzyme and form inactive enzyme. High concentration of heavy metal salt coagulates cytoplasmic proteins of microorganisms that cause death of the cells.

Advantages:

- They are powerful biocides.
- They form complex with proteins of microorganisms and converts inactive form of microorganisms.

Disadvantage:

- They are highly toxic to the animals.

Surface Active Agents:

Soaps and detergents are mainly used as surfactant. It depends on the alkali content in the soap how they show their action. They act as germicidal for pneumococci, streptococci, gonococci etc. Detergents are ionized in water and are made up of fats. They quickly dissolve in cold and hard water. Quaternary ammonium compounds are important cationic detergents which act as germicidal as well as bactericidal, mainly against Gram positive bacteria. They also show fungicidal activity and destroy the pathogenic protozoa. Benzalkonium chloride is used as disinfectant. Diaparene chloride acts as bacteriostatic against *Brevibacterium ammoniagenes*.

Principle:

They denature proteins, interference with glycolysis and damage membrane of the microorganisms. They also damage the cell cytoplasmic membrane and alter the cell structure.

Advantages:

- They are effective against vegetative bacteria.
- They are widely available.
- They are less expensive.
- They are non-irritant.

Disadvantages:

- They are ineffective against spores.
- They are not effective against non-enveloped viruses.
- They may become contaminated.
- They are easily inactivated by presence of anionic detergents, soaps and hard water.

Dyes:

There are two types of dyes exhibiting antimicrobial activity viz. triphenylmethane and acridine. Triphenylmethane dyes such as brilliant green, crystal violet, malachite green inhibit gram positive bacteria and fungi. Malachite green is used to inhibit *Staphylococcus aureus*.

Acridine dyes are the compounds that are derived from acridine such as acriflavine, tryptoflavine, proflavine etc. They inhibit bacteria especially Staphylococci and gonococci. Gonococci specially are inhibited by tryptoflavine. Acriflavine, proflavine are used to inhibit the growth of Gram negative bacteria.

Principle:

They have their inhibitory effect by interfering with cellular oxidation processes.

Advantages:

- They are bacteriostatic in high dilution.
- They are more active against Gram positive bacteria.

Disadvantages:

- They are less active against Gram negative bacteria.
- They have low bactericidal activity.

2.7 GASEOUS AGENTS FOR STERILIZATION

There are some important medical devices which are required for sterilization but not possible through heat sterilization due to damage of the materials. In such cases, gaseous sterilization is required. Like Plastic syringe, blood transfusion apparatus, plastic pipettes, petri dishes etc. The main gaseous agents are used for sterilization are ethylene oxide, formaldehyde, beta-propiolactone etc. They act on principle of denaturing proteins and DNA by cross linking functional groups. But this method has some disadvantages.

Disadvantages:

- Gas can be hazardous to people.
- They often highly explosive.
- Some time gases are extremely poisonous.
- They are potentially carcinogenic.

Factors affecting gaseous sterilization:

- Efficiency of sterilization method is influenced by the concentration of ethylene oxide.
- The humidity of the sterilizing atmosphere.

- The temperature of sterilization.
- Time of exposure.
- Physical nature and permeability of the load.
- Atmospheric preconditioning of the load before sterilization.

Formaldehydes:

It is also a group of alkylating agent. It inactivates microorganisms by alkylating the amino acid and sulfhydryl groups of proteins and ring nitrogen atoms of purine bases. It is a gaseous disinfectant and biocide. It is a strong, broad-spectrum disinfectant and biocide that has the ability to kill bacteria, viruses, fungi and endospores. It is very irritating to living tissues and also carcinogenic, therefore it is not used as an antiseptic.

Ethylene Oxides:

It is a type of alkylating agent that is used for gaseous sterilization. It is highly penetrating and can sterilize items within plastic bags such as catheters, disposable items in laboratories and clinical settings.

- It is gaseous above 10.8°C.
- Ethylene oxide have high antimicrobial activity, it kills even endospores.
- It is used for sterilization of heat sensitive materials such as spices, oils, plastics etc.
- Ethylene oxide is used in formulation with CO_2 as Freon (CClFe).

β-Propiolactone:

It binds to DNA, thereby inactivating it. It is a clear liquid with a strong odor and has the ability to kill endospores. It has been used in either liquid form or as a vapor for the sterilization of medical instruments and tissue grafts, and it is a common component of vaccines, used to maintain their sterility. It is also used for the sterilization of nutrient broth, as well as blood plasma, milk and water.

- It is gas above 15.5°C.
- Penetration power of β-propiolactone is less than ethylene oxide but it is more active in killing microorganisms.
- Due to its carcinogenic effects, it is not commonly used.

Applications of Gaseous Sterilization:

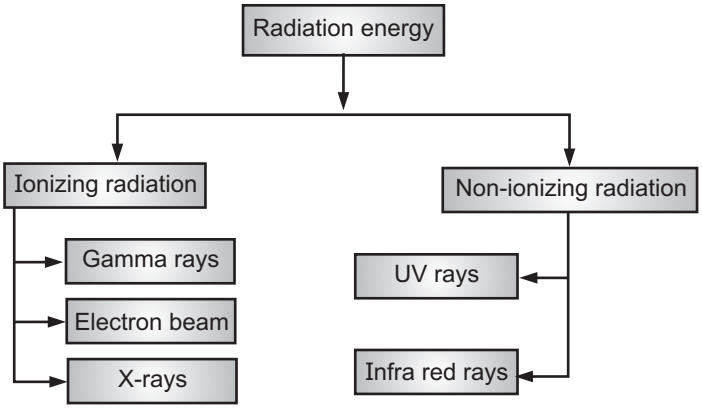
- This method is used for sterilizing thermolabile substances like hormones, proteins, various heat sensitive drugs etc.

2.8 RADIATION METHOD OF STERILIZATION

This type of method is carried out for surface sterilizations. Many types of radiations are used for sterilization like electromagnetic radiation (examples: UV rays and Gamma rays). There are two types of radiations used for sterilization, ionizing radiation and non-ionizing radiation. Ionizing radiation is the use of short wavelength, high-intensity radiation to destroy microorganisms. This radiation is comes in the form of gamma or X-rays that react with DNA resulting in a damaged cell. Non-ionizing radiation uses longer wavelength and lower energy, and is only used for sterilizing surfaces. The most common form of non-

ionizing radiation is ultraviolet light. Gamma radiation is very penetrating and are commonly used for sterilization of medical equipments like syringes, needles etc. It is generated by cobalt 60. X-ray is the form of ionize energy allowing to irradiate large packages for sterilization of medical equipments. E-beam radiation converts X-ray for more penetration but sometimes E-beam radiation is not applied for complex products.

Infrared radiation is considered as a form of hot air sterilization. It is used for rapid mass sterilization of pre packed items like syringes, catheters etc.



Flowchart 5: Radiation sterilization

Principle of Ionized Radiation:

It causes death or mutations in microorganisms as they damage the DNA and protein.

Principle of Non-ionized Radiation:

Cellular DNA absorbs the energy by purine and pyrimidine bases when microorganisms are comes in contact with UV light. During that time linked thymine molecules are unable to encode adenine on messenger RNA molecules during protein synthesis and finally no longer produce protein. UV light also inactivates viruses. UV wavelength in the 200 nm to 265 nm range is most effective in killing microorganisms.

Advantages:

- It effectively reduces the number of airborne microorganisms and kills them.
- No degradation of media during sterilization, thus it can be used for thermally labile media.
- It leaves no chemical residue.
- Its administration of precise dosage and uniform dosage distribution.
- Its immediate availability of the media after sterilization.

Disadvantages:

- This method is a more costly alternative to heat sterilization.
- This method requires highly specialized equipment.

Applications of Radiation Sterilization:

- UV light is the most lethal component in ordinary sun light used in sanitation of cloths and utensils.
- Gamma rays are used to sterilize antibiotics, hormones, sutures, plastics etc.

Table 2.5 depicts the advantages and disadvantages of overall methods with their mechanisms.

Table 2.5: Advantages and disadvantages of overall methods

Methods	Mechanism	Advantages	Disadvantages	Applications
Physical: Heat sterilization	Destroys bacterial endotoxins by denaturation of proteins and oxidative damage	Most widely used and reliable method of sterilization, involving destruction of enzymes and other essential cell constituents.	Can be applied only to the thermostable products	Dry heat is applicable for sterilizing glasswares and metal surgical instruments and moist heat is the most dependable method for decontamination of laboratory waste and the sterilization of laboratory glassware, media and reagents.
Chemical: Gaseous sterilization	Alkylation	Penetrating ability of gases	Gases being alkylating agents are potentially mutagenic and carcinogenic	Ethylene oxide gas has been used widely to process heat-sensitive devices.
Radiation: Radiation sterilization	Ionization of nucleic acids	It is a useful method for the industrial sterilization of heat sensitive products.	Undesirable changes occur in irradiated products, an example is aqueous solution where radiolysis of water occurs.	Radiation sterilization is generally applied to articles in the dry state; including surgical instruments, sutures, prostheses, unit dose ointments, plastics
Mechanical: Filtration sterilization	Does not destroy but removes the microorganisms	It is used for both the clarification and sterilization of liquids and gases as it is capable of preventing the passage of both viable and non-viable particles.	Does not differentiate between viable and non-viable particles	This method is Sterilizing grade filters are used in the treatment of heat sensitive injections and ophthalmic solutions, biological products, air and other gases for supply to aseptic areas.

2.9 EVALUATION OF THE EFFICIENCY OF STERILIZATION METHODS

Evaluation of sterilization methods is carried out to ensure that the product produced by design process should be of best quality. Evaluation of sterilization methods provide a high degree of assurance that indicates whether a specific process will consistently produce a product that will meet it predetermined specifications and quality assurance. So this action proves that any procedure, process, equipments, material activity or system actually leads to the expected result and produce quality product.

Process of Microbial Destruction:

Destruction of microbes are carried out by several methods such as heat, chemical, and radiation sterilization are used. Upon exposure of such treatment, microorganisms die according to logarithmic relationship between concentration or population of the living cells and the time exposure or radiation dose. The relationship between microbial population and time may be linear or non-linear. The D value or time required or dose required for one log reduction in microbial population is calculated from the plots (Fig. 2.6).

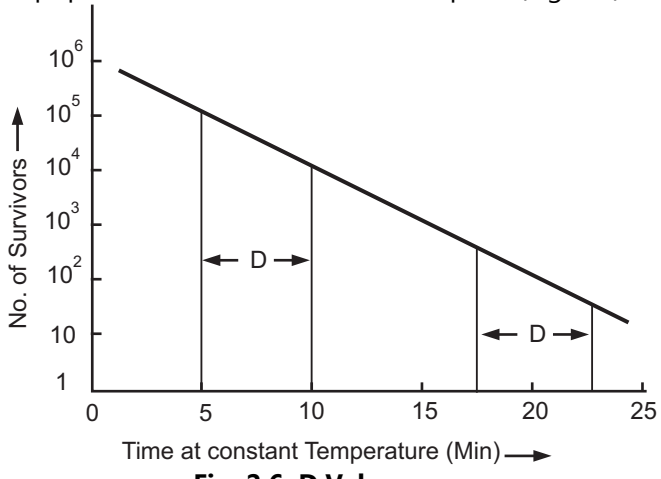


Fig. 2.6: D Value curve

D value:

It is the rate of killing of microorganism. It is used to determine the time of contact for any sterilization process. It determines the time required to reduce the microbial population by one decimal point i.e. it is the time required for 90% reduction in the microbial population. Hence, the time or dose it takes to reduce thousand microbial cells to hundred cells is the D value.

Importance of D value validation:

- It is specific for each microorganism in environment subjected to specific sterilizing agent or condition.
- The knowledge of D value at different temperature in heat sterilization is necessary for the calculation of Z value.
- The D value is used in the calculation of biological factor F.
- Extra-polation of D value predicts number of log reduction of microbial population.

Factors affecting D value:

- The type of microorganism used as biological indicator.
- The formulation component and characteristics.
- The surface on which the microorganism is exposed.
- The temperature, gas concentration and dose of radiation.

D value is determined by:

- **Survival curve method:** The survival curve method is based on plotting the log number of the surviving organism versus independent variable such as time, gas concentration or radiation dose.

- **Fraction negative method:** In this method, sample containing similar spore population are treated in an identical environment and the number of sample still showing microbial growth after treatment and incubation are determined. Data obtained by survival curve method are plotted semi-logarithmically.

  Data points are connected by least square analysis.

$$\textbf{Log N = a + bt}$$

Where, N is number of surviving organism, t is time, a is γ intercept and b is slope of line as determined by linear regression.

D value is the reciprocal of linear slope **D = 1/ b**.

Z value:

This value is used in the validation of heat sterilization process. The Z value is the reciprocal of slope resulting from the plot of the logarithm of D value versus the temperature at which the D value was obtained. The Z value may be defined as the temperature required for one log reduction in the D value. The accepted standard (Z value) for steam sterilization of *Bacillus stearothermophilus* spores and dried heat sterilization for *Bacillus subtilis* are 10°C and 22°C respectively. These plots are important because one can determine D value of the indicator microorganism at any temperature of interest. The magnitude of slope indicates the relative degree of lethality as temperature is increased or decreased (Fig. 2.7).

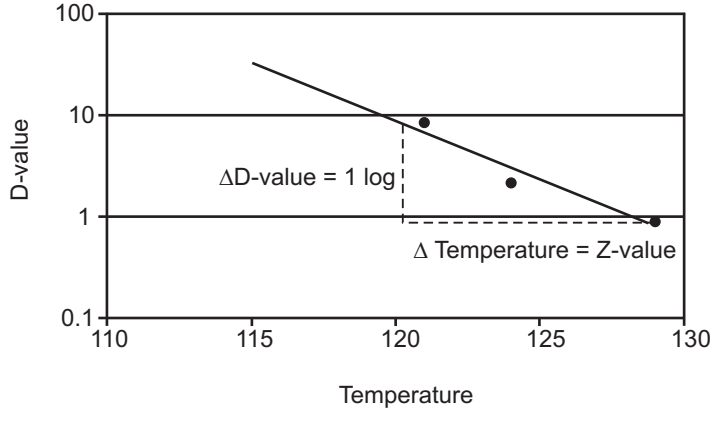


Fig. 2.7: Z-value determination

F value:

It is the time in minutes at a specific temperature is needed to kill a population of cells or spores. The F value measures equivalent time, not clock time that a monitored article is exposed to the desired temperature e.g. 121.1°C. Mathematically, the F value is expressed by the rate of motility per minute in function of temperature for a given pressure.

F value is calculated from the following equation:

$$\textbf{F} = \Delta t \sum 10^{(T-To)/Z}$$

Where, Δt is the time interval for the measurement of product temperature t, T is reference temperature T_o is 121.1°C for steam sterilization.

Example: Food heated at 121.1°C for 2 minutes will give a value of F = 2. To get the same F-value of 2 using 111.1°C, the food need to heat for 20 minutes.

2.10 EQUIPMENTS EMPLOYED IN LARGE SCALE STERILIZATION

(A) Moist heat sterilization:

Autoclave: The object to be sterilized is exposed to saturated steam under 1 Atmosphere pressure at a minimum temperature of 121°C for 15 minutes where microorganisms are destroyed by cellular protein coagulation. It is a device to sterilize equipments and supplies by subjecting them to high pressure saturated steam at various temperatures and times (Table 2.6). Two types of autoclaves are used likely portable autoclave and stationary autoclave. Stationary autoclave is used as large scale sterilizer.

Table 2.6: Working condition of an Autoclave at various temperatures and times

Temperature (degree C)	Pressure (lb/sq.inch)	Time (Minutes)
115-118	10	30
121-124	15	15
126-129	20	10
135-138	30	3

Working of Autoclave:

All autoclaves operate on similar principles that they share with a common kitchen pressure cooker - the door is locked to form a sealed chamber, and all air within the chamber is replaced by steam. The steam is then pressurized to reach the desired sterilization temperature and time, before exhausting the steam and allowing the goods to be removed. The various phases of a sterilization cycle are given below (Fig. 2.8).

1. **Purge Phase:** Steam flows through the sterilizer beginning the process of displacing the air; temperature and pressure ramp slightly to a continuous flow purge.
2. **Exposure (Sterilization) Phase:** During this phase, the autoclaves' control system is programmed to close the exhaust valve causing the interior temperature and pressure to ramp up to the desired set point. The program then maintains the desired temperature (dwells) until the desired time is reached.
3. **Exhaust Phase:** The pressure is released from the chamber through an exhaust valve and the interior is restored to ambient pressure, although contents remain relatively hot.

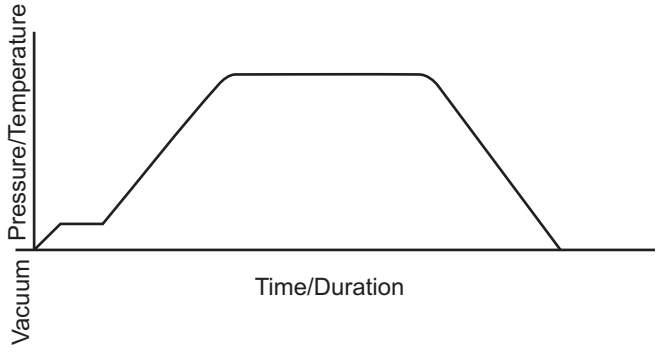


Fig. 2.8: Phases of sterilization cycle

(B) Dry heat sterilization:

Hot air oven: It is an electrical device, and uses dry heat to sterilize. It was originally developed by Pasteur. Generally, it can be operated from 50 to 300°C, using a thermostat to control the temperature. It requires direct heat at different times and temperatures (Table 2.7).

Table 2.7: Working condition of hot air oven

Temperature (degree C)	Time (Minutes)
170	60
160	120
150	150
140	180

Working:

The working of the hot air oven is based on the hot air inside the chamber of oven by the forced circulation. When hot air reaches top of chamber, it is circulated back to bottom by a fan installed inside the chamber and hence optimum amount of heat is achieved gradually inside the hot air oven. After heating the content of the oven for two hours at 160°C, the articles are allowed to remain there, till the temperature comes down to 40°C. then the sterilized materials is then removed from the oven.

Pharmaceutical Applications:

1. It is mainly used for the sterilization of glasswares such as pestle and motar, Petri dishes, flasks, pipettes, bottles, test tubes etc.
2. It is used for the sterilization of powders such as sulphacetamides, sulphadiazenes, kaolin, zinc oxide, starch etc.
3. Injections where fixed oils is used as the vehicle, are sterilized by the dry heat method like, injections of progestrone, injection of testosterone propionate and injections of oestradiols dipropionate.
4. It is also used for sterilization of scalpels, scissors, spatula, blades and glass syringes.
5. The chemicals, glassware in laboratories, research institutions, industries, hospitals and R&D centers used hot air ovens are suitable for temperature upto 250°C.
6. Hot air ovens suits to various applications like heating, drying, sterilizing and baking.

Microwave: Electromagnetic radiation is transmitted in waves or particles at different wavelengths and frequencies. This broad range of wavelengths is known as the electromagnetic spectrum. The spectrum is generally divided into seven regions in order of decreasing wavelength and increasing energy and frequency. Electromagnetic energy in the microwave region (225 MHz to 100 GHz, especially 2,450 MHz) is extensively used as an alternative energy sources for sterilization. In microwave oven heat is a byproduct and is controlled by temperature probes where sterilization is also achieved at 40°C.

Sterilization methods are carried out with the use of microwaves where a bactericidal solution is used for heating. The technique is being promoted for use with dental instruments

and relies on heating a solution of a quaternary ammonium compound (benzylkonium chloride) to approximately 100°C. At present, these processes are applicable only to unpackaged instruments. Dry spores of *Bacillus subtilis* and *B. niger* are treated with heat simultaneously in a convection dry-heat oven and a microwave oven.

Principle:

Microwaves are radio waves and the commonly used radio wave frequency is roughly 2,450 megahertz (2.4 gigahertz). These waves are absorbed by water and other nutrients and then converted directly into atomic motion and motion is converted into heat.

Working:

The microwaves are generated in a microwave by a device called a magnetron, which converts electricity from the power outlet into microwaves. These microwaves are absorbed by water molecules from the inside materials, which are rotated by the electric field from the microwaves. As these molecules rub against each other, they heat up, and then they heat up molecules around them (Fig. 2.9).

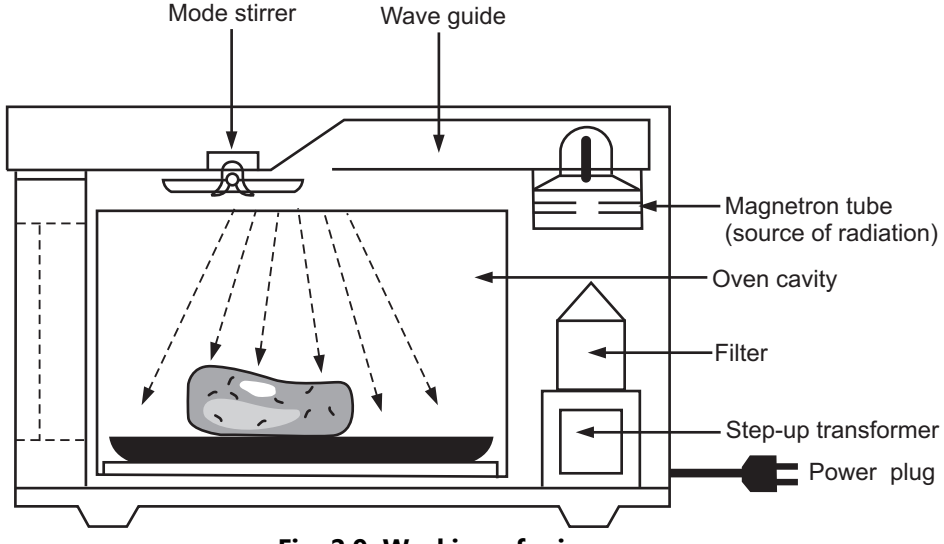


Fig. 2.9: Working of microwave

(C) Mechanical sterilization

Air filter: HEPA filter (discussed already).

(D) Colour coded biomedical waste bags: Various coloured coded bags are used for particular materials for disposal. They are discussed below (Table 2.8).

Table 2.8: Functions of various coloured bags

Yellow plastic bags	For human anatomical, animal, microbiological waste
Red disinfected container or plastic bag	Microbial waste
Blue or white plastic bag	Disposable tubing
Black plastic bags	Discarded medicines, incineration ash

2.11 STERILITY INDICATORS

Sterility Testing is defined as a test that confirms that the products are free from the presence of viable microorganisms. It is very important for medical devices, pharmaceuticals, preparations, tissue materials and other materials that claim to be free from viable microorganisms. Such conditions of materials are known as sterile materials or devices or any articles. It is an essential part of every sterilization validation. Sterility testing for products are mainly carried out by direct inoculation and by membrane filtration.

In direct inoculation method, the test article is directly inoculated into two types of media to allow for the detection of both aerobic and anaerobic microorganisms. Then after inoculation the media is incubated for 14 days and finally observed for any microbial contamination.

In membrane filtration method, the sterile enclosed units are allowed for the simultaneous filtration of equal volumes of test samples through two membrane filters. Then samples are incubated in two types of media for 14 days and finally observed for growth of any aerobic and anaerobic microorganisms. Hence, sterility indicators are required to check whether microbial growth occurs or not in terms of sterilization quality and process. So, as definition of **Sterility indicators,** are the indicators that are used for check the quality of sterilization and monitoring of the sterilization process.

2.11.1 Dry Heat Sterilization

Physical indicator: In this process, temperature record chart is made of each sterilization cycle with dry heat sterilization. This chart forms the batch documentation and is compared against a master temperature records. The temperature should be taken as the coolest part of the loaded sterilizer, further thermocouple is used for heat distribution and penetration within sterilizer.

Chemical indicator: It is based on the ability of heat to alter the chemical or physical characteristics of variety of chemical substances. Chemical indicators generally undergo melting or colour change. This change should take place only when satisfactory condition for sterilization prevails.

Biological indicator: The biological indicators are the standardized bacterial spore preparations which are usually in the form of suspension in water or culture medium or of spore dried on paper or plastic carriers, they are placed in sterilizer (Table 2.9).

Example: Clostridium species is generally used for dry heat sterilization indicator.

Table 2.9: Indicators in dry heat sterilization

Indicators	Sterilization Method	Principle	Device	Parameter monitored
Physical	Dry heat	Temperature recording charts	Temperature recording charts	Temperature
Chemical	Dry heat	Temperature sensitive coloured solution	Browne's tube	Temperature, Time
		Temperature sensitive chemical	A temperature sensitive white wax concealing a black marked	Temperature
Biological	Dry heat	Temperature sensitive microbes	*Bacillus subtilis*	D-value

2.11.2 Moist Heat Sterilization

Physical Indicator: In this process, temperature record chart is made of each sterilization cycle with dry heat sterilization. This chart of the batch documentation is compared against a master temperature records.

Chemical Indicator: It is based on the ability of heat to alter the chemical or physical characteristics of variety of chemical substances. This change should take place only when satisfactory condition for sterilization prevails. Thus, conforming that sterilization cycle has been successfully completed chemical indicator generally undergo melting or colour change.

Biological Indicator: Spores of *B. Steareothermophylus* in sealed ampoules of culture medium are used for moist heat sterilization monitoring and these may be incubated directly at 55°C, thus may eliminate the need of aseptic transfer (Table 2.10).

Table 2.10: Indicators in moist heat sterilization

Indicator	Sterilization Method	Principle	Device	Parameter monitored
Physical	Moist heat	Temperature recording charts	Temperature recording charts	Temperature
Chemical	Moist heat	Temperature sensitive coloured solution	Browne's tube	Temperature, Time
		Steam sensitive chemical	A device which is impregnated into a carrier material.	Saturated steam
Biological	Moist heat	Temperature sensitive microbes	*Bacillus Stearothermophilus*	D-value

2.11.3 Gaseous Sterilization

Physical Indicator: Gas concentration is measured independently of pressure rise, often by reference to weight of gas used.

Chemical Indicator: The Royach Sacket paper is used as chemical indicator. The indicator paper impregnated with reactive chemical which undergoes a distinct colour change on reaction. Chemical indicators are valuable monitors of the condition prevailing at the coolest of most in accessible part of a sterilizer.

Biological Indicator: They are packed in dummy packs located at strategic sites in the sterilizer. Alternatively for gaseous sterilization, these may also be placed in tubular helix device. The species of bacteria generally used for gaseous sterilization *are B. subtilis* var. niger and *B. subtilis var. golbigii* (Table 2.11).

Table 2.11: Indicators in gaseous sterilization

Indicator	Sterilization Method	Principle	Device	Parameter monitored
Physical	Gaseous	Temperature recording charts	Temperature recording charts	Temperature
Chemical	Gaseous	Reactive chemical	Indicator paper impregnated with reactive chemical	Gas concentration, Temperature, Time
		Capillary principle	Based on same migration along wick principle	Gas concentration, Temperature, Time
		Temperature sensitive chemical	A temperature sensitive white wax concealing a black marked	Temperature
Biological	Gaseous	Temperature sensitive microbes	*Bacillus subtilis*	D-value

2.11.4 Radiation Sterilization

Physical Indicator: In radiation sterilization, a plastic or perspex dosimeter which gradually darkens in proportion to the radiation it absorbs give an accurate measure of the radiation dose.

Chemical Indicator: Chemical dosimeter acidified with cerric ammonium sulphate or cerric sulphate solution is used as chemical indicator. These respond to irradiation by dose change in the applied density.

Biological Indicator: It consists of standardized bacterial spore preparation which is usually in the form of suspension in water or culture medium or of spore dried on paper or plastic carriers, it is placed in a sterilizer (Table 2.12).

Table 2.12: Indicators used in radiation sterilization

Indicator	Sterilization Method	Principle	Device	Parameter monitored
Physical	Radiation	Recording charts	Recording charts	Radiation Dose
Chemical	Radiation	Radio chromic chemicals	Plastic device impregnated with radio sensitive	Only indicates exposure to radiation

Indicator	Sterilization Method	Principle	Device	Parameter monitored
			chemicals which undergo colour changes at relative low radiation doses	
		Dosimeter device	Acidified ferric ammonium sulphate solutions responds to irradiation by dose related changes in their optical density	Accurately measures radiation doses
Biological	Radiation	Radiation sensitive microbes	*Bacillus pumilus*	D-value

Filtration Sterilization

Physical Indicator: Sterilizing filters are subjected to a bubble point pressure test. This is a technique for determining the pore size of a filter, and may also be used to check the integrity of certain types of filters. The principle of the test is that the wetted filter in its assembled unit is subjected to an increasing air or nitrogen gas pressure difference. The pressure difference recorded when the first bubble of gas breaks away from the filter is related to maximum pore size.

Biological Indicator: The test effectively measures in the ability of a filter to produce a sterile filtrate from a culture of suitable organism *S. marcesence*, a small gram negative rod shape bacterium. *B. diminuta* used as a biological indicator having a dimension 0.5 micrometer and 0.3 micrometer respectively has been used for filters of 0.45 micrometer and 0.22 micrometer (Table 2.13).

Table 2.13: Indicators in filtration sterilization

Indicator	Sterilization Method	Principle	Device	Parameter monitored
Physical	Filtration sterlization	Forcibly passing of solution through the membrane	Bubble point pressure test	Pressure
Biological	Filtration sterlization	Retention of bacteria	*B. diminuta*	Size of microorganism

QUESTIONS

Long Essays:

1. Explain various types of staining technique for bacteria.
2. Explain physical method of sterilization.
3. Explain chemical method of sterilization.
4. Explain various evaluation methods for efficiency of sterilization.
5. Explain mechanical method of sterilization.
6. Explain various sterility indicators.

Short Essays:

1. Explain IMViC test.
2. Explain acid fast staining.
3. Explain Gram staining.
4. Explain steps involved in simple staining.
5. Explain gaseous method of sterilization.
6. Explain radiation method of sterilization.
7. Explain dry heat sterilization.
8. Explain principle, working, advantages and disadvantages of hot air oven.
9. Explain principle, working, advantages and disadvantages of autoclave.
10. Explain principle, working, advantages and disadvantages of HEPA filter.
11. Explain the role of halogens as sterilizing agent.
12. Explain D-value for evaluation method for efficiency of sterilization method.

MULTIPLE CHOICE QUESTIONS

1. With Gram Iodine stain, Gram negative bacteria shows ……
   (a) Colourless (b) Purple
   (c) Red (d) None of these
2. With Safranin stain, Gram negative bacteria shows ……
   (a) Purple (b) Colourless
   (c) Red (d) Blue
3. In simple staining, the colour of Cocci is ……
   (a) Blue (b) Red
   (c) Colourless (d) Pink
4. In Gram staining, acetone is used as ……
   (a) Primary stain (b) Primary dye
   (c) Counter stain (d) Decolourizer
5. In Gram Staining Safranin is used as ……
   (a) Primary stain (b) Primary dye
   (c) Counter stain (d) Decolourizer

6. In acid fast staining, the counter stain is ……
   (a) Methylene blue (b) Alcohol
   (c) Crystal violet (d) Carbol Fuchsin
7. In acid fast staining, the primary dye is ……
   (a) Methylene blue (b) Alcohol
   (c) Crystal violet (d) Carbol Fuchsin
8. Example of acid fast bacteria ……
   (a) *Straphyllococcus aureus* (b) *E. coli*
   (c) *Mycobacterium tuberculosis* (d) *Salmonella typhi*
9. IMViC test is carried out for ……
   (a) Cocci (b) Algae
   (c) Enterobacteria (d) Protozoa
10. Methyl red test is for production of ……
   (a) Indole (b) Acid
   (c) Citrate (d) Acetoin
11. Reagent used for Indole test ……
   (a) Alpha naphthol (b) Methyl red
   (c) Kovac reagent (d) KOH
12. Alpha naphthol and KOH is used for ……
   (a) Voges-Proskauer test (b) Indole test
   (c) Citrate test (d) Methyl red test
13. Alpha naphthol is also known as ……
   (a) Kovac reagent (b) Barritt's A reagent
   (c) Barritt's B reagent (d) Van Ark's reagent
14. Bromothymol blue indicator is used for ……
   (a) Citrate (b) Indole
   (c) Acetoin (d) Acid
15. In MR-VP test, pink colour produced by ……
   (a) *E. coli* (b) *Mycobacterium*
   (c) *Klebsiella* (d) *Cocci*
16. pH of Simmons citrate Agar medium is ……
   (a) 4.9 (b) 3.9
   (c) 7.9 (d) 6.9
17. Hot air oven is example of ……
   (a) Moist heat (b) Dry heat
   (c) Both (d) None of these
18. Equipment used in moist heat sterilization is ……
   (a) Hot air oven (b) Autoclave
   (c) HEPA filter (d) None of these
19. Pasteurization is carried out at ……
   (a) < 100°C (b) > 100°C
   (c) At 100°C (d) None of these

20. Flash method is carried out at ……
    (a) 60°C for 30 mins (b) 15-20 secs at 72°C
    (c) 15-20 secs at 60°C (d) 72°C for 30 mins
21. Tyndallization is carried out at ……
    (a) 100°C for 20 mins (b) 100°C for 10 mins
    (c) 100°C for 40 mins (d) 100°C for 20 sec
22. Protein denaturation is the basic principle of ……
    (a) Dry heat (b) Moist heat
    (c) Filtration (d) None
23. Cidex is known as ……
    (a) 1% solution of glutaraldehyde (b) 2% solution of glutaraldehyde
    (c) 0.2% solution of glutaraldehyde (d) 0.1% solution of glutaraldehyde
24. Bordeaux mixture contains ……
    (a) $CuSO_4$ (b) $FeSO_4$
    (c) $(Ag)_2\ SO_4$ (d) $ZnSO_4$
25. Example of triphenyl methane dye is ……
    (a) Acriiflavin (b) Tryptoflavin
    (c) Malachite green (c) None of these
26. Freon is a Compound for ……
    (a) Ethylene oxide + O_2 (b) Ethylene oxide + CO_2
    (c) Formaldehyde + O_2 (d) Formaldehyde + CO_2
27. Z-value is used in the validation of ……
    (a) Filter sterilization (b) Gaseous sterilization
    (c) Heat sterilization (d) Radiation sterelization
28. F-value is expressed as ……
    (a) Rate of death of microorganism (b) Rate of survival of microorganisms
    (c) Rate of motility of microorganisms (d) None of these
29. Electromagnetic energy in the microwave region is ……
    (a) 2800 MHz (b) 2000 MHz
    (c) 1450 MHz (d) 2450 MHz
30. Device used as biological indicator in dry heat sterilization is ……
    (a) *Bacillus substilis* (b) *Bacillus stearothermophilus*
    (c) *Bacillus pumilus* (d) None of these

Answers:

1. (b)	2. (c)	3. (a)	4. (d)	5. (c)	6. (a)	7. (d)	8. (c)	9. (c)	10. (b)
11. (c)	12. (a)	13. (b)	14. (a)	15. (c)	16. (d)	17. (b)	18. (b)	19. (a)	20. (b)
21. (a)	22. (a)	23. (b)	24. (a)	25. (c)	26. (b)	27. (c)	28. (c)	29. (d)	30. (a)

***

Unit ... 3

STUDY OF DISINFECTANTS, THEIR EVALUATION, STERILITY TESTING OF PRODUCTS AND STUDY OF FUNGI AND VIRUSES

♦ LEARNING OBJECTIVES ♦

After completing this chapter, reader should be able to understand:

- About morphology, classification and reproduction methods of fungi and viruses.
- About disinfectants and their classifications.
- About mechanism of various disinfectants.
- About various factor affecting disinfection and antiseptics.
- About evaluation method for disinfection and antiseptics.
- About the methods for sterility testing of products as per IP, BP and USP.

3.1 FUNGI

Fungi are widely distributed organisms on earth. The study on fungi is known as **mycology**. They have great environmental and medical importance like penicillium as antibiotic but some fungi cause diseases in plants and animals (like wheat rust causes puccinia). Many fungi are free-living in soil and water. Some other fungi form parasitic or symbiotic relationships with plants or animals because they lack chlorophyll. They are eukaryotic organisms. They can grow anywhere but preferably grow in warm and humid places. Their cells contain membrane-bound organelles and well defined clear nuclei. They are unicellular as well as multicellular. They reproduce by both sexual and asexual methods. Many fungi are harmful; they cause infections of plants known as rots, rusts, blights, wilts and smuts.

Examples: Yeasts (Unicellular), Molds (Multicellular), Mushrooms (Multicellular) etc.

Importance of Fungi:

- They are decomposers and recyclers of nutrients.
- Plants live in a symbiotic relationship with mycorrhizae fungi.
- Yeasts are used to make beer and breads.
- They produce penicillin antibiotic.
- Mushroom fungi are eaten as food.
- *Aspergillus* is used to make soy sauce.
- Yeasts ferment sugar to produce alcohol.
- Trichoderma is used to produce cellulose which is used to make fruit juice.
- Genetically, engineered yeast strains are used to make proteins (Hepatitis B vaccine).

3.1.1 Morphology

Fungi are divided into two morphological forms viz. yeasts and hyphae (mold). Yeasts are unicellular nucleated and rounded fungi which reproduce asexually by budding formation (blastocondia) or fission. Hyphae are multi-cellular and filamentous fungi which reproduce asexually or sexually. Dimorphism is another condition whereby a fungus can exhibit either the yeast form or the hyphal form, depending on growth conditions.

Generally, Yeast cells are 1-5 micrometre in width and 5-30 micrometre in length which are larger size than bacteria. Commonly they are oval or egg shaped but sometimes they are elongated and spherical. Yeast cells do not have flagella (Fig. 3.1).

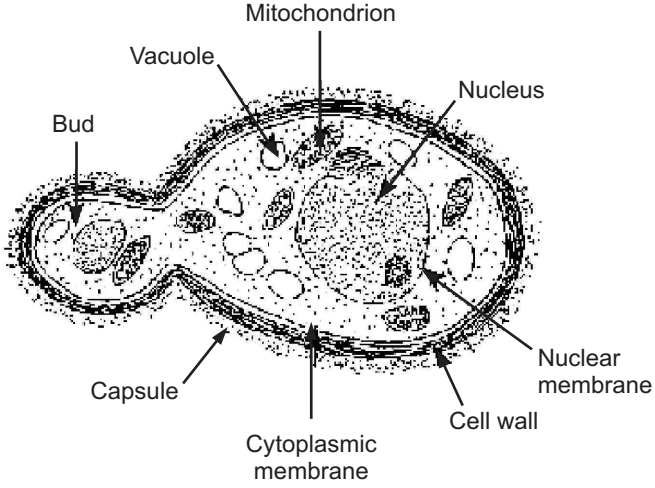


Fig. 3.1: General structure of Yeast cell

They have granular cytoplasm enclosed with cell wall which contains reserved materials like oil globules, glycogen, and volutin but the content of glycogen accumulation is increased with the decrease in fermentation. They have large nuclear vacuole with prominent nucleus and nucleolus. The cell wall is composed with chitin (1-10%) along with protein, lipid, mannan (15-23%) and glucan (50-60%). Glucan is the main structural component of cell wall which is the branched polymer of glucose i.e. beta 1, 6-glucan, beta 1, 3-glucan and beta 1, 3-beta 1, 6-complexed with chitin. Glucan and chitin are the important components that regulate cell division as well as provide rigidity to the cell wall. Mode of nutrition of fungi is

organotropic and heterotrophs and mostly are saprophytic and some are parasitic. They grow best in acidic environment. They can tolerate high sugar concentration and dry condition. Most of the fungi are obligate aerobes (molds) and few are facultative anaerobes (yeasts). Optimum temperature of growth for most saprophytic fungi is 20-30°C while parasitic fungi grow in 30-37°C.

Mold is a type of fungus which has two parts like mycelium and spores. Mycellium is made up of several filaments known as **hyphae**. The hypha is generated from spores. Each hypha is about 5-10 micrometer wide. Hyphae are composed of another tuber wall surrounded with cavity known as **lumen**. This lumen is filled with protoplasm. Between the protoplasm and wall there is a plasmalemma. Hyphae are of three types.

(i) In some molds like coenocytic fungi, the cytoplasm passes through and among cells of the hypha uninterrupted by cross wall which is known as **non-septate fungi.**

(ii) Septate with unicellular cells and

(iii) Septate with multicellular cells which contain more than one nucleus (Figs. 3.2 and 3.3).

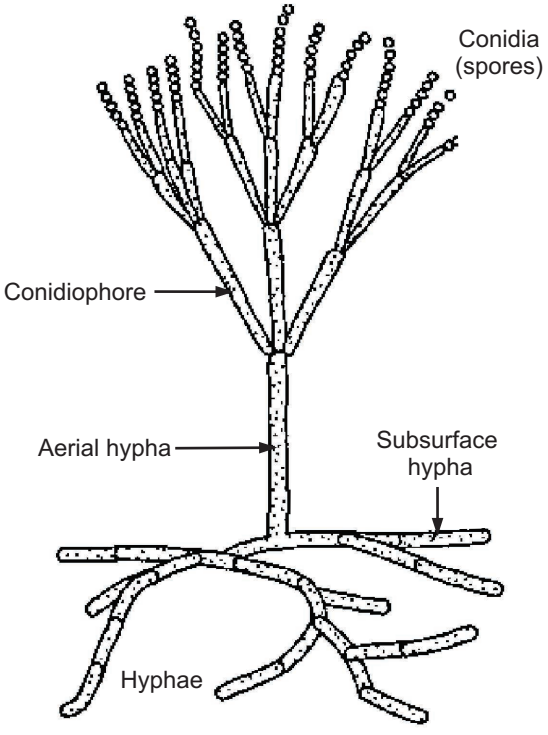
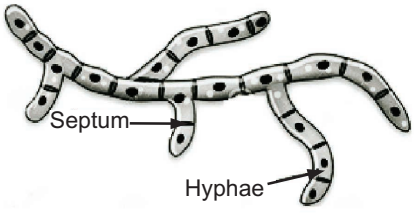
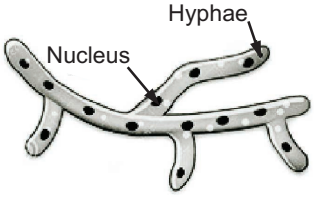


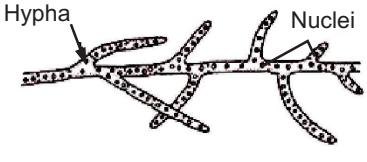
Fig. 3.2: Structure of Mold



Septate hyphae



Coenocytic hyphae



Septate with multicellular cell

Fig. 3.3: Various types of Hyphae

Dimorphic Fungi:

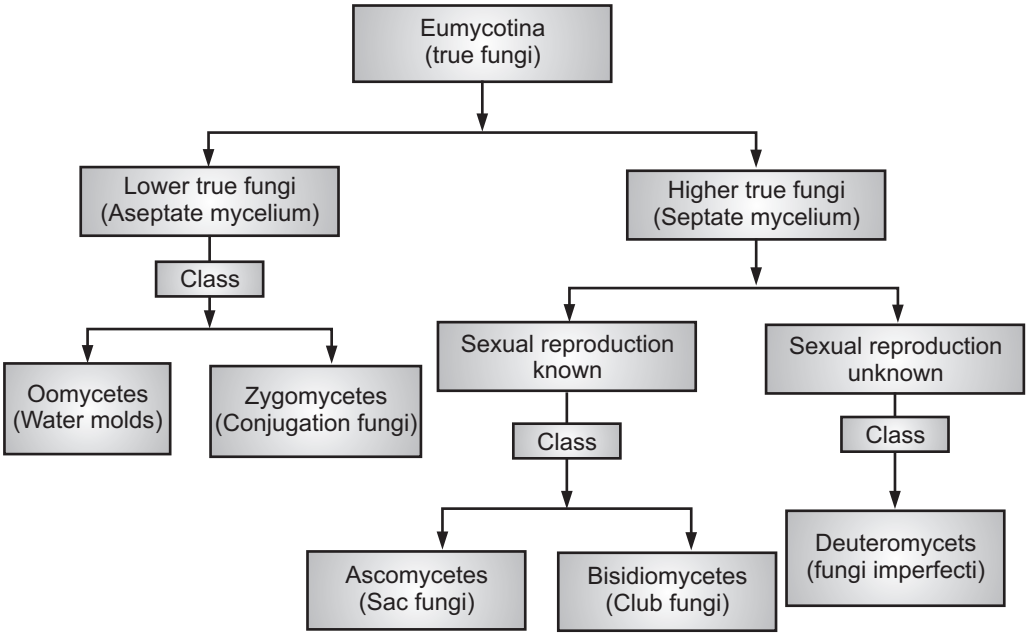
Dimorphic fungi are fungi that can exist in the form of both mold and yeast. Dimorphic fungi are fungi that can reproduce as either a mycelial or a yeast-like state. Generally, the mycelial saprotrophic form grows at 25°C, and the yeast-like pathogenic form at 37°C.

The dimorphism is important in the identification of mycoses. These fungal pathogens generally overcome the physiological and cellular defenses of the normal human host by changing their morphological form. An example: *Penicillium marneffei*, a human pathogen that grows as a mold at room temperature, and as yeast at human body temperature.

Some diseases cause by the dimorphic fungi is like blastomycosis, histoplasmosis, sporotrichosis etc. *Candida albicans* cause oral and genital infections.

3.1.2 Classification

True fungi are classified into two major classes viz. Lower true fungi and higher true fungi (Flow Chart 1).



Flowchart 1: Classification of Fungi

(a) Lower True Fungi:

They are also known as **water fungi**. They have aseptate mycelium. Based on the category they are of two types: Oomycetes and Zygomycetes.

Oomycetes are eukaryotic cells and pathogenic in nature and hence they are having disreputable properties. They have well defined cell wall, composed of β-1,3, and β-1,6 glucans and the body composed of mycelium formed by hyphal networks. They absorb nutrition by heterotrophic mode. They cannot do photosynthesis due to lack of chlorophyll and hence they are parasitic. They undergo sexual reproduction and produce spores as a mode of reproduction. They cause seedling blights, damping-off (*Pythium aphanidermatum* and *P. ultimum*), root rots (*Phytophthora cinnamomi*), foliar blights and downy mildews (*Plasmopara viticola*) diseases.

Saprolegnia is the only genus of oomycete pathogens that does not contain plant pathogens but contains pathogens of different water-borne organisms such as crayfish.

Zygomycetes:

They are conjugated fungi made up of fusion of the protoplasts of two equal or unequal gametangia. They are having mainly three parasitic orders viz. Mucorales, Entomophthorales and Zoopagales. They reproduce by both sexual and asexual methods. They have sexual spores which are known as **Zygospore** whereas asexual reproduction occurs by sporangiospore. Zygospore germinates to produce a hypha and the hyphae are generally aseptated. The hypha wall is composed of chitinchitosan. Example: Black bread mold (*Rhizopus stolonifer*), Mucor, Basidiobolus, Conidiobolus.

Higher Fungi:

They have septate mycelium. The septa have each a central pore and no motile cells are produced in the life cycle. They have three classes viz. Ascomycetes, Bisidiomycetes and Deuteromycetes.

(i) Ascomycetes:

- Sexual spores are produced within a sac like structure called ascus.
- Sexual spores are called ascospore.
- Asexual reproduction occurs by single celled or multi-celled conidia.
- Ascomycetes are also known as sac mycetes.
- Hyphae are generally septated.
- Examples: *Saccharomyces, Arthroderma, Gibberella*.

(ii) Bisidiomycetes:

- Sexual spores are produced externally on a basidium.
- Sexual spores are known as basidiospore.
- Asexual reproduction occurs by budding, fragmentation or conidia formation.
- They are commonly called as mushroom group.
- Hyphae are generally septated.
- Examples: *Amanita, Agaricus, Filobasidiella*.

(iii) Deuteromycetes:

- No sexual stage is present.
- Deuteromycetes are also known as fungi imperfection.
- Asexual reproduction occurs by means of conidia.
- Most of the human and animal pathogens are present in this class.
- Examples: *Candida, Cryptococcus, Trichophyton, Epidermophyton, Histoplasma*.

3.1.3 Cultivation of Fungi

Most fungi grow more slowly than bacteria. They grow on usual bacteriological culture media at temperature 20-30°C. High sugar content in acidic media (pH 5.6) are tolerant to molds. Generally, glucose and peptones content media is useful for isolation of molds and some yeast. Some other well known media such as solid Sabouraud agar media is used for the culture of fungi which content maltose and peptone with less agar content. Media is acidic with pH 5.5 which inhibits bacterial growth. Potato Dextrose agar, brain heart infusion and Czapekdox agar media are also used for isolation of fungi. Yeast grows in colonies whereas filamentous fungi grow as a mycelium. Yeast forms colony: Example: yeast, *Cryptococcus neoformans*. Yeast like colony: Example: *Candida albicans*. Hyphomycete form colony: Example: Dermatophytes. Saprophytic fungi can grow in the media contains chloramphenicol and cyclohexamid. Papanicolaou stain is good for initial differentiation of dimorphic fungi. Most of the fungi are gram positive but Nocardia and actinomyces are gram variable.

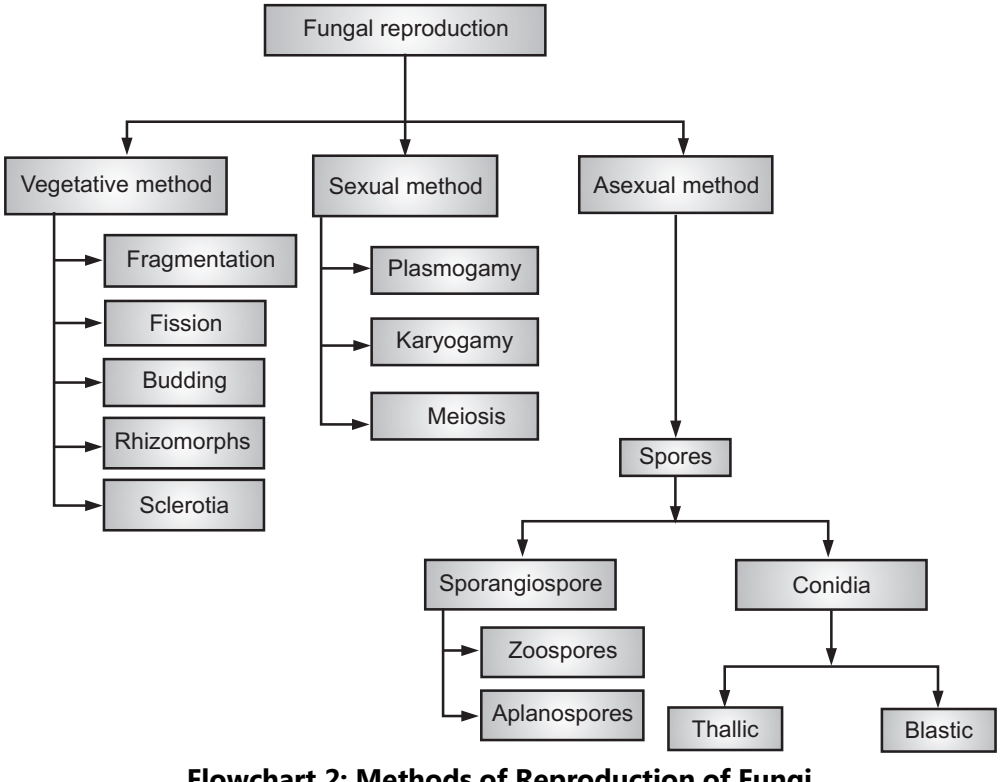
3.1.4 Reproduction of Fungi

The fungi have three main types of reproduction viz. vegetative, sexual and asexual type. Some fungi show only one known reproduction type. Asexual forms (anamorph) were often described separately and given different names than the sexual form (teleomorph). The complete form having both reproductive forms is called a holomorph (Flowchart 2 and Fig. 3.4).

(A) Vegetative method:

In this type of reproduction, the new individual is formed from the somatic portion of the fungal thallus without seed production. There are some methods by which this propagation method is carried out viz. Frgamentation, fission, budding, rhizomorphs and sclerotia.

- **Fragmentation:** In this process, the mycelium breaks into similar fragments with the help of external force and gradually turns into new mycelium.
- **Fission:** In case of unicellular fungi, the single cell is multiplied by this method. The parent cell is divided into two daughter cells by division of nucleus followed by division of cytoplasm and cell wall formation.
- **Budding:** Sometimes the parent cells produce the outer growth known as buds. These buds are generally enlarged and slowly separated out from the parent cells by a cross wall and finally new yeast cell is formed.
- **Rhizomorphs:** In some higher fungi, hyphae are interwoven to form rope like structure known as rhizomorphs. Under favorable condition, they grow and form fruiting bodies.
- **Sclerotia:** It occurs in ergot plant where hyphae become interwoven to form compact mass surrounded by hard coat. This structure is known as sclerotia. Under favorable condition these sclerotia germinate and form new mycelia.



Flowchart 2: Methods of Reproduction of Fungi

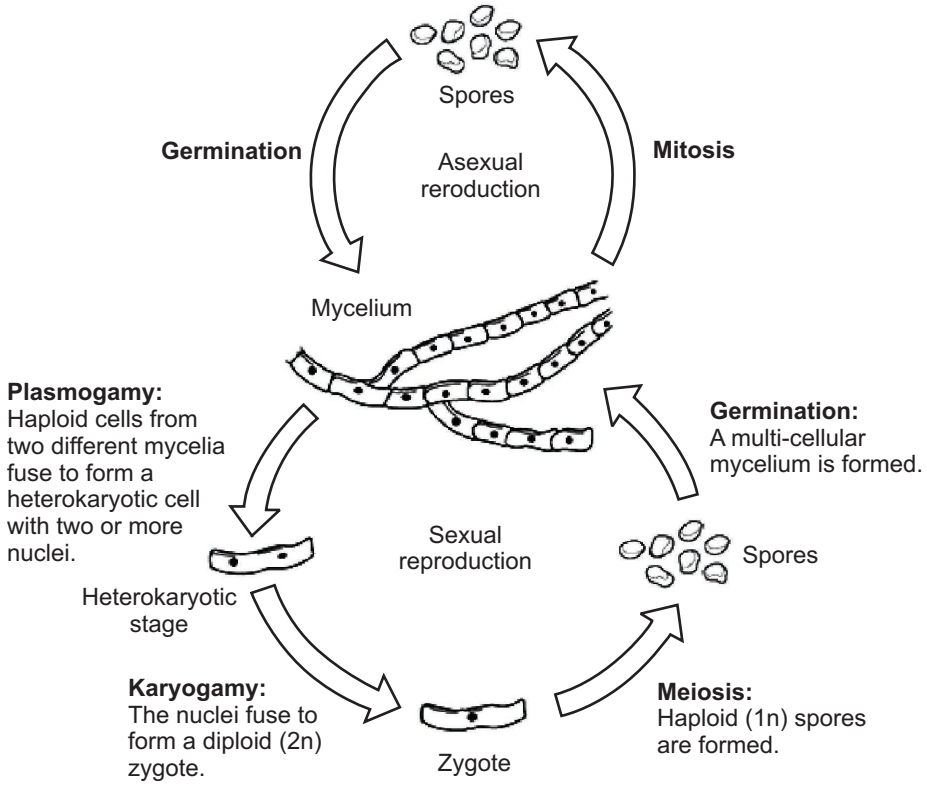


Fig. 3.4: Reproduction Method of General Fungi

(B) Asexual reproduction:

This method is carried out by sporulation where new spores are formed in reproductive cells by mitosis process. Each spore develops in to a new mycelium. Usually spores are unicellular. There are different types of spores form in various fungi.

- **Zoospores:** They are flagellated motile spores produced inside structure known as **zoosporangia**. They do not have cell wall and produced in the lower fungi. Examples: Achyla and Saprolegnia (Fig. 3.5).

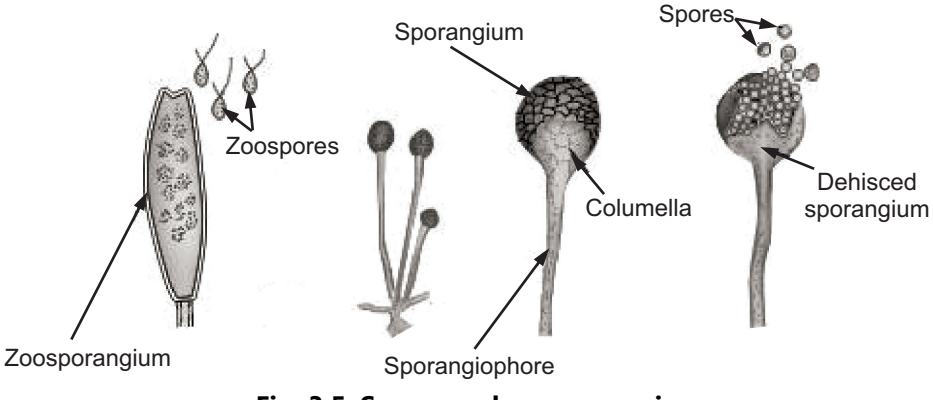


Fig. 3.5: Spores and zoosporangia

- **Sporangiospores:** They produce inside the sac like structure known as sporangium. They are non-motile and branched. They can disperse by wind. Examples: Rhizopus and Mucor.
- **Chlamydospores:** They develop thick wall from hyphal cells. They store reserve foods and with favorable condition they develop into a new mycelium. Examples: Mucor, and Fusarium.
- **Oidia:** In some filamentous fungi, the hyphae break up into individual cell, known as oidia. They are oval shaped and do not store reserve food. Example: Rhizopus.

Fungi produce two types of asexual spores viz. sporangiospores and conidia. Sporangiospores are formed inside the sporangium. They are of two types viz. motile and non-motile spores. Motile spores are known as Zoospores and non-motile spores are known as Aplanospores. Zoospores are three types namely Chytridiomycota, Hypochytridiomycota and Oomycota.

Conidia are formed at a tip of supporting hyphae known as **conidiophores**. They are mainly of two types namely Thallic conidia and Blastic conidia. Thallic conidia develop by septation and fragmentation of a hypha. They are of two types like Arthrospores (formed by septation and fragmentation of an existing hyphae) and Chlamydospores (resting spores). Blastic conidia develop by budding method. They develop as single spores and are classified as Blastospores (formed by budding of a hyphae), Porospores (developed through distinct pore in the hyphal wall), Aleuriospores (single and terminal spores), Annellospores (a chain spores), Phialospores (pushed up from the tip of the conidiophore).

Sexual Reproduction:

Sexual reproduction occurs via fusion of gametes and gametangia, seen in all groups of fungi. This is the method where fusion occurs between two compatible sex cells or gametes of opposite strains. Fungal sex organs are known as gametangia. The reproduction occurs by three phases namely Plasmogamy, Karyogamy and Meiosis.

- **Plasmogamy:** It is the accumulation of protoplast cells of reproductive hyphae. The nucleui from one male and other female are closed together as a pair without fusion. These types of cells are known as **dikaryon**. This phase has several methods like Planogametic copulation where motile gametes undergoes fusion; Gametangial contact where gametangia come closer to each other and forms a fertilization tube, Gametangial copulation where gametangia fuses together and forms zygospore, Spermatization where spermatia are formed externally on special hyphae and spermatia are develops inside the cavities known as spermatogonia and finally Somatogamy where fusion between two somatic cells are occurred.
- **Karyogamy:** This is the step where two parent nuclei are fused together. The fusion occurs very fast in lower fungi whereas delayed in higher fungi so that hyphae remain dikaryotic. This phase is known as **dikaryophase**.
- **Meiosis:** After dikaryophase the next step is meiosis where after cell division, produce four daughter cells each with half the number of chromosomes of the parent cell, as in the production of gametes and plant spores. The production of meiotic spores is known as **meiospores**.

3.2 VIRUSES

Virus is a small nucleoprotein complex and infectious agent, replicates only inside the living cells of other organisms such as animals and plants. They are such smaller than bacteria (20-300 nm). They are obligate intracellular parasites of bacteria, protozoa, fungi, algae, plants and animals. In 1890, D. Ivanovski and M. Beijerinck discovered Tobacco mosaic virus that caused disease in tobacco plant. Friedrich Leoffler and Paul Frosch have discovered animal viruses that cause foot and mouth disease in cattle. They cannot be grown in sterile medium but grow in specific host cells. They have only a single type of nucleic acid that serves as genetic material but lack cellular structure and enzymes for metabolite processes. They are highly species specific.

Properties of Viruses:

- They do not have cellular organization.
- They contain only one type of nucleic acid either DNA or RNA.
- They are obligate intracellular parasites.
- They lack enzymes for protein synthesis.
- They multiply by complex process but not by binary fission.
- They are unaffected by antibacterial antibiotics.

3.2.1 Morphology

Viruses are acellular that means they do not have cells as it has no nucleus, cytoplasm or organelles. They consist of two main parts viz. nucleic acid and capsid. In nucleic acid, DNA or RNA is found at the core of virus. They need living host to survive. Smallest infectious virus is 20 nm to 300 nm. The smallest RNA virus is picornavirus, the smallest DNA virus is parvovirus (20 nm) and the largest virus is poxvirus (300 nm). They may be single or double stranded. The nucleic acid is linear or circular and either segmented or non-segmented. Capsid is a protein coat which is surrounded by the genetic material. Protein coat is made up of repeating sub-units known as **capsomeres**. Based on the arrangement of the morphological sub-unit, viruses are grouped in to three types namely Icosahedral symmetry, Helical symmetry and Complex symmetry.

(a) Icosahedral symmetry: Capsomeres are arranged in 20 triangles (Fig. 3.6), Example: Adeno virus.

(b) Helical symmetry: They are arranged in a hollow coil that appears rod shaped (Fig. 3.7), Examples: Influenza virus, Rabies virus etc. and

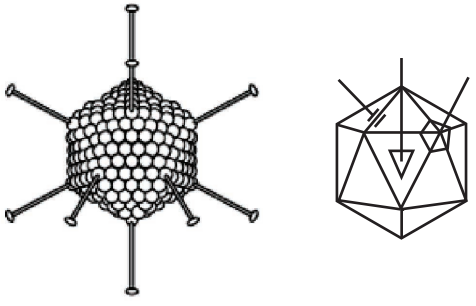
(c) Complex symmetry: They have complexicity in their structure of viruses and they do not show icosahedral or helical symmetry. Example: Pox viruses.

Some viruses have an outer envelope which is made of lipids and proteins covering the capsid. They are parasites. Most of viruses are spherical shaped but some are different types like pox virus is brick shaped, bacteriophage is tadpole shaped, tobacco mosaic virus is rod shaped. They do not have ribosome and do not undergo mitosis or binary fission.

Functions of Viral Capsid:

- It protects the genetic materials.
- It serves as antigenic determinants.

- It induces antibody production.
- It provides the structural symmetry of the virus.
- It facilitates the assembly and packaging of viral genetic information.
- It serves as a vehicle of transmission from host to another.



Icosahedral (adenovirus) Isosahedral symmetry

Fig. 3.6: Icosahedral symmetry

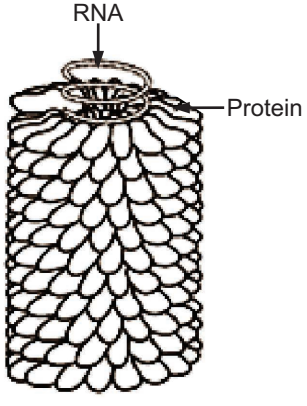


Fig. 3.7: Tobacco mosaic virus (rod shaped)

Viral Envelope: It is a lipoprotein derived from the host cell membrane and virus specific protein. It is composed of glycoproteins which look like spikes. It confers instability to the virus because of the loss of infectivity due to disruption of lipid. They are more sensitive to heat, lipid solvents and detergents.

Functions:

- They help to attach to host cell receptors.
- They are antigenic determinants.
- They stimulate antibody production.

Examples: Herpes virus, HIV virus, Hepatitis virus etc.

Virion: It is an entire virus particle, consisting of an outer protein shell called a capsid and an inner core of nucleic acid. They are present in naked viruses, identical to nucleo capsid whereas in enveloped viruses develops envelop.

Peplomers: In mature virus particle, the glycoprotein often appears as projecting spikes on the outer surface of the envelope, that are known as peplomers. Example: Influenza virus carries two types of peplomers viz. hemagglutinin and neuraminidase.

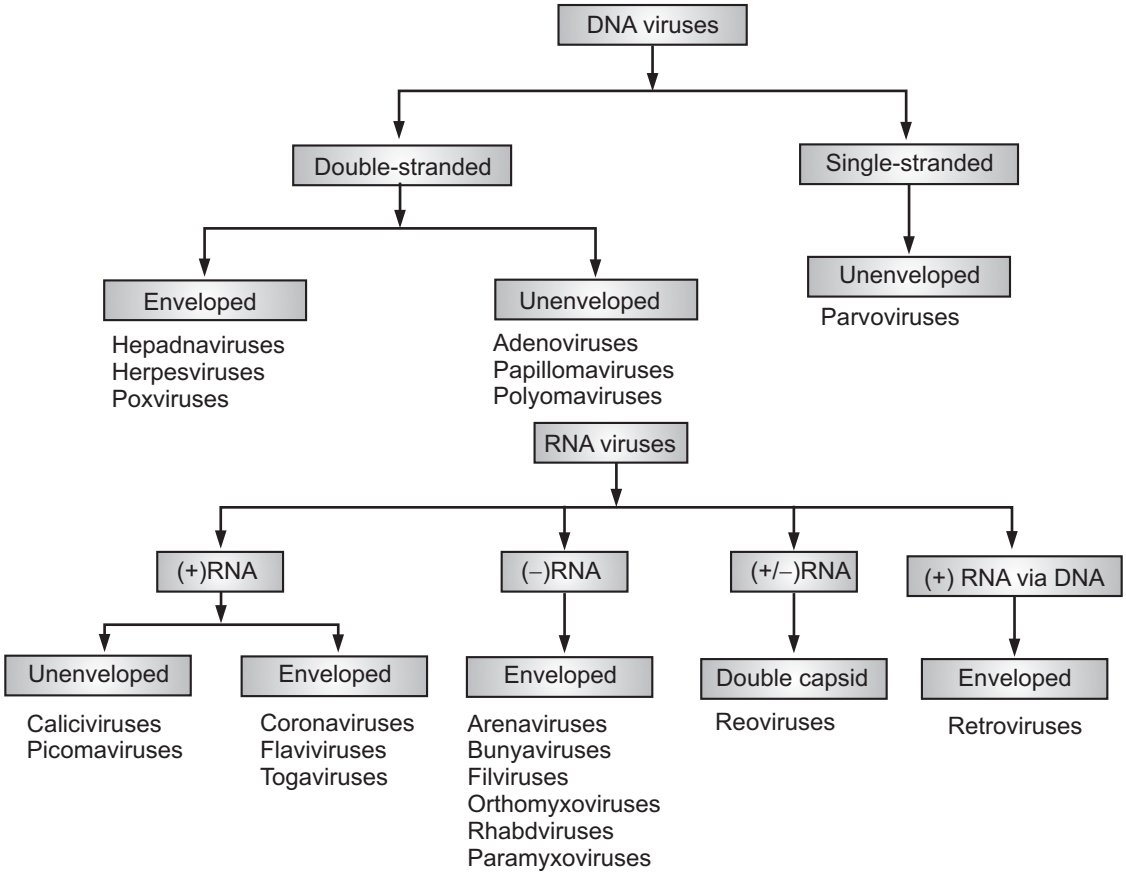
Functions:

- It helps for attachment of virus to the host cell receptors to initiate the entrance of the virion into the cell.
- It has antigentic properties.
- It has enzymatic activity like neuraminidase which cleaves neuraminic acid from host cell glycoproteins.
- It attaches to the receptors on the RBC, causes these cells to agglutinate.

3.2.2 Classification of Viruses (Flowchart 3)

Viruses are classified by phenotypic characteristics, such as morphology, nucleic acid type, mode of replication, host organisms, and the type of disease they cause. They are as follows:

1. Classification on the basis of nucleic acid
2. Classification on the basis of structure or symmetry
3. Classification on the basis of replication properties and site of replication
4. Classification on the basis of host range
5. Classification on the basis of mode of transmission



Flowchart 3: Classification of viruses

1. Classification on the Basis of Nucleic Acid

(a) DNA virus:

- Viral genome is DNA.
  - (i) Double stranded DNA virus: Examples: Adenovirus, Herpesvirus.
  - (ii) Single stranded DNA virus: Example: Parvovirus.

(b) RNA virus:

- Genome is RNA.
  - (i) Double stranded RNA virus: Example: Reo virus.
  - (ii) Single stranded RNA virus: These are further classified into two groups.
    - Positive sense RNA (+RNA): Polio virus, Influenza virus.
    - Negative sense RNA (−RNA): Rabies virus.

2. Classification of Virus on the Basis of Structure

(a) Cubical virus:

- They are also known as icosahedral symmetry virus.
- Examples: Reo virus, Picorna virus.

(b) Spiral virus:

- They are also known as helical symmetry virus.
- Examples: Paramyxovirus, orthomyxovirus.

(c) Radial symmetry virus:

- They are look like spoke or wheel type.
- Example: Bacteriophage.

(d) Complex virus:

- They possess a capsid that is neither purely helical nor purely icosahedral.
- Example: Pox virus.

3. Classification of virus on the basis of replication properties and site of replication

(a) Replication and assembly in cytoplasm of host:

- Example: All RNA virus replicate and assemble in cytoplasm of host cell except Influenza virus.

(b) Replication in nucleus and assembly in cytoplasm of host:

- Examples: Influenza virus, Pox virus.

(c) Replication and assembly in nucleus of host:

- All DNA viruses replicate and assemble in nucleus of host cell except Pox virus.

(d) Virus replication through ds DNA intermediate:

- Examples: All DNA virus, Retro virus and some tumor causing RNA virus replicates through ds DNA as intermediates.

(e) Virus replication through ss RNA intermediate:

- Example: All RNA virus except Reo virus and tumor causing RNA viruses.

4. Classification of Virus on the basis of Host Range:

(a) Bacteriophage:

- Phages are virus infecting bacteria. Examples: λ phage, T2, T4, ϕ174, MV-11.

(b) Plant virus:

- Those viruses that infects plants. Examples: TMV, cauliflower mosaic virus.

(c) Animal virus:

- Those viruses that infects animals. Examples: Polio virus, Retro virus, Herpes virus, Adeno virus.

(d) Insect virus:

- Virus that infects insects. Examples: Baculovirus, Sacbrood virus, Entomopox virus, Granulosis virus.

5. Classification of virus on the basis of mode of transmission:

(a) Virus transmitted through respiratory route:

- Examples: Swine flu, Rhino virus.

(b) Virus transmitted through faeco-oral route:

- Examples: Hepatitis A virus, Polio virus, Rota virus.

(c) Virus transmitted through sexual contacts:

- Example: Retro virus.

(d) Virus transmitted through blood transfusion:

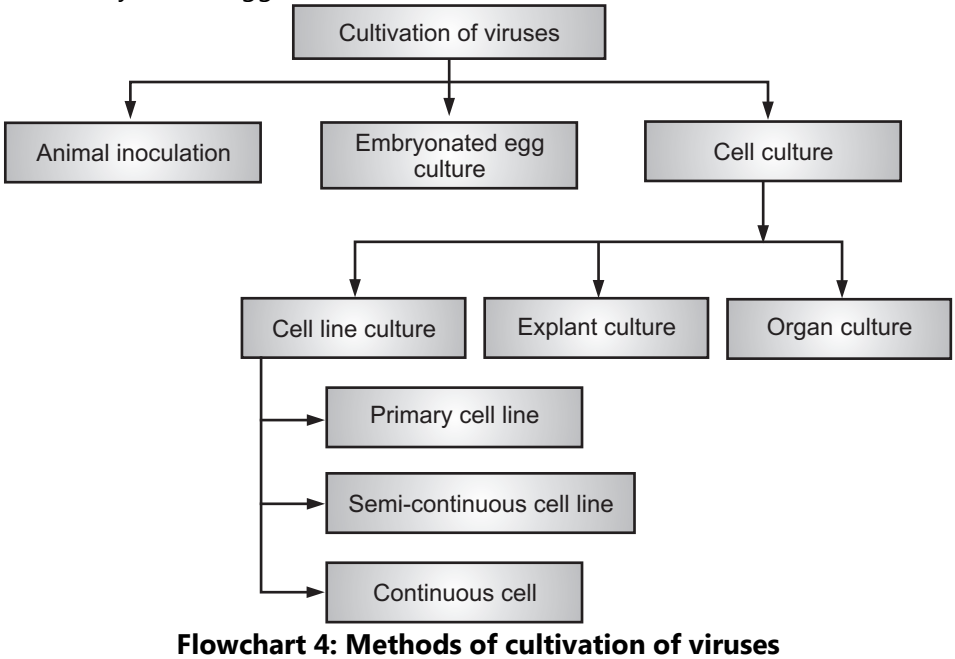
- Examples: Hepatitis B virus, HIV

(e) Zoonotic virus:

- Virus transmitted through biting of infected animals.
- Examples: Rabies virus, Alpha virus, Flavi virus.

3.2.3 Cultivation of Viruses

Viruses are cultured in embryonated egg, cell line culture and animal inoculation because they can only replicate inside host cell, so viruses cannot be cultured in non-living medium as bacteria and fungi (Bacteria and fungi are living creatures). The main purposes of virus cultivation are identification and isolation of viruses in clinical samples, research on viral structure, replication, genetics and their effects on host cell and production of viral vaccines. Hence, cultivation of viruses is essential which is carried out by three methods viz. Animal inoculation, Embryonated egg culture and Cell culture (Flowchart 4).



Flowchart 4: Methods of cultivation of viruses

1. Animal inoculation:

Animal inoculation is one of the primary methods for isolation of certain viruses and for study of pathogenesis of certain viral diseases. This method is carried out for those viruses which are not cultivated in embryonated egg and tissue culture methods. In this method,

healthy and disease free laboratory mice (white mice) are particularly used for virus cultivation. Suckling mice of age less than 48 hours are used for culture of Toga virus and Coxsackie virus. The inoculation of viruses is carried out by intracerebral and intranasal route. The inoculation is also carried out by intraperitoneal and subcutaneous routes. Other healthy and disease free animals such as hamsters, Guinae pig, rabbits, Chimpanzee and primates are also used as alternative for virus culture. After inoculation of virus sample in to the host cell, the animals are observed for symptoms of disease till death and then virus is isolated from tissue of animal. Live inoculation was first used to study of yellow fever virus on human volunteers.

Advantages:

- Diagnosis, Pathogenesis and clinical symptoms are determined.
- Antibodies productions are identified.
- Mice are the reliable model for virus replication.
- This method helps to study of immune responses, epidemiology and oncogenesis.

Disadvantages:

- Animal experiment is costly and difficult to maintain.
- Animal selection for specific viruses is difficult.
- Some human viruses are not grown in animals.
- Vaccine production is not possible with mice model.

2. Embryonated egg culture:

For virus cultivation, an egg embryo of 7-12 days is used. At first egg is kept in incubator for embryo development up to 7-12 days and then virus sample is inoculated into the egg. For inoculation, eggs are first prepared for cultivation and the shell surface is first disinfected with iodine and penetrated with a small sterile drill. After inoculation, the opening is sealed with paraffin and incubated at 36°C for 2-3 days. The egg is broken after the incubation and virus is isolated from tissue of egg. Virus can be cultured in different parts of embryonated egg, such as choriallontoic membrane (Pox virus are cultured), amniotic sac (Influenza virus, Mumps virus, Yellow fever virus and Rabies virus are cultivated), amniotic cavity (Influenza virus is cultured) or yolk sac (Herpes virus is cultured) depending upon types of virus. In 1931, Scientist Pasture was first used the embryonated hen's egg for the cultivation of virus (Fig. 3.8).

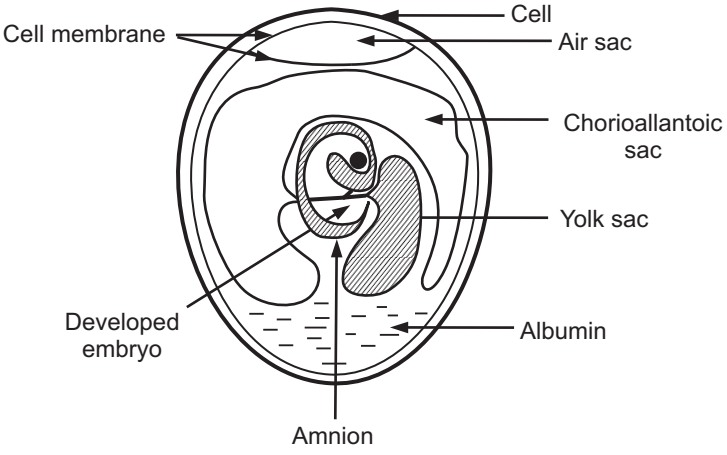


Fig. 3.8: Technique of embryonated egg culture

Advantages:

- This method is widely used method for the isolation of virus.
- It is an ideal substrate for the viral growth and replication.
- This method is cost effective and maintenance is much easier.
- Less labor is needed.
- The embryonated eggs are readily available.
- They are free from contaminating bacteria and many latent viruses.

Disadvantages:

- Each virus has different sites for their growth and replication hence the site of inoculation varies with the viruses.

3. Cell culture (tissue culture) technique:

This technique is most commonly used technique for cultivation of virus. There are three types of cell culture technique viz. organ culture, explant culture and cell culture.

(i) Organ culture: This culture method is mainly done for highly specialized parasites of certain organs. Like, tracheal ring culture is done for isolation of coronavirus.

(ii) Explant culture: In this method, small fragment of tissue is extracted from human or animal and used for virus culture but this technique is very rarely used.

(iii) Cell line culture: This is the most commonly used technique. Cell line culture is routinely used in lab for virus culture, isolation and identification. In this method, the growth media is prepared by maintaining balanced salt concentration, all essential aminoacids, glucose, buffering agents, some antibiotic, serum etc. Thereafter some tissue fragment is obtained which is trypsionized to dissociate cells. These cells are washed and suspended in culture media in petriplates and incubated for sufficient time. On incubation, cell divides and spread out on the glass surface to form a confluent mono-layer of cells that is used for virus culture.

On the basis of origin, chromosomal characteristics and number of generation through which cell culture can be maintained, cell line culture is classified in three types viz. Primary cell line, Semi-continuous cell line and Continuous cell line.

I. Primary cell line:

These are normal cells, obtained from fresh organ of animals or human and cultured. These primary cell line cultures are used for isolation of viruses and for preparation of vaccines. These cells are capable of limited growth for limited generation. They cannot be maintained in serial sub-culture. Examples: Monkey kidney cell line, Human amnion cell line, etc.

II. Semi-continuous cell line:

These cells are fibroblastic cells or also known as diploid cell because they contain same number of chromosome as the parent cell. These diploid cells are sub-cultured for limited generation. They are susceptible for wide range of Human virus culture and also used for vaccine production. Examples: Rhesus embryo cell, human embryonic lung strain, etc.

III. Continuous cell line:

These are cells of single type capable of infinite growth *in-vitro*. These are usually cancer cells derived from cancerous tissue. These cells grow faster and they are haploid cells. These cells are maintained by serial sub-culture or by deep freezing at −7°C, so that these cells are reused when necessary. This cell line is used for virus culture but not used for vaccine preparation. Examples: **HeLa** cell is obtained from cervical cancer, **HEP-2** (Human Epithelioma of larynx cell line), **Vero** (Vervet monkey) kidney cell lines, **BHK-21** (Baby Hamster Kidney cell line).

Advantage of Cell Culture:

- The method is easy, broad spectrum, cheaper and good sensitivity.

Disadvantages of Cell Culture:

- The process requires skilled person.
- Tissue or serum is sent central laboratories for analysis to identify virus.

3.2.4 Cultivation of Bacteriophages

Bacteriophages are cultivated in either broth or agar cultures of young, actively growing bacterial cells.

Cultivation of Plant Viruses:

Plant viruses are cultivated by plant tissue cultures, cultures of separated cells, or cultures of protoplasts, etc. and viruses are grown in whole plants. Leaves are mechanically inoculated by rubbing with a mixture of viruses and an abrasive and that time cell wall is broken and the viruses directly contact the plasma membrane and infect the exposed host cells. A localized necrotic lesion often develops due to the rapid death of cells in the infected area.

3.2.5 Reproduction of Viruses

Viruses do not reproduce. The living cell in which the virus reproduces is called a host cell. They use these host cells to replicate themselves by creating an exact copy of the virus. Two main types of reproductive cycle are observed in virus namely Lytic Cycle and Lysogenic Cycle.

Lytic Cycle (Fig. 3.9): This cycle is completed with several steps which are as follows:

1. **Attachment:** In this first step, Virus is attached to the host cell.
2. **Entry:** Then genetic material is injected into the host cell.
3. **Replication:** The virus takes over the cell's metabolism, causing the creation of new proteins and nucleic acids by the host cell's organelles.
4. **Assembly:** Then proteins and nucleic acids are assembled into new viruses.
5. **Release:** Finally virus enzymes cause the cell to burst and viruses are released from the host cell. These new viruses further infect to other cells and this process is continuing.

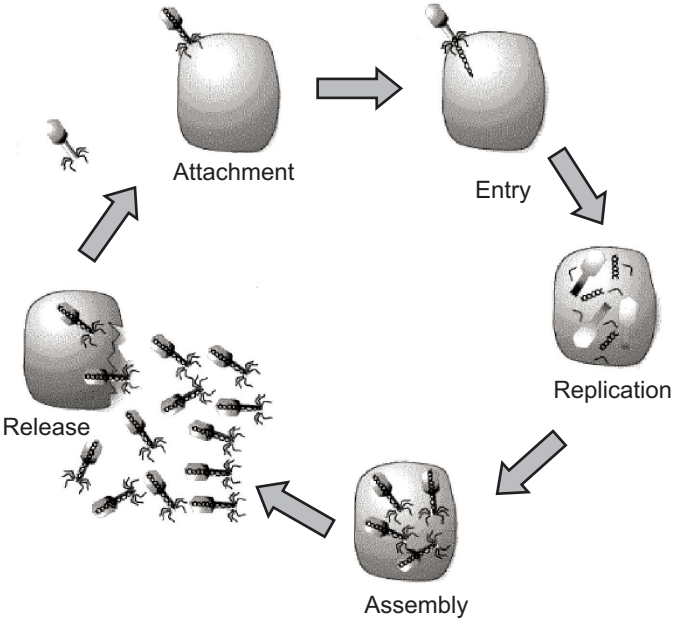


Fig. 3.9: Reproduction of virus through Lytic cycle

Lysogenic Cycle (Fig. 3.10): This cycle is completed with several steps which are as follows:

1. **Attachment:** In the first step, virus is attached to the host cell.
2. **Entry:** Then genetic material is injected into the host cell.
3. **Integration:** Viral DNA integrates into the host cell's genome.
4. **Replication (lysogenic cycle):** When the host cell replicates, viral DNA is copied along with host cell DNA. Each new daughter cell is infected with the virus.
5. **Induction:** When the infected cells are exposed to certain environmental conditions, viral DNA is activated and enters the lytic cycle.
6. **Replication (lytic cycle):** The virus takes over the cell's metabolism, causing the creation of new proteins and nucleic acids by the host cell's organelles.
7. **Assembly:** Proteins and nucleic acids are assembled into new viruses.
8. **Release:** Then finally virus enzymes cause the cell to burst and viruses are released from the host cell. These new viruses can infect other cells.

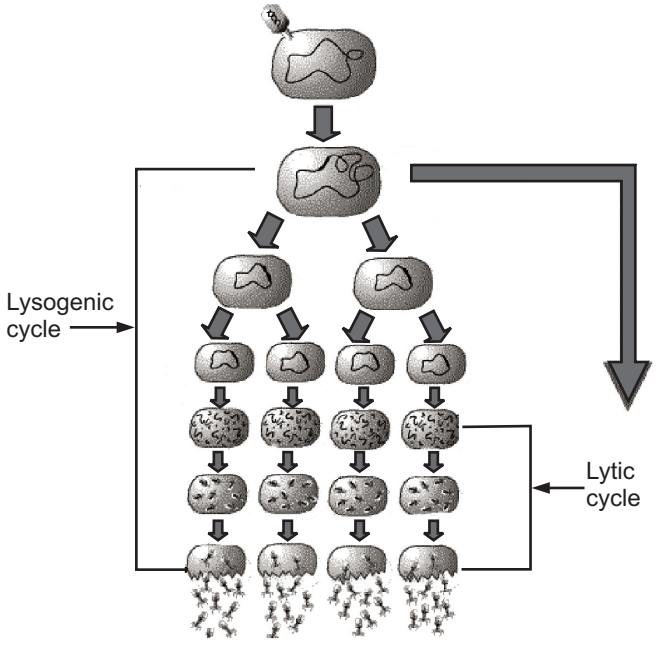


Fig. 3.10: Reproduction of virus through Lysogenic cycle

3.3 CLASSIFICATION AND MODE OF ACTION OF DISINFECTANTS

Disinfectants: They are chemical agents that are used to kill microorganisms (except spores, viruses and prions) on inanimate objects (the things that are not alive) such as instruments and surfaces by means of physical or chemical processes. But they are not to be used on living tissues such as skin or mucous membranes.

The process by which destruction or removal and killing of all pathogenic organisms are carried out is known as disinfection.

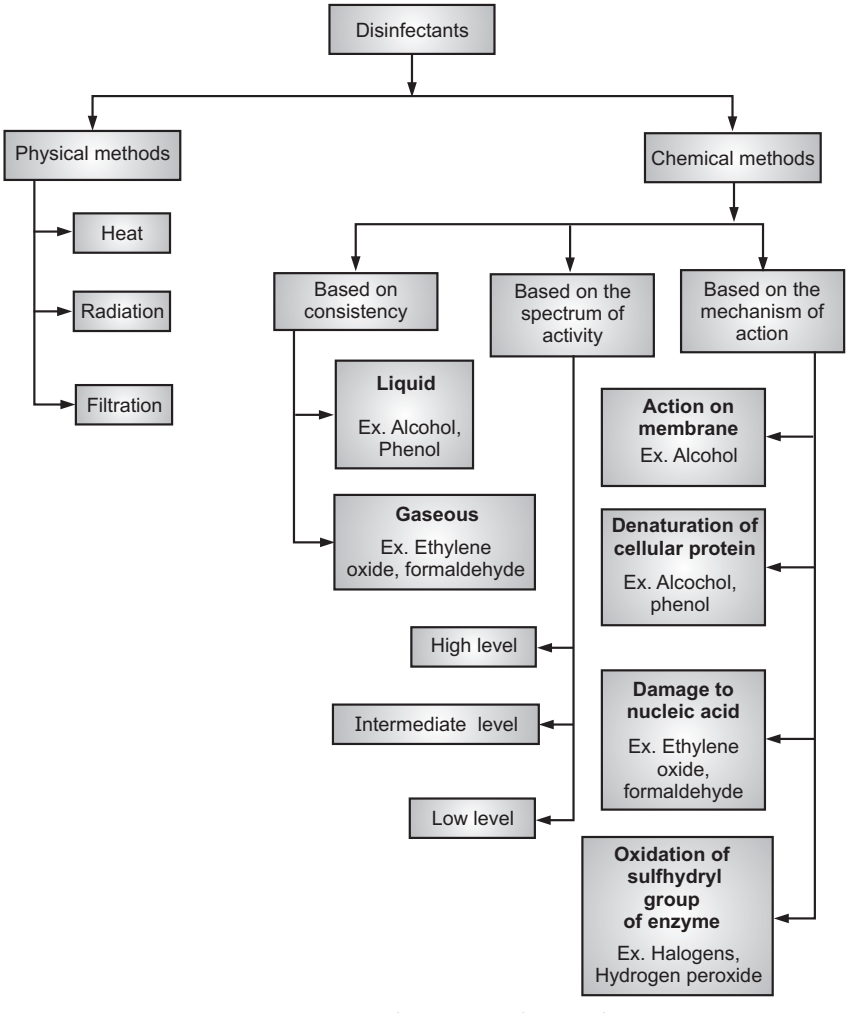
The main purpose is to prevent transmission of certain microorganisms with objects, hands or skin and prevent spreading of infection.

Ideal Properties:

- They should have wide spectrum of activity, should be active against all pathogens.
- They should be cidal (capable of killing bacteria) and chemically stable.
- They should be cheap and should not produce any stain.
- They should not damage any non-living materials.
- They should act in presence of organic matters.
- They should have high penetrating power and rapid action.
- They should be non-toxic and non-corrosive.
- They should be relatively safe to human and other animals.
- They should be easily soluble in hard water and should be active at any pH.
- They should have good cleaning properties and should be non-inflammable.
- They should not damage environment on disposal.

3.3.1 Classification

Disinfectants are broadly classified into two categories viz. physical methods and chemical methods. The overall classification is listed in Flowchart 5.



Flowchart 5: Classification of disinfectants

3.3.1.1 Physical Method

Heat:

Mainly dry and moist heats are used for the disinfection action. Flaming is used for dry heat process whereas temperatures at various levels are used in moist heat sterilization. In moist heat sterilization, pasteurization is mainly used for disinfection where temperature below 100°C is applied. This pasteurization is a process that is intended to reduce spoilage organisms and eliminate vegetative bacteria but not bacterial spores in certain foods and beverages. Pasteurization of milk was used first at temperatures of about 63°C for 30 minutes or heating to a higher temperature, 72°C and holding for 15 seconds for disinfection.

Filtration:

This method is carried out for fluids that are heat labile like antibiotics, vitamins and other growth factors with very fine pore membrane filters below 0.45 microns in diameter. These fluids are checked for sterility by subculture before use. Filtration of air is necessary for the disinfection of the laboratory. Sterile filters are used for separation of toxins and other soluble products of bacterial growth and also used for purification of water. Sometimes syringe filters are used for disinfection of small volume of fluids. Membrane filters (pore size of 0.015 to 12 μM) are used for preparation of parenterals and remove bacteria and yeast from the solution. HEPA filter is used for disinfection of air. The HEPA filtration reduces the number of organisms on dust particles by 99.9% down to 0.3 micron size.

Radiation:

Ultra-violet lights are used in laboratories that reduce the number of organisms to low level in the air and on surfaces.

3.3.1.2 Chemical Methods

Based on Consistency:

Mainly liquids and gaseous substances are used for the disinfection activity. Liquids like alcohol and phenol are widely used as disinfectant. Alcohols like ethanol or isopropanol at a concentration of 50-70% are used for disinfectants. They are used for the disinfection of the surfaces and other laboratory equipment. Alcohols are low in sporocidal activity.

The phenolic chemicals contain a benzene ring with hydroxyl (OH) group which are more common disinfectants for environmental surfaces. Cresol is more powerful than phenol and more commonly used compound which are diluted out further (1 : 128 - 1 : 256) to achieve their bactericidal activity.

Gaseous agents like Ethylene oxide and aldehydes are used as disinfectant agents. Ethylene oxide is used as a sterilant, but in liquids, it shows antimicrobial action. Formaldehyde and glutaraldehyde are used as powerful disinfectants. The glutaraldehyde is the basic chemical for various marketed compounds like Cidex, or Sonacide, Sporocidin or Glutacide. These are used for disinfectants of instruments, pipettes and clinical thermometers, etc.

Based on the Spectrum of Activity:

Based on the activity, disinfectants are classified as high, intermediate and low levels. High level disinfectants such as aldehydes and gases, are used for endoscope and

disinfectants for surgical instruments. Some examples of high level disinfectants are like 2% glutaraldehyde for 20 minutes, 6% hydrogen peroxide for 30 minutes, 0.2% per acetic acid for 30-45 minutes. Intermediate level disinfectants are like alcohols and iodophore, used for disinfectant of laryngoscope. Thereafter low level disinfectants are like quaternary ammonium compounds. They are also known as **quats**. They irreversibly bind to the phospholipids and proteins of the membrane. Earlier experiments utilized benzalkonium or cetylpyridium chlorides which act as good disinfectants but in low concentrated solution they become contaminated. Quaternary ammonium compounds are used as disinfectants of electrocardiogram and stethoscope. Some examples of low level disinfectants are like 3% hydrogen peroxide solution for 10 minutes, 1000 ppm hypochlorite solution, 60-95% alcohol for 10 minutes.

Based on Mechanism of Action:

(i) Action on membrane: Mainly alcohols such as ethanol, isopropanol and methanol are used for this purpose. They act on membrane of microorganisms and destroy the cell membrane. They are highly active in combination with water. 70% solution of Ethanol and isopropanol are used as hand disinfectants in pharmaceuticals. Example: Triclogel contains 75% ethanol.

(ii) Denaturation of cellular proteins: Alcohols also denatures the cell wall proteins. Phenols such as chlorocresol and chloroxylenol are used as disinfectants. They also denature the proteins and enzymes of the cell. Examples: Dettol contains chloroxylenol and Lysol contains para chloroorthobenzylphenol.

(iii) Damage to nucleic acid: Ethylene oxide is a colourless gas which is soluble in water. It has alkylating action on proteins and damages the nucleic acid. Inhibition produced by it is irreversible, resulting inhibition of enzyme activity. Formaldehyde gas acts on proteins by denaturation and on nucleic acids by alkylation of amino acid and sulfhydryl group of proteins and ring nitrogen atoms of purine bases. The reaction is irreversible. The 5′dGMP (deoxyguanosine monophosphate) interacts more rapidly with formaldehyde than 5′ GMP.

(iv) Oxidation of sulfhydryl group of enzyme: Hydrogen peroxide acts by producing destructive hydroxyl free radicals that attack membrane lipid, DNA and other cell components. Halogen compounds such as chlorine and chlorinated compounds and iodine compounds act as disinfectants. Inactivation of microorganisms with chlorine compounds depends on various mechanisms such as oxidation of sulfhydryl enzymes, ring chlorination of amino acids; loss of intracellular contents; decreased uptake of nutrients; inhibition of protein synthesis; decreased oxygen uptake; oxidation of respiratory components; decreased adenosine triphosphate production and depressed DNA synthesis. In other way, iodine compounds can penetrate the cell wall of microorganisms quickly, and results disruption of protein and nucleic acid structure and synthesis of the microorganisms. Actually, the iodine compound inhibits protein synthesis and oxidizes –SH group of amino acids. Example: Povidone-iodine solution is used to rapid kill (seconds to minutes) of *S. aureus* and *M. chelonae* at a 1 : 100 dilution.

3.3.2 Mode of Action of Disinfectants

The main function of disinfectants is to act on microorganisms and the action is two ways either growth inhibition (bacteriostasis, fungistasis) or lethal action (bactericidal, fungicidal or virucidal effects). But the main objective of the disinfectants is the lethal action or lethality.

Generally, disinfectants are either complex formulations of active molecules or containing co-solvents, chelating agents, acidic or alkaline agents or surface-active products. Hence, these disinfectants are working on various mechanisms likely, acting on membrane, leakage of membrane, denaturation of cellular proteins, oxidation of –SH group of enzymes, Alkylation of amino, carboxyl and hydroxyl groups and nucleic acid damage. These activities are performed by various types of chemical agents which are acting as disinfectants. In below diagram showed the various compounds and their target locations in the cells by which disinfection activity is occur (Fig. 3.11, Table 3.1).

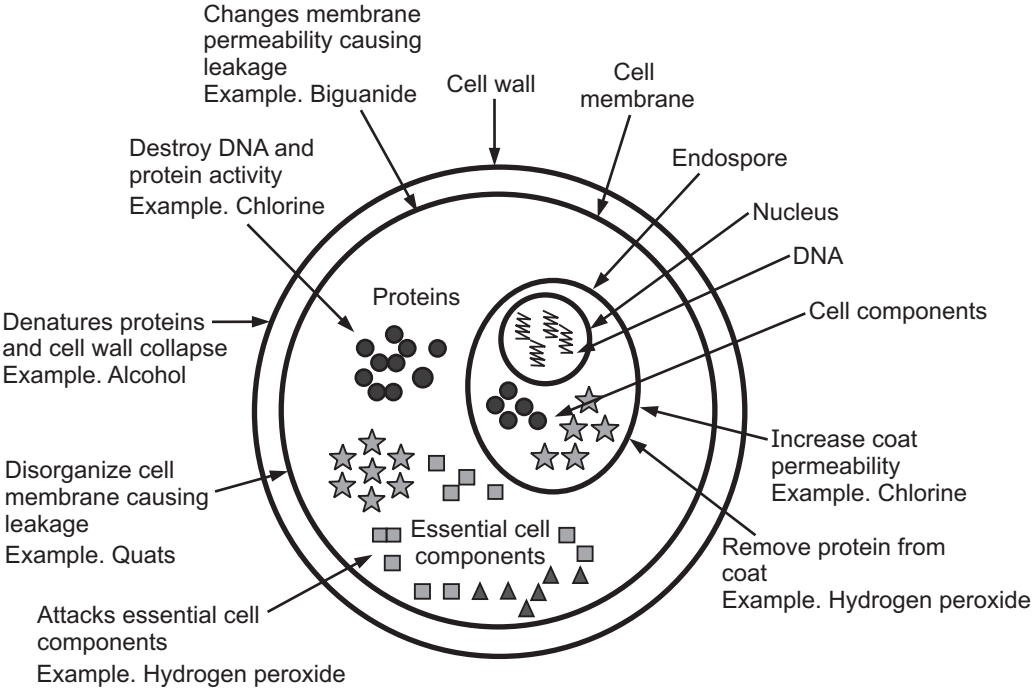


Fig. 3.11: Mechanism action of Chemical compounds acts as disinfectants

Table 3.1: Mechanisms of actions of various disinfectants in the specific targeted cells

Disinfectants	Target cells	Mechanism of action
Glutaraldehyde	Cell envelop (Cell wall)	Cross linking of proteins
Quats	Cytoplasmic membrane	Membrane damage
Chlorhexidine		Low concentration cause membrane integrity

Disinfectants	Target cells	Mechanism of action
Biguanide		Phase separation of membrane lipids
Phenols		Membrane leakage
Formaldehyde	Cross linking of macromolecules	Cross linking of proteins, DNA, RNA
Glutaraldehyde		Cross linking of proteins in cell envelop
Halogens	Effect on DNA	Inhibition of DNA synthesis
Hydrogen peroxide		DNA strand breakage
Halogens	Oxidizing agents	Oxidation of thiol group to disulfides or sulfoxides
Ethylene oxide	Effect on nucleic acid	Alkylates proteins and nucleic acid

Different types of disinfectants are listed in Table 3.2 with their marketed products.

Table 3.2: Types of disinfectants

Disinfectants		
Type	**Category**	**Examples**
Liquids	Quaternary ammonium	A-33, Hi-Tor
	Chlorine	Chloramine T, Clorox
	Ethanol	Ethyl alcohol
	Formaldehyde	Sterac
	Glutaraldehyde	Cidex
	Isopropanol	Hy-Sine, Ioprep, Mikroklene
	Phenolic	Matar, Hil-Phene, O-syl
Gas	Ethylene oxide	Carboxide, Cryoxide, Steroxide
	Paraformaldehyde	----

Difference between sterilization and disinfection are tabulated in Table 3.3.

Table 3.3: Difference between sterilization and disinfection

Sterilization	Disinfection
1. It is the method of complete removal of microorganisms.	1. It only reduces the number of microorganisms without killing.
2. It requires strict protocol to remove microbes.	2. It do not require any strict protocol.
3. It kills bacteria along with their spores.	3. It does not kills bacterial spores.

Sterilization	Disinfection
4. It is require in surgical operations or in sterile labs.	4. It is commonly use in daily life.
5. Usually physical methods are used for destruction of microorganisms (heat, chemical sterilants and gases).	5. Usually chemical methods are used.
6. Sterilization is an absolute condition.	6. Disinfection is an adequate method.
7. Some sterilants are like dry heat, steam, radiation, filtration.	7. Disinfectants are like phenol, chlorine, iodine, alcohol, hydrogen peroxide.

3.4 ANTISEPTICS

They are the agents that cause destruction or inhibition of growth of microorganisms such as bacteria, viruses and fungi on living surfaces such as skin and mucous membranes. In the 19th century, the discovery and development of antiseptic chemicals and principles began. They are different from antibiotics and antiviral drugs that work inside the body, and from disinfectants, which are chemicals applied to non-living items and surfaces.

Mechanisms of Action:

All antiseptics interfere with life processes of microorganisms. Example: Benzylalkonium interferes with intracellular signaling and chemistry to the point that the cell membrane begins to disintegrate. Iodine compounds irreversibly deform, or denature, critical proteins in bacteria, starting at the cell membrane and moving inwards. Alcohol also denatures and destroys cell membranes. Some soaps and detergents cause bacterial cell membranes to lose integrity or denature critical proteins. Other antiseptics directly interfere with critical enzymes inside a microbe.

Types of Antiseptics:

Many antiseptics are used today and they are classified based on their active chemical ingredients.

1. **Alcohol Antiseptics:** They are generally composed of isopropyl or methyl alcohols and are commonly used to sterilize hard surfaces such as medical tools at hospitals. Medical personnel also use alcohol gels as hand sanitizers in order to prevent the spread of disease.
2. **Chlorhexidine Antiseptics:** It is used as both oral and skin cleansing products. It is the active ingredient in prescription mouthwashes like Peridex (According to the FDA Professional Drug Information database). It is applied either directly onto wounds or to unbroken skin before surgery in order to proactively prevent infections.
3. **Idochlor Antiseptics:** They are applied as a topical treatment to unbroken skin or mucus membranes to prevent infection before surgery. Example: Betadine.

4. **Peroxygen Antiseptics:** They are used in bubbling reaction to blood and damaged cells. Example: hydrogen peroxide. Hydrogen peroxide is used to cleanse wounds and disinfect skin. It is also used as a gargle or mouthwash.
5. **Phenol Antiseptics:** They are used primarily as skin disinfectants and in medicated soaps and scrubs. In addition, phenol can also be used in mouthwashes and throat lozenges.
6. **Quaternary Ammonium Compound Antiseptics:** They are also known as "Quats,". They are the group of chemicals used in antiseptics commonly used to sterilize the skin before surgery. They are also used as a preservative in eye drops. Example: Benzalkonium chloride.

Ideal Properties of Antiseptics:

- They should have broad spectrum activity, should destroy microorganisms.
- They should have rapid bactericidal activity.
- They should be active at any pH.
- They should be non-irritant, non-corrosive.
- They should not stain the instruments.
- They should be soluble in water.
- They should penetrate deeply to the bacterial cells.
- They should not damage materials or living tissues when they are come in contact.

Differences between Antiseptics and Disinfectants:

Table 3.4: Differences between antiseptics and disinfectants

Disinfectants	Antiseptics
1. They are manly used to kill the microbes on the non-living objects.	1. They are used to kill the microbes on the skin.
2. They have power to kill all the microorganisms and bacteria on the surfaces.	2. They do not have power to kill all the microorganisms and bacteria on the surfaces.
3. They can cause skin corrosion and inflammation.	3. They do not cause any skin damage and inflammation.
4. They are used in laboratory and house hold items cleaning.	4. They are used in surgery and hand washing.
5. They destroy the cell wall of microorganisms or interferes with the metabolism of microbes thrives on the surface of an object.	5. They transport through the lymphatic system and destroy bacteria within the living body.

Common antiseptics are tabulated in Table 3.5.

Table 3.5: Various antiseptics and their uses

Antiseptics	Composition
Bactin liquid	Benzalkonium chloride 0.13 % w/w Lidocane hydrochloride 2.5% w/w
Bactin pain relief spray	Benzalkonium chloride 0.13 % w/w Lidocane hydrochloride 2.5% w/w
Betadine	Povidone-Iodine 10%
Camphophenique pain relief liquid	Camphor 10.8% Phenol 4.7%
Dermoplast antibacterial spray	Benzocaine USP 20% Benzalkonium chloride USP 0.2%
Antibacterial wound wash saline	Benzalkonium chloride 0.13%

3.5 FACTORS INFLUENCING DISINFECTION AND ANTISEPTICS

Disinfectants are used to remove the germs and other contaminants on the surfaces of the area. They are used daily for cleaning of homes, laboratory as well as food preparation areas. Hence, it is required to select the proper disinfectant according to the type of contaminants present but several physical and chemical factors also influence disinfectant procedures namely temperature, pH, relative humidity and water hardness.

Temperature: It is one of the major factors that influence disinfection activity. Generally, disinfectant activity increases with the increased temperature but there are some exceptions. Generally, higher temperature decreases the surface tension and also the viscosity and enhances the germicidal action. Furthermore very high temperature causes the degradation of disinfectant and weakens its germicidal activity and may produce health problems. Example: Chlorine compounds are more corrosive in higher temperature and iodine tends to sublime at temperature 49°C or above.

pH: An increase in pH improves the antimicrobial activity of some disinfectants (e.g., glutaraldehyde, quaternary ammonium compounds) but decreases the antimicrobial activity of others (e.g., phenols, hypochlorites, and iodine). The pH influences the antimicrobial activity by altering the disinfectant molecule or the cell surface.

Relative humidity: It is the most important factor influencing the activity of gaseous disinfectants, such as ethylene oxide, chlorine dioxide, and formaldehyde.

Water hardness: Hardness of water means high concentration of divalent cations present in water. Hardness reduces the rate of kill of certain disinfectants because divalent cations (e.g., magnesium, calcium) in the hard water interact with the disinfectant to form insoluble precipitates.

Other factors like duration, age of disinfectants, concentration of disinfectants, biofilms and number of pathogens are also play an important role.

Duration: The effectiveness of a disinfectant is greatly depends on the length of time that is exposed to the area to be disinfected. The duration of the exposure depends on targeted organisms as well as concentration of appropriate disinfectants. In general, longer contact times are more effective than shorter contact times.

Age: The age of the disinfectant solution being used also affects its effectiveness. Many chemicals have diminished potency with the passage of time and their exposure to air and the other chemicals in the solution.

Concentration: The chemical makeup and the potency of the solution will affect how effective the disinfectant is at eliminating the contaminants present. The higher the concentration of the solution, disinfectant is more potent and therefore more effective. The disinfectant rate by the chemical agents is depends on Chick's law:

$$-\frac{dN}{dt} = kN$$

where, dN/dt = Rate of cell destruction,
k = Rate constant,
N = Number of living cells remaining at time "t".

"k" depends on microorganism species, disinfectant nature and concentration of disinfectants.

Disinfectant's rate is described in another equation,

$$C^n t_c = K$$

where, C = Concentration of disinfectants at time t = 0,
t_c = Time of contact required to kill a given percentage of microbes; K,
n = Exponential constants.

Further, "n" depends on the nature of the disinfectants.

If n > 1, then disinfection depends on the concentration of the disinfectant,

If n < 1, then disinfection depends on the time of contact

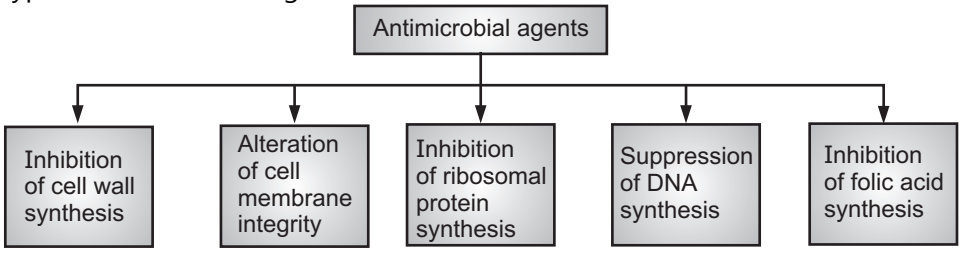
"K" depends on the type of microorganisms and other environmental factors such as pH and temperature.

Biofilms: Biofilms are thick masses of cells and extracellular materials of microorganisms. They are protected from disinfectants by production of this biofilms. These biofilms are tightly attached to surfaces of microorganisms and forms resistant to disinfectants by multiple mechanisms, including physical characteristics of older biofilms, genotypic variation of the bacteria, microbial production of neutralizing enzymes, pH etc.

Number of microbes: Disinfection activity is directly proportional with the number of microbes. The more number of microbes present indicates to take more time to eliminate microorganisms. It is also further dependent on the type of microbes. Endospores are more difficult to destroy whereas vegetative organisms are widely vary in susceptibility to different methods of microbial control.

3.6 EVALUATION OF BACTERICIDAL AND BACTERIOSTATIC

Antimicrobial agents are the agents that are used to prevent the infections by killing or by preventing the growth of living organisms. They are of various types like antibacterial, antifungal, antiprotozoan, antihelminthic etc. As per spectrum activity antimicrobials are of two types namely broad spectrum antimicrobial agents (both for gram positive and gram negative bacteria, example: Tetracyclins) and narrow spectrum antimicrobial agents (acts on either gram positive or gram negative organisms, example: Penicillin G). Based on mode of action, antimicrobials are mainly of two types either bactericidal or bacteriostatic. Bactericidal are the agents which have lethal action on bacteria whereas bacteriostatic are the drugs that prevent the growth of microbial cell. Hence, mode of action of antimicrobials is divided into various types that are listed in given chart.



Flowchart 6: Mode of action of antimicrobial activity

Factors Affecting Antimicrobial Activity:

Some antimicrobial agents are having cidal action under one set of conditions and static action under others. There are several factors that influence the activity of antimicrobial agents are: (1) the susceptibility of the microorganism, (2) the concentration or dose of the agent, (3) the length of exposure, (4) the number of microorganisms, and (5) environmental conditions.

1. **Microbial susceptibility:** Responses of microbes varies on different disinfectants. For example: vegetative cells of the Mycobacteria that cause tuberculosis and leprosy, are covered by a waxy coating that protects them from many antimicrobial chemicals. The hepatitis B virus and some fungal spores are resistant to most disinfectants. Bacillus and Clostridium are especially difficult to eliminate etc.
2. **Concentration of the agent:** Diluted microbicidal chemicals have usually weak antimicrobial activity. At lower concentrations they become microbistatic or lose antimicrobial activity altogether. The antimicrobial effects of temperature or radiation also depend on intensity of the exposure. Low dose may inhibit growth, whereas high doses may result in sterilization.
3. **Length of exposure:** Microorganisms die when physical or chemical conditions irreversibly damage essential cell components. All organisms are do not die rapidly and simultaneously when a critical exposure is achieved, because microbial death is a function of time-the longer microbes are exposed to potentially lethal conditions, the more microbes will be killed. Microbistatic agents are effective only as long as they are present and must be used during the entire time that inhibition is to be maintained.

4. **Number of microorganisms:** Antimicrobial effectiveness also depends on the initial concentration of the microbial population. As the number of microbial contaminants increases, either the exposure period to or concentration of the agent must increase to achieve acceptable levels of decontamination.
5. **Environmental conditions:** Temperature, pH, and moisture affect the efficiency of most antimicrobial agents. Some chemical agents are absorbed by organic materials such as blood, mucus and tissue are severely reduce antimicrobial effectiveness and hence they are not used on the skin.

Antibiotics:

Antibiotics are the chemical drugs (naturally occurring, semi-synthetic or synthetic) that kill or inhibit the growth of microorganism.

Ideal Properties of Antibiotics:

- They have the appropriate spectrum of activity.
- They have no toxicity to the host.
- They should have low development of resistance.
- They should have rapid and extensive tissue distribution.
- They should have relatively long half-life.
- They should be free of interaction with other drugs.
- They should be easy to administer.

Mode of Action of Antibiotics:

Mode of action of various antibiotics is listed in Table 3.6.

Table 3.6: Mechanism of action of antibiotics

Mechanisms	Antibiotics
Inhibition of synthesis or damage to cell wall	Penicillins, Cephalosporins
Inhibition of synthesis or damage to Cytoplasmic membrane	Polymyxins
Inhibition of synthesis or metabolism of nucleic acids	Rifampin, Quinolones
Protein biosynthesis	Tetracyclins, Chloramphenicol
Modification of energy metabolism	Sulfonamides, Trimethoprim

3.7 ANTIBIOTIC SUSCEPTIBILITY TEST

***In-vitro* test:** This test is carried out to know the antibiotic susceptibility. Because many bacteria have unpredictable susceptibilities to antimicrobial agents, it is measured by MIC method. Susceptibility means the microorganism is inhibited by a concentration of antimicrobial agent that can be attained in blood with the normally recommended dose of the antimicrobial agent.

Determination of MIC (Minimum Inhibitory Concentration): It is lowest concentration of disinfectant that will inhibit the growth of a micobe in culture. Similar to test-tube serial dilution used in antibiotic susceptibility test (Fig. 3.12). This test is performed using Mueller Hinton Agar (MHA) medium because it has good reproducibility, low in sulfonamide, trimethoprim and tetracycline inhibitors. It gives satisfactory growth of most bacterial pathogens. The MIC is carried out by various methods, (A) Serial dilution method and (B) Agar diffusion method. But serial dilution method is more advantageous because:

- The method evaluates the agent against many microorganisms at once.
- Turbid agents are evaluated by this method.
- It is most suitable for evaluation of antifungal agents.
- It is easy to detect resistance cells.

Procedure:

- Prepared graded doses of test substance (disinfectant) as well as stock solution of antibiotics by the following formula:

$$\frac{1000}{P} \times V \times C = W$$

where, P = Potency (mcg/mg),
V = Volume required,
C = Final concentration of solution,
W = Weight of antibiotic (mg)

- To this added suitable liquid medium e.g. Nutrient Broth or MHA medium.
- Aseptically distributed 1 ml of double strength nutrient broth in to each tube.
- 1 ml of antibiotic is added to the first tube then transfer 1 ml from first tube to next tube and repeated up to last tube.
- Tubes are inoculated with 0.5 ml of standardized test organism (McFarland standard: 0.5 ml of 0.048 M barium chloride is added to 99.5 ml of 0.18 M sulphuric acid).
- Tubes incubated at appropriate temperature at 37°C for 24 hours.
- Finally, turbidity is measured.

Example: In case of broth dilution, 2 fold dilutions: 1, 2, 4, 8, 16, 32, 64, 128 mcg/ml, now up to 4 mcg/ml there are growth of organism and from 8 to 128 mcg/ml no growth, then MIC = 8 mcg/ml.

Minimum Bactericidal Concentration (MBC):

It is the lowest concentration of the antimicrobial agent that kills 99.9% of the test organism.

Procedure:

- A pure culture of a specified microorganism grown overnight, then diluted in growth-supporting broth (typically Mueller Hinton Broth) to a concentration between 1×10^5 and 1×10^6 cfu/ml.
- A stock dilution of the antimicrobial test substance is made.

- Further 1 : 1 dilutions are made in test tubes.
- All dilutions of the test product(s) are inoculated with equal volumes of the specified microorganism.
- A positive and negative control tube or well is included for every test microorganism to demonstrate adequate microbial growth over the course of the incubation period and media sterility, respectively.
- An aliquot of the positive control is plated and used to establish a baseline concentration of the microorganism used.
- The tubes are then incubated at the appropriate time and temperature.
- Turbidity indicates growth of the microorganism and the MIC is the lowest concentration where no growth is visually observed.
- To determine the MBC, the dilution representing the MIC and at least two of the more concentrated test product dilutions are plated and enumerated to determine viable CFU/ml.
- The MBC is the lowest concentration that indicated a pre-determined reduction (such as 99.9%) in CFU/ml when compared to the MIC dilution.

Example: In case of broth dilution, 2 fold dilutions: 1, 2, 4, 8, 16, 32, 64, 128 mcg/ml, now up to 16 mcg/ml there are growth of organism and from 32 to 128 mcg/ml no growth, then MBC = 32 mcg/ml.

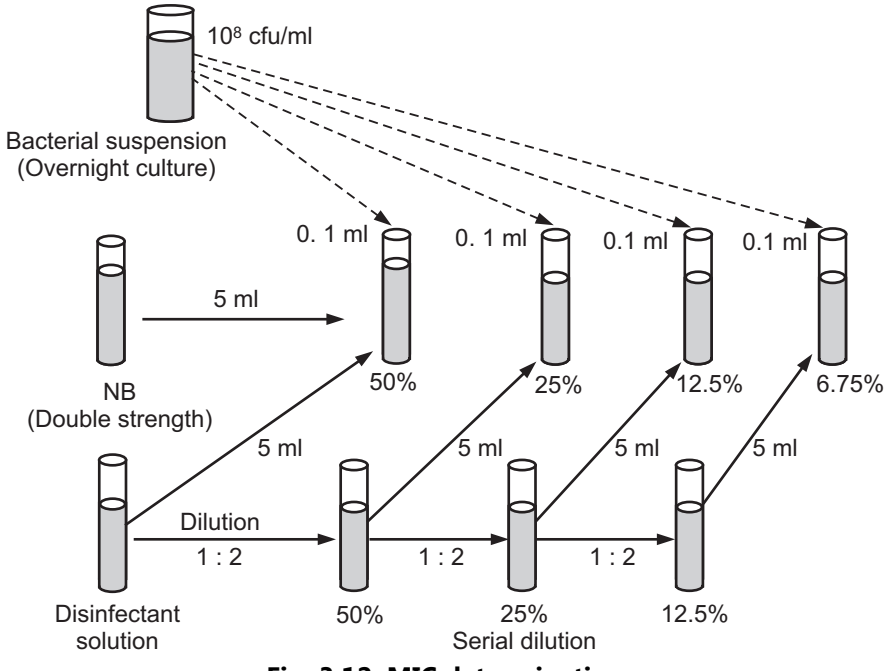


Fig. 3.12: MIC determination

Bactericidal:

They are the agents that disrupt the cell wall synthesis and kill the bacteria. They are less reliant on host resistance. They have more flexibility with dosage interval and hence more

powerful than static drugs. MBC is mainly done for bactericidal activity. It is defined as the minimum concentration of the drug or antibiotic that kills the given test organism. Examples: Penicillins, cephalosporins.

Bacteriostatic:

They are the agents that inhibit the RNA synthesis or reproduction and hence inhibit the growth and reproduction of bacteria. They help the host defenses to take over. MIC is mainly done for bacteriostatic activity. MIC is defined as the lowest concentration of drug or antibiotics that inhibits the growth of the test organism. Examples: Tetracyclins, chloramphenicol, Trimethoprim.

Some of other examples of bacteriostatic and bactericidal drugs are given below Table 3.7.

Table 3.7: Example: of Bactericidal and bacteriostatic drugs

Bacteriostatic	Bactericidal
▪ Clindamycin ▪ Macrolides ▪ Sulfonamides ▪ Tetracyclines	▪ Isoniazid ▪ Metronidazole ▪ Polymyxins ▪ Rifampin ▪ Vancomycin ▪ Aminoglycosides (at high doses) ▪ Bacitracin ▪ Quinolones

The figure below indicates that when antibacterial drugs are added at particular time it effects either removal or killing action based on the nature of drugs (Fig. 3.13).

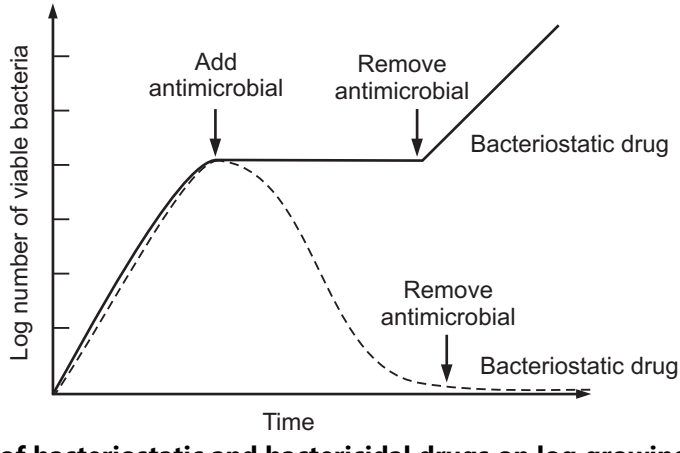
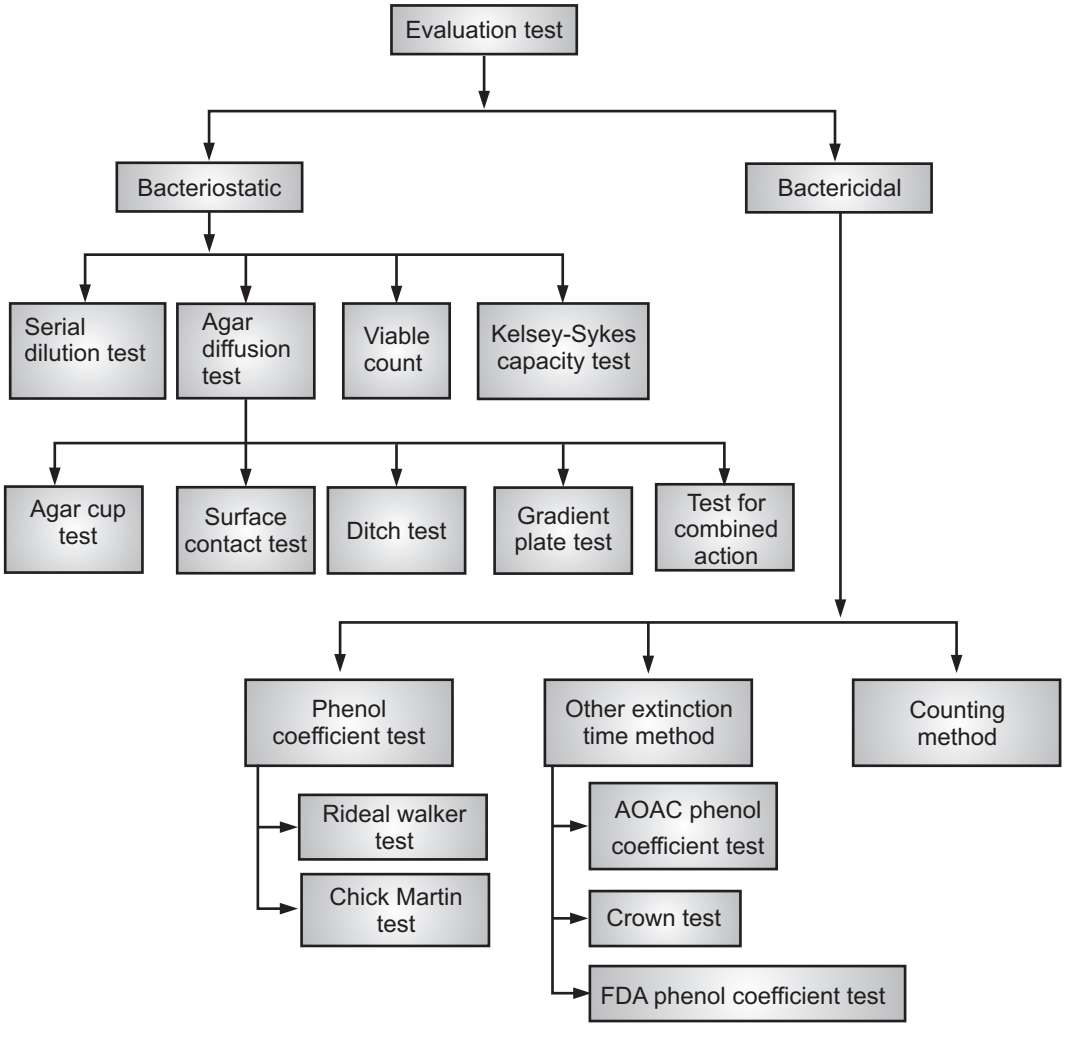


Fig. 3.13: Effects of bacteriostatic and bactericidal drugs on log growing bacteria Vs time

All the methods are used for disinfection of various types of substances. In particular which evaluation tests are carried out for what type of substances is listed in below Table 3.8.

The organisms are used in ATCC grade which stands for the "American Type Culture Collection". It is a non-profit organization which collects, stores, and distributes standard reference microorganisms, cell lines and other materials for research and development. Another type is NCTC, "The National Collection of Type Cultures". They supplies reference bacterial cultures, including many type strains, of medical, scientific and veterinary importance worldwide. Examples: *E. coli*: NCTC 8196 (ATCC 11229); *Staphylococcus aureus* NCTC 4163 (ATCC 6538) etc.

The activity of bactericidal and bacteriostatic is evaluated by various microbial methods which are enlisted in below flowchart 7.



Flowchart 7: Various evaluation methods for bactericidal and bacteriostatic disinfectants

Table 3.8: Antimicrobial test for various substances

Test substances	Bacteriostatic test	Bactericidal test
Liquid disinfectants	Serial dilution in fluid and solid media, cup plate, filter paper method, gradient plate method, Ditch plate method.	End point or extinction time methods, counting method, turbidometric assessment.
Semi-solid antibacterial formulations like cream, ointments and gels	Cup plate and Ditch plate method	Extinction time method, sometimes skin tests are performed
Solid disinfectants like powers	Inhibition on seeded agar	Extinction method
Aerial disinfectant	Use of slit sampler in test chamber	

Disinfectant Evaluation Testing Schemes:

The antimicrobial efficacy of a disinfectant is examined at three stages:

Phase 1:

- Laboratory tests.
- Verifies whether a chemical compound or a preparation possesses antimicrobial activity.
- Preliminary screening tests essentially quantitative suspension tests.
- Absence of organic matter.
- One strain of *S. aureus* and *P. aeruginosa*.
- Determines basic bacteriostatic activity.

Phase 2:

- Carried out in the laboratory.
- Simulating real-life conditions (in-use).
- Organic matter load.
- Several test microorganisms.
- Either as a suspension test.
- On surfaces.
- Determined in the Practical tests.
- Conditions at which use-dilution after a given contact time the preparation is active.

Phase 3:

- "In-use" trials.
- Field tests or pilot studies.
- Tests verify whether, after a normal period of use, germs in the disinfectant solution are still killed.

Evaluation Test of Bacteriostatic Disinfectant:

- **Serial Dilution Test:** This test is carried out in liquid media as well as in solid media. In liquid media, serial dilution is any dilution where the concentration decreases by the same quantity in each successive step. Hence, serial dilutions are multiplicative. In this method, graded concentration of the test substance in a nutrient medium are inoculated

with the test organism and incubated (Fig. 3.14). The medium concentration preventing detectable growth (MIC) is measured as bacteriostatic activity. MIC is defined as the lowest concentration of drug or antibiotics that inhibits the growth of the test organism. MIC mainly done for bacteriostatic activity.

In solid media, a suitable volume of double strength nutrient agar is diluted with an equal volume of bacteriostatic solution and poured into sterile petridish and incubated at 37°C for drying. Then culture broth of test organisms are placed on the dried surface and incubated for 2-3 days (Fig. 3.15). This method is mainly used for solutions that give turbidity with fluid nutrient media.

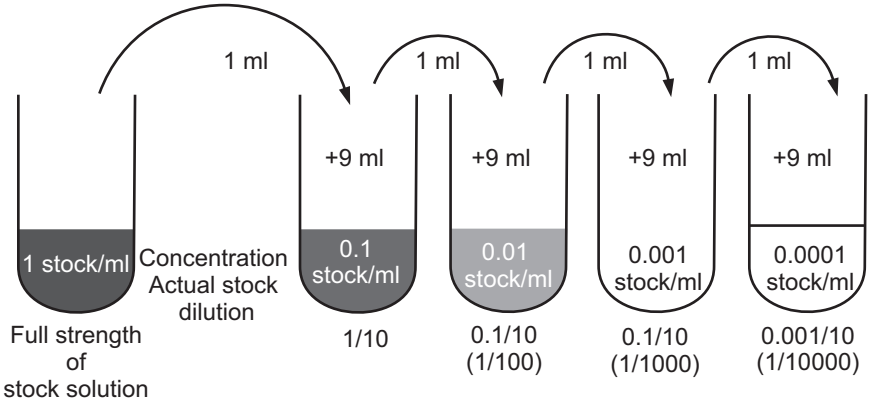


Fig. 3.14: Serial dilution method

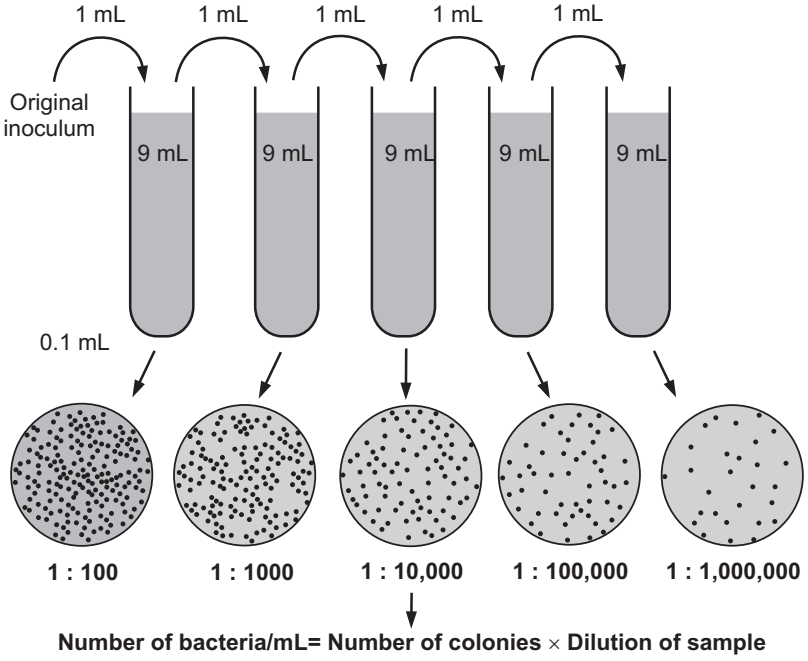


Fig. 3.15: Serial dilution in solid media

- **Cup Plate Method:** This method depends on the diffusion of an antibiotic from a vertical cavity through the solidified agar layer in a petri plate. In this method, agar is melted, cooled and then inoculated with the test organism and poured into a sterile petridish. In this method when the inoculated agar is solidified, holes about 8 mm in diameter are cut in the medium with a sterile cork borer. Using the proper buffer solutions, prepared solutions of known concentrations of the antibiotic are examined and poured into cup of agar plate and then incubated at 37°C for 24 hours. In this method, zones of inhibition may be observed, the diameter of the zones giving a rough indication of the relative activities of different antimicrobial substance. (Fig. 3.16). If the antibiotic has any anti-bacterial effect it will show zone of inhibition.

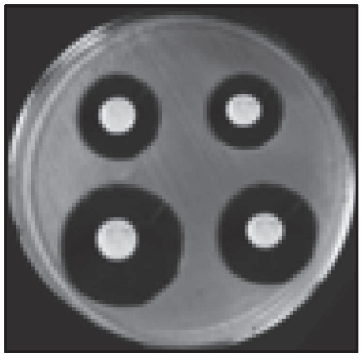


Fig. 3.16: Cup plate method

- **Ditch Plate Method:** In this method the agar is poured in a petri plate and then allowed to solidify. After that ditch cut out is made of the agar. A solution of the antimicrobial substance or a mixture of this with agar is carefully run into the ditch at about three quarters fill it. A loopful of each test organism is then streaked outwards from the ditch on the agar surface. The drug is poured very carefully into the ditch. Various microorganisms are streaked on the sides of the ditch. The width of the zone of inhibition indicates antimicrobial activity. The method is used to find out the potency of the drug against various microorganisms by the means of inhibition of growth on streaked area (Fig. 3.17).

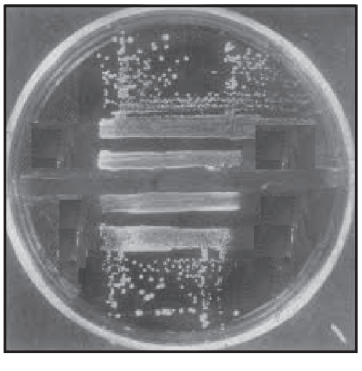


Fig. 3.17: Ditch plate method

- **Gradient Plate Test:** In this method, two layers of agar is poured. The petri dish is kept in slanting position and a sufficient amount of melted nutrient agar is poured and solidified in slanting position. Another layer of agar is poured over it which content antibiotic solution and solidified it (Fig. 3.18). After solidification the bacterial culture is spreaded over the solid surface and the plates are then incubated over night at 37°C to allow diffusion of antimicrobial substance. The microorganisms will grow on the lower concentration of drug. The antibiotics get diluted on the lower layer and concentration gradient is produced. Finally, MIC is determined with the following formula given below. This method is used to isolate the resistant mutants.

$$MIC = C(x/y) \text{ mg/ml}$$

where, C = Concentration in mg/ml, in total volume
x = Length of growth (cm)
y = Total length of possible growth (cm)

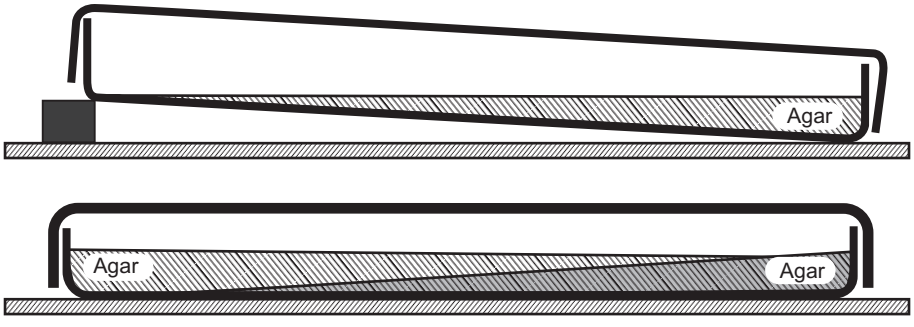


Fig. 3.18: Method of preparation of gradient plate

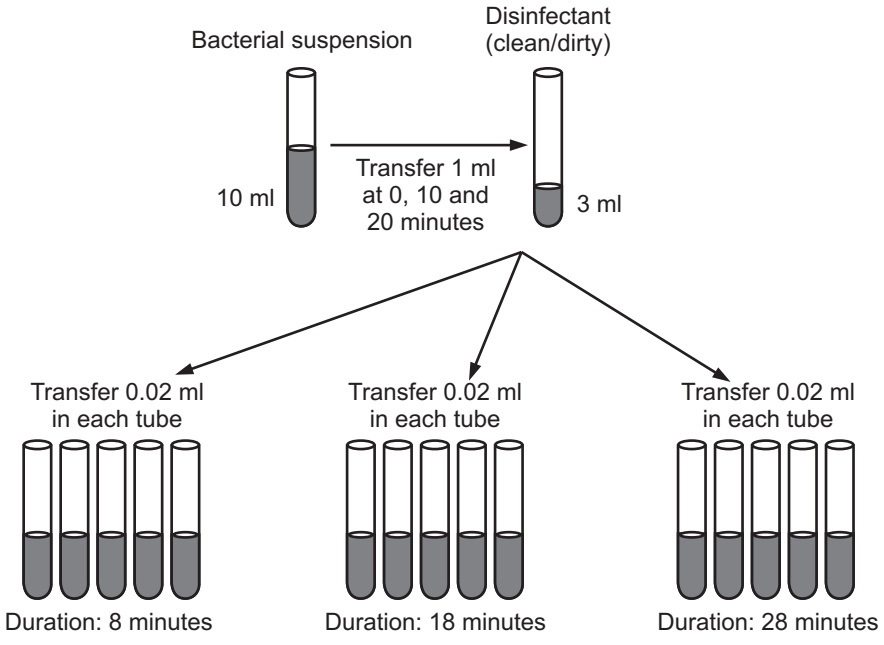
- **Surface contact test:** This method is used to assess the effectiveness of the disinfectant against surface adhered to microorganisms. The test substance is contaminated with a standardized inoculum of the test bacteria and dried. Then a known volume of disinfectant solution is distributed over the carrier. After certain time the number of survivors is determined by impression on contact plate and the number of bacteria is determined. This method is used for determination of reduction rate of *S. aureus*. The disinfectant is applied on the carrier for the application time and thereafter the carrier continues to dry during the exposure. This test reflects in-use conditions like contact times, temperatures, use-dilutions, and surface properties.
- **Test for Combined Action:** This test helps to prevent emergence of resistant strains. The test was described by **Maurer** in 1985 that can be used in hospitals and laboratories to detect contamination of disinfections.

- **Viable count:** A viable cell is able to divide and form offspring. Viable count determines the number of cells in a sample capable of forming colonies on a suitable agar medium. Hence, viable count is also known as plate count. Each viable cell can grow and divide to yield one colony. Hence, colony numbers are a reflection of cell numbers. It is done by two methods likely spread plate method and pour plate method. The main advantages are, the method is very sensitive and one can count subsets of the population. Some disadvantages are, colony forming units may underestimate cell numbers because of chains of cells and also it requires at least few hours or overnight for incubation.
- **Kelsey-Sykes Capacity Test:** The ability to retain activity in the presence of an increasing contamination load is the capacity of the disinfectant. In a capacity test, the disinfectant is challenged repeatedly by successive additions of bacterial suspension until its capacity to kill has been exhausted. Capacity tests simulate the practical situations of housekeeping and instrument disinfection. This capacity test is known as **Kelsey-Sykes test**.

It is a triple challenge test to determine concentrations of disinfectant that will be effective in clean and dirty conditions. The disinfectant is challenged by three successive additions of a bacterial suspension during the course of the test. The duration of test is for 30 minutes. The concentration of the disinfectant is reduced by half by the addition of autoclaved yeast cells, which builds up to a final concentration of 0.5%. Four organisms are used namely *S. aureus, E. coli, P. aeruginosa* and *Proteus vulgaris* based on the type of disinfectant. The method can be carried out under 'clean' or 'dirty' conditions. The dilutions of the disinfectant are made in hard water (10% w/v of $MgSO_4$, $7H_2O$) for clean conditions and in yeast suspension for dirty conditions. Test organism alone or with yeast is added at 0, 10 and 20 minutes interval. The contact time of disinfectant and test organism is 8 minutes. The three sets of five replicate cultures are prepared and incubated at 37°C for 48 hours and growth is assessed by turbidity (Fig. 3.19). The disinfectant is evaluated on its ability to kill microorganisms or absent of it and the result is reported qualitatively as a pass or a fail. Sets that contain two or more negative cultures are recorded as a negative result. Further the dilution tested if negative results are obtained after the first and second challenges then the disinfectant is passed. The third challenge is not included in the pass/fail criterion but positive cultures serve as inbuilt controls. If there are no positive cultures after the third challenge, a lower concentration of the disinfectant further is tested (Table 3.9).

Table 3.9: Result of the experiment is as follows (as an example)

Conc.	Inoculum count	Challenge number			Result
		1	2	3	
1.0	2×10^9	+++++	+++++	+++++	Fail
1.5	2×10^9	−−−−+	--+++	+++++	Pass
2.0	2×10^9	−−−−−	−−−−−	−−−−+	Pass



Each tube incubated at 37° C for 48 hours

Fig. 3.19: Kelsey-Sykes dilution

Evaluation Tests for Bactericidal Disinfectants:

- **Phenol Coefficient Test:** The test is for the measurement of bactericidal activity of a chemical compound in relation to phenol. This test is carried out by measured the concentration at which a chemical is equal in effectiveness to phenol. *Staphylococcus aureus* or *Salmonella typhi* are used for this test which are inoculated at 20-37°C for 2-3 days. Phenol coefficient is calculated by:

$$\text{Phenol coefficient} = \frac{\text{Concentration of chemical}}{\text{Concentration of phenol}}$$

If a chemical is equal in effectiveness to phenol at the same concentration then its phenol coefficient is = 1. If the concentration of chemical is less than phenol then phenol coefficient is greater than 1.

Example: Phenol dilution = 1/50 and the maximum effective dilution for disinfectant (x) = 1/ 450.

Then phenol coefficient = 9.

It is determined by two methods:

(a) Rideal Walker Method

(b) Chick Martin Test

(a) Rideal Walker Test (R-W Test): It is the ratio of the dilution of the disinfectant that kills a microorganism to the dilution of phenol that kills the organism in the same time under

identical conditions. The test is based on the principle of dilutions of the test disinfectant is compared with the standard dilutions of the phenol (usually 1 in 95) further activity against *Salmonella typhi*.

Procedure:

Preparation of sample: The disinfectant fluids sample is mixed thoroughly and is withdrawn from the middle of the sample without formed air bubble.

Apparatus: A loop, 4 mm in internal diameter is made at end of a 0.376 mm wire of platinum or platinum iridium alloy, 38 mm long from the loop to the holder. The loop is bent at such an angle to the length of the wire as will facilitate in removal vertically from the surface of the liquid by the loop horizontal.

Incubator: Maintained at 37°C ± 1°C.

Pipettes: Standard graduated pipettes of capacity 10 ml, 5 ml and 1 ml are required.

Dropping Pipette: Made to deliver 0.2 ml.

Medication Tubes: 5 sterile plugged rimless test tubes 125 mm × 22 mm made of hard neutral glass.

Broth Tubes: About two dozens of standard measuring cylinders, stoppered and graduated are used.

Standard measuring cylinders stoppered and graduated: 500 ml graduated in 10 ml: one 100 ml graduated 1 ml: five.

Reagents:

(a) Broth: Broth is prepared with the mixture of the following ingredients:

Meat extract (Microbiological grade) 20 g.

Peptone (Microbiological grade) 20 g.

Sodium Chloride (Reagent Quality) 10 g.

Distilled Water: 1000 ml.

Dissolved the solids in distilled water then sodium hydroxide is added to neutralize the solution and boiled to bring down phosphates and filter while hot. pH 7.6 is adjusted with normal hydrochloric acid. The broth is then sterilized by autoclaving at 15 Ibs with pressure for 20 minutes. It is then filtered and placed in 5 ml quantities in sterilized broth tubes and is sterilized by autoclaving at 15 Ibs pressure for 10 minutes.

(b) Test Organism: The test organism *Salmonella typhi* (NCTC 786) is used. This culture is maintained by weekly sub-culture on a nutrient agar slope and incubated the sub-culture for 24 hours at 37°C and then stored in refrigerator at a temperature below 22°C. For the purpose of the test a little of the growth from the most recent sub-culture in nutrient agar slope is placed in tube of R.W. broth and incubated for 23 hours at 38°C. A standard loopful is then transferred to a second tube and incubated. This is done for at least three times before a test is carried out.

(c) Standard Phenol: Phenol of 5 per cent w/v solution is prepared at 40.5°C in sterile distilled water. Test dilutions are prepared from this stock solution containing 1 g of phenol in each 95, 100, 105, 155 ml of the solution made.

(d) Sample dilutions: A test portion of 5 ml is withdrawn and discharged into about 480 ml of sterile distilled water in a 500 ml glass stoppered sterile measuring cylinder and the pipette is rinsed three times or more in the clear liquid. The whole is then made up to 500 ml with sterile distilled water. The cylinder is stoppered and the contents are mixed thoroughly. Suitable test dilutions in sterile distilled water are then immediately prepared.

Procedure: 5 ml of four chosen dilutions of the disinfectant are placed in four medication tubes and then placed in water bath at temperature between 17°C and 19°C, with the strongest dilution on the left. The fifth medication tube containing 5 ml of the particular phenol dilution is placed on the right. When the content of the medication tubes and broth culture of the test organism have reached the temperature of the water bath, starting at Zero time, 0.2 ml of the culture is added to the left hand medication tube and the tube is shaken gently. After 30 seconds the next tube is inoculated similarly and the process is repeated with each successive tube at an interval of 30 seconds until the phenol control has been inoculated. A loopful of the well shaken content of the tube at the extreme left is withdrawn after 30 second and placed in tube containing 5 ml of broth medium. Thirty seconds after this, similar operation is performed on the second medication tube.

The procedure is repeated at an interval of 30 seconds with each of the five medication tubes working from left to right until four sets of cultures have been made i.e. at 2.5, 5, 7.5 and 10 minutes respectively after exposure. Then the inoculated broth tubes are incubated for 48 hours at 37°C and observed for the growth of the test organisms by formed turbidity of the broth.

A typical result and the method of calculating the coefficient is given below.

Dilution		**Time of exposure in minutes**			
		2.5	**5**	**7.5**	**10**
Disinfectant	**1 in 400**	**–**	**–**	**–**	**–**
	1 in 500	**+**	**–**	**–**	**–**
	1 in 600	**+**	**+**	**–**	**–**
	1 in 700	**+**	**+**	**+**	**–**
Phenol	**1 in 100**	**+**	**+**	**–**	**–**

(+) = growth; (−) = no growth

Calculation of the Coefficient :

The Rideal-Walker coefficient is obtained by dividing the dilution of test disinfectant which does not kill all the organisms after 2.5 and 5 minutes but after 7.5 and 10 minutes, by the dilution of phenol that produces similar effect.

$$\text{RW coefficient} = \frac{\text{Dilution of disinfectant killing in 7.5 minutes but not in 5 minutes}}{\text{Dilution of phenol killing in 7.5 minutes but not in 5 minutes}}$$

$$= \frac{600}{100}$$

$$= 6$$

The result indicated that a phenol coefficient greater than 1 means that the disinfectant is better than phenol.

Disadvantages of the Rideal-Walker Test:

1. No organic matter is included.
2. Microorganism *Salmonella typhi* may not be appropriate.
3. Time allowed for disinfection is short.
4. Used to evaluate phenolic typed is infectants only.

- **Chick Martin Test:** This test is carried out in the presence of organic matter like 3% human faecas or dried yeast. This test is modified method of RW test or qualitative suspension test.

Procedure (Fig. 3.20):

- Serial dilution of test solution and phenol is prepared in distilled water.
- Prepared 5% solution of phenol.
- To this 5% yeast suspension is added after 30 minutes.
- Set up a row of nine test tubes. To each test tube added 2.5 ml of diluted disinfectant at 30 seconds intervals. Allow the reaction to proceed at 20°C (room temperature) in a water bath.
- To this solution *Salmonella typhi* is added.
- After contact time of 30 minutes the above mixture is transferred to the freshly prepared 10 ml of broth.
- Set up two rows of 10 ml nutrient broth, one for each dilution of disinfectant under test and of the Phenol (i.e. two at each disinfectant and phenol dilution).
- The test tubes are incubated at 37°C for 48 hours.
- Finally presence of microorganisms is calculated.

Calculation:

$$\text{Chick Martin Test} = \frac{\text{Mean of highest phenol concentration inhibiting and lower permitting growth}}{\text{Mean of highest disinfectant concentration inhibiting and lowest permitting growth}}$$

Sample result:

	Decreasing concentration (%) ⟶								
	A	B	C	D	E	F	G	H	I
Phenol series	–	–	–	+	+	+	+	+	+
Disinfectant series	–	–	–	–	–	+	+	+	+

(+) = Growth; (−) = No growth

Average of phenol series = C + D/2

Average of disinfectant series = E + F/2

$$\text{Chick Martin coefficient} = \frac{C + D/2}{E + F/2} = \frac{C + D}{E + F}$$

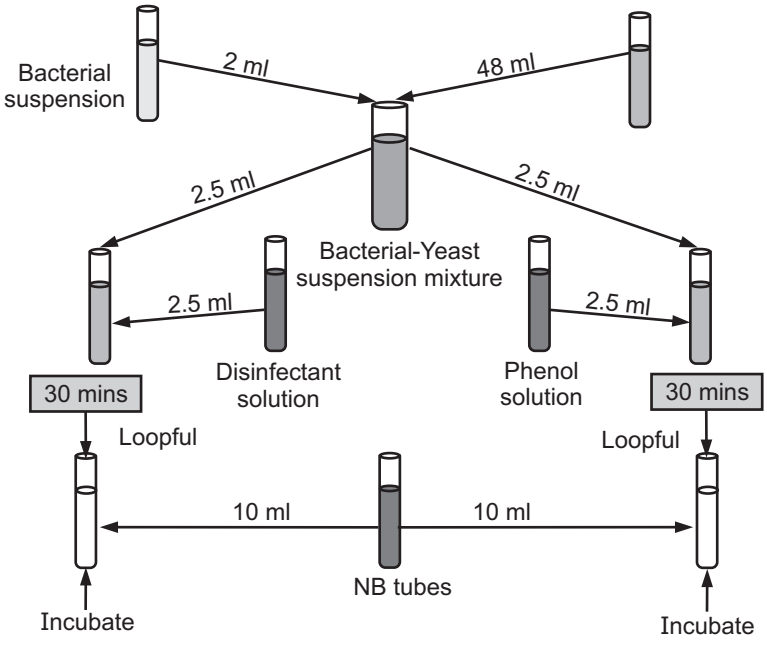


Fig. 3.20: Schematic diagram of Chick Martin test dilution

There are some differences between RW test and Chick Martin test which are tabulated in Table 3.10.

Table 3.10: Difference between RW test and Chick Martin test

Parameters	RW test	Chick Martin test
Medium volume	5.0 ml	10.0 ml
Diluent for test disinfectant	Distilled water	Water with yeast suspension of faeces
Reaction temperature	17.5 ± 0.5°C	30°C
Organism used	*Salmonella typhi*	*Salmonella typhi* and *Staphylococcus aureus*
Sampling times	2.5, 5.0, 7.5, 10 minutes	30 minute
Calculation of coefficient	Dilution test killing in 7.5 minutes divided by same for phenol	Mean concentration of phenol showed no growth after 30 minutes divided by same for test.

- **AOAC phenol coefficient test:** AOAC mean American Association of Official Analytical Chemists.

Procedure:

- Dilutions of disinfectant are made from 1% stock solution.
- From 5% stock solution of phenol, made further dilutions.

- Used 1 : 80 and 1 : 90 for *Pseudomonas aeruginosa*.
- Used 1 : 90 and 1 : 100 for *Salmonella typhi*.
- Used 1 : 60 and 1 : 70 for *Staphylococcus aureus*.
- Placed tubes with 5 ml of each final disinfectant dilution and phenol in a water bath at 20°C.
- Placed tubes with standardized suspension of test organisms in the water bath.
- Added 0.5 ml of test culture to each dilution and phenol.
- Mixed gently and transfer a loopful to neutralizing media at 5, 10, and 15 minutes interval.

In this method, Phenol Coefficient is given by the highest dilution of disinfectant killing test organism in 10 minutes but not in 5 minutes divided by the highest dilution of phenol showing the same result. The calculation of the coefficient is tabulated in Table 3.11.

Table 3.11: Calculation of coefficient test

Dilutions	**Disinfectant**		
	5 minutes	**10 minutes**	**15 minutes**
1 : 300	–	–	–
1 : 325	+	–	–
1 : 350	+	–	–
1 : 375	+	+	–
Phenol			
1 : 90	+	–	–
1 : 100	+	+	+

- **Crown Test:** This test is intended for testing white disinfectants in finely dispersed emulsions of coal tar acids or similar acids from petroleum. This test is nearly similar to Chick-Martin test, but in this test disinfectant is diluted in sterile artificial seawater and gelatin and rice starch are included as organic matter.
- **FDA Phenol Coefficient Test:** In this technique, water is used as diluents for disinfectant. The reaction volume mixture is 10 ml and the medium pH is 6.8. *Salmonella typhi* is used as standard organism in this test. The test sample is inoculated in the medium at 5, 10, 15 minutes intervals. Coefficient is calculated by dilution test killing in 10 but not in 5 minutes divided by same for phenol.

Below table reflected the comparative study between all the biocidal evaluation tests (Table 3.12).

Table 3.12: Comparative study between all the bactericidal activities (Phenol coefficient tests)

	RW test	Chick Martin Test	AOAC Test	FDA Test	Crown Test
Medium pH	7.5	7.5	6.8	6.8	7.4
Medium volume	5 ml	10 ml	10 ml	10 ml	5 ml
Volume of reaction mixture	5 ml	5 ml	5 ml	10 ml	5 ml
Organism used	*S. typhi*	*S. typhi*	*S. typhi, S. aureus*	*S. typhi*	*S. typhi*
Sampling time	2.5, 5, 7.5 and 10 minutes	30 minutes	5, 10 15 minutes	5, 10 15 minutes	10 minutes
Reaction temperature	17.5 ± 0.5°C	30°C	20-37°C	20°C	19.0 ± 0.1°C
Diluent for disinfectant	Water	Yeast suspension	Water	Water	Artificial Sea water
Coefficient calculation	Dilution test killing in 7.5 but not in 5 minutes divided by same for phenol	Mean highest phenol concentration inhibiting and lowest permitting growth divided by the same for test	Greatest dilution of the test killing in 10 minutes divided by same for phenol	Dilution test killing in 10 but not in 5 minutes divided by same for phenol	Dilution test killing in 10 but not in 5 minutes divided by same for phenol

- **Counting Method:**

Mainly viable count method is used which is known as quantitative suspension test. The count is taken after suitable contact time and proportion of viable cells are observed. The main advantages of viable count are:

- It counts viable cells only i.e. growing cells.
- It is possible to see cell morphology.
- It can differentiate between different microorganisms.
- This method is used for different types of sample (milk and milk products).

Some of disadvantages are like:

- The method takes longer time.
- The method is tedious and requires lot of test tubes.

Types of Viable Count:

The method is two types namely spread plate method and pour plate method. All these methods have three basic steps viz. dilution, Plating and incubation.

Spread Plate Method:

Principle:

The method involves using a sterilized spreader with a smooth surface made of metal or glass to apply a small amount of bacteria suspended in a solution over a plate. The plate is dried at room temperature to absorb the bacteria in agar very fast. A successful spread plate will have a countable number of isolated bacterial colonies evenly distributed on the plate.

Method (Fig. 3.21):

1. Dilution series are made from a sample.
2. 0.1 ml of sample is pipetted out from the appropriate desired dilution series onto the center of the surface of an agar plate.
3. Dipped the L-shaped glass spreader into alcohol and steriled.
4. Spreaded the sample evenly over the surface of agar using the sterile glass spreader.
5. Incubated the plate at 37°C for 24 hours.
6. Calculate the Colony Forming Unit (CFU) value of the sample.
7. Counted colonies further multiply by the dilution factor to determine the number of CFU/mL in the original sample.

Advantages:

- With this method the total number of colony forming units on a single plate is enumerated.
- It is used to calculate the concentration of cells in the tube from the plated sample.
- The method is used in enrichment, selection and screening experiments.

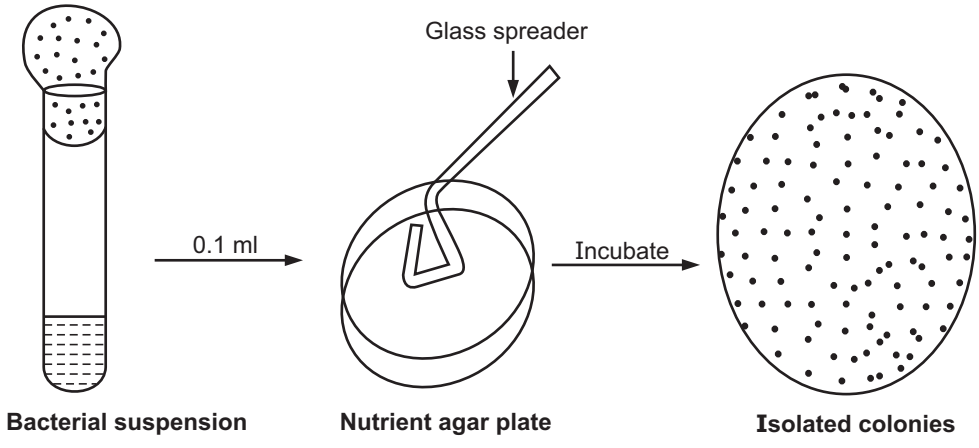


Fig. 3.21: Spread plate counting methods

Pour Plate Method:

Principle:

In this method successive dilution of inoculums is added to molten agar in respective petri plates and the individual colonies are counted.

Method (Fig. 3.22):

- The sample is diluted in sterile distilled water and the agar medium is maintained in molten stage at 45°C.
- 1 ml of each dilution is added to each sterile petri dish.
- Further added 9 ml of molten agar in to the same petri dishes.
- The contents are mixed properly and allowed to solidify.
- The petri dishes are incubated at suitable temperature of 37°C for overnight.
- After incubation there are different kinds of microbes are grown as colonies.
- The colonies are then counted.

Advantages:

- The method is simple and useful.
- The method is sensitive.
- It gives the count of viable cells.

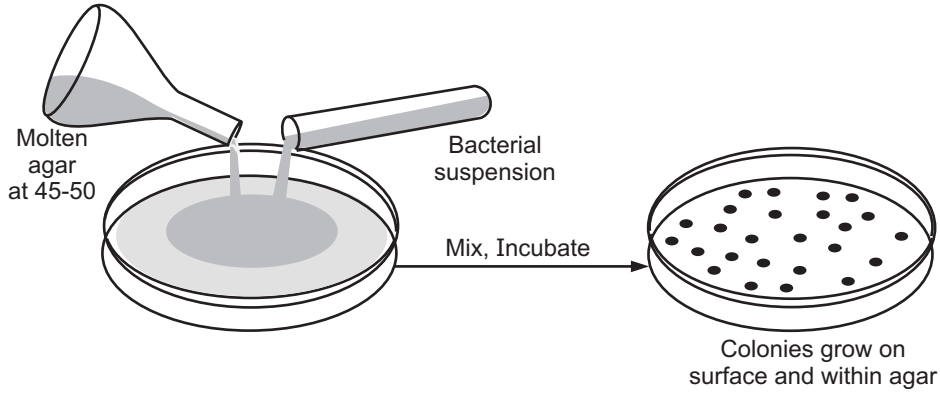


Fig. 3.22: Pour plate counting method

3.8 STERILITY TESTING OF PRODUCTS ACCORDING TO IP, BP AND USP

Sterility Testing:

It is the method required for all articles or substances to be introduced in to raw tissue (injections, ophthalmics etc.). The method is reveals the presence or absence of viable microorganisms in any articles. All the products labeled sterile indicate that the product must pass through the sterility testing methods as per IP, BP and USP. There are two methods are used namely membrane filtration and direct inoculation. Minimum number of items are recommended as per IP to be tested in a batch are depicted in Table 3.13.

Table 3.13: Number of test item in a batch as per IP, BP and USP

Preparations	Number of Items in a batch	Recommended number of items
Injectable preparations	Not more than 100 containers	4 containers (10%)
	More than 100 but not more than 500 containers	10 containers
	More than 500 containers	20 containers (2%)

Preparations	Number of Items in a batch	Recommended number of items
Ophthalmic and other non-injectable preparations	Not more than 200 containers	2 containers (5%)
	More than 200 containers	10 containers
Devices	Catgut and other surgical sutures	5 packets (2%)
	Not more than 100 articles	4 articles (10%)
	More than 100 but not more than 500 articles	10 articles
	More than 500 articles	20 articles (2%)
Bulk solid products	Up to 4 containers	Each containers
	More than 4 containers but not more than 50 containers	4 containers (20%)
	More than 50 containers	10 containers (2%)
Liquids (other than antibiotics)	Less than 1 ml	Whole contents of container
	1-40 ml	Half the content in each container
	More than 40 ml but not more than 100 ml	20 ml
	More than 100 ml	10% of the content of the container but not less than 20 ml
	Antibiotic liquids	1 ml
	Insoluble preparations, creams and ointments	Not less than 200 mg
Solids	Less than 50 mg	Whole content of the container
	More than 50 mg but less than 300 mg	Not less than 50 mg
Added as per BP	300 mg to 5 g	150 mg
	More than 5 g	500 mg

(A) Membrane Filtration:

This method is used for those substances that are put in to the solution like oil, ointment, non-bacteriostatic solids and a soluble powder or liquids that have themselves bacteriostatic properties etc. This method is used for the liquid products which have the volume of the container 100 ml or more.

Apparatus:

The apparatus contains a reservoir and a container to collect the filtrate. In the junction of these two, a membrane is placed which has an proper porosity (0.45 μM) and filter diameter is 47 mm. The flow rate of fluid is 55-75 ml/minute at a pressure of 70 mm of mercury. Complete unit should be free from microorganisms (Fig. 3.23).

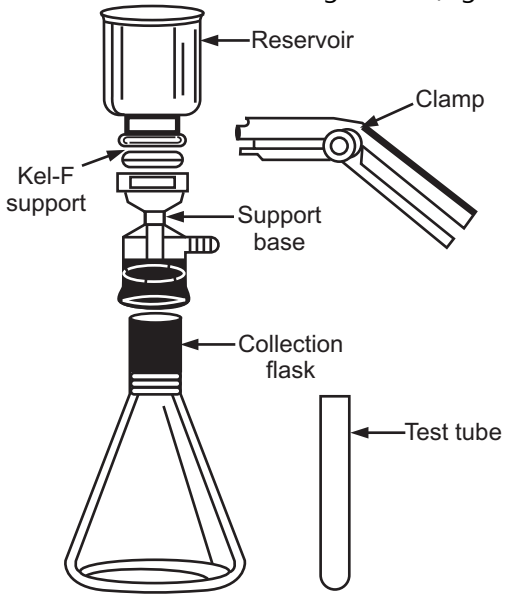


Fig. 3.23: Membrane filter

Dilution Fluids:

Mainly two types of dilution fluids are used likely Fluid-A, which is prepared by dissolved 1 g of peptic digest animal tissue in 1 litre of water. Filtered and pH adjusted 7.1. Poured the fluid into 100 ml flask and then sterilized at 121°C for 20 minutes. Fluid-B, which is prepared by using fluid A, to each litre, is added 1 ml of polysorbate 80. This is prepared if the sample contains lecithin or oil. Then poured the fluid into 100 ml flask and then sterilized at 121°C for 20 minutes in autoclave.

Quantities of Sample:

1. For injectable preparations:

The uses of the contents of the containers are listed in below Table 3.14.

Table 3.14: Quantities of injectable preparations for sterility testing

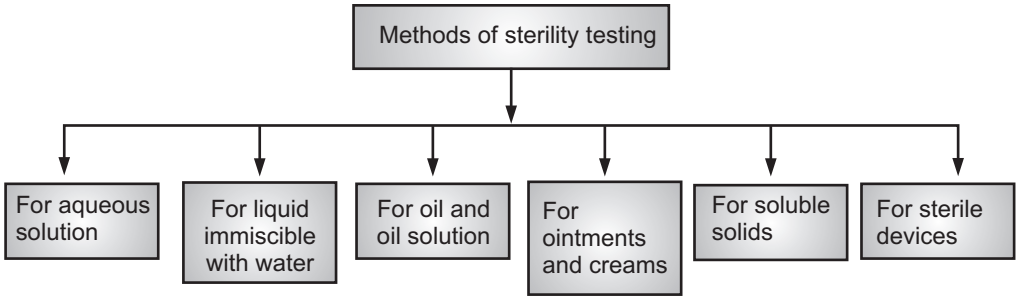
Preparations	Quantities of each containers	Minimum quantities to be used
Liquids	Less than 1 ml	Total content of the container
	1 ml to 4 ml	Half of the content
	4 ml to 20 ml	2 ml
	20 ml to 100 ml	10% of the content of the container
	100 ml or more	Not less than half of the content
Solids	Less than 50 mg	Whole content
	50 mg to 200 mg	Half the content
	200 mg or more	100 mg

2. For ophthalmic and other non-injectable preparations:

The uses of the contents of the containers are listed in below Table 3.15.

Table 3.15: Quantities of ophthalmic and other non-injectable preparations for sterility testing

Preparations	Quantities to be mixed	Minimum quantities to be used
Ophthalmic and other non-injectable preparations	10 to 100 ml	5 to 10 ml
Insoluble preparations for emulsification or suspension (cream, ointments), preparation soluble in water	1 to 10 gm	0.5 to 1 gm
Absorbent cotton	–	Not less than 1 gm



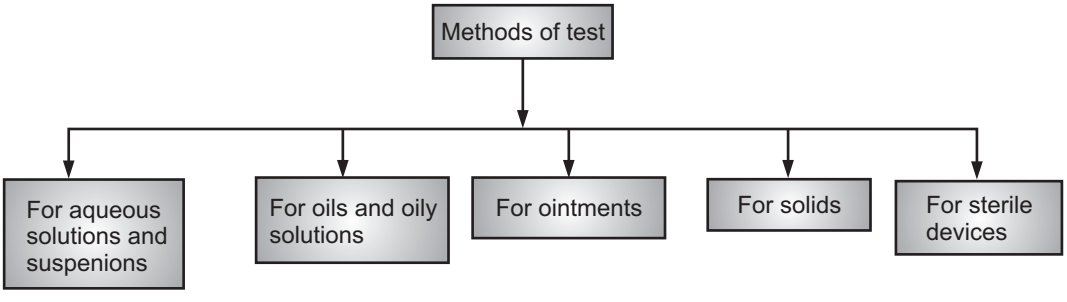
Flowchart 8

- **For aqueous solution:** Aseptically transferred the quantities of test preparations in the two media on to one membrane. Collected the filtrate through the vacuum filtration. The membrane is removed aseptically and cutted in to two pieces. One piece is then soaked in 100 ml soyabean casein digest medium and incubated at 25°C for 7 days. Other part of the paper is immersed in 100 ml of liquid thioglycollate medium and incubated at 35°C for 7 days.
- **For liquid immiscible with water:** This test is carried out with sufficient quantity of fluid A to the sample to get fast filtration.
- **For oils and oil solutions:** Low viscosity of oils and their solutions are filtered through drug membrane without dilution whereas viscus oils are filtered through filter membrane with suitable diluents.
- **For ointments and creams:** Ointments are dilute in a fatty base and water in oil type emulsions formed by heating at 40°C by added sterile isopropyl myristate as sterile diluents.

- **For soluble solids:** Solids are dissolved in a suitable sterile solvent like fluid A and then filtered.
- **For sterile devices:** Aseptically passed a sufficient volume of fluid B and then collected the liquid in sterile container and filtered through membrane filter.

(B) Direct Inoculation:

This method is used for small volume samples. The volume of the product is not more than 10% of the volume of the medium. It is suitable method for aqueous solutions, oily liquids, ointments and creams. The method involves aseptically opened each sample container from a sterilized batch of product. Then using sterile syringe and needle the required volume of sample is withdrawn for both media from the container. Half of the volume of the sample is injected in to a test tube containing the required volume of fluid thioglycollate medium and the other half volume of sample into a second test tube containing the required volume of Soybean Casein Digest Medium.



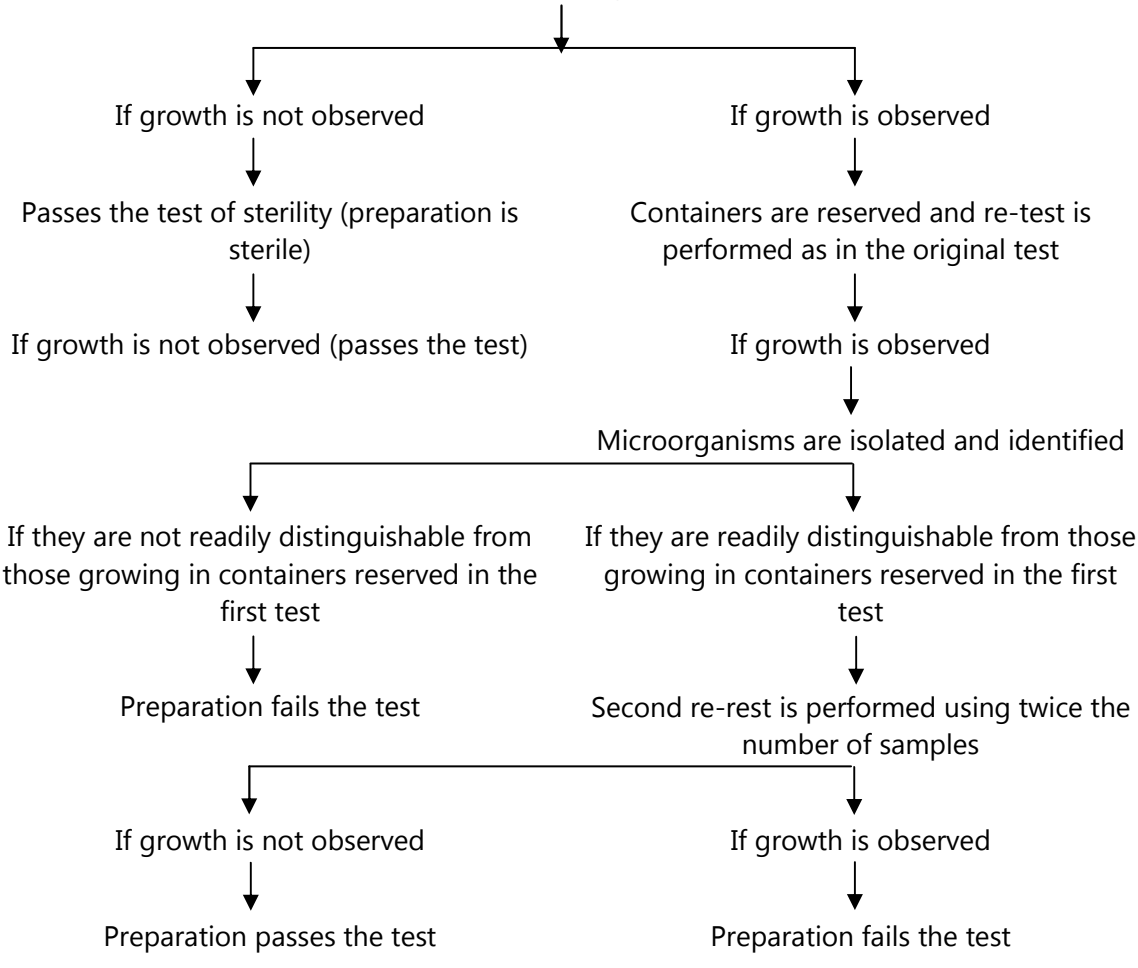
Flowchart 9

- **For aqueous solutions and suspensions:** The liquid from the test container is removed and specified volume is aseptically transferred to the vessel of culture medium. The inoculated medium is then incubated for 35°C for 14 days in fluid thioglycolate medium and at 25°C in case of soyabean casein digest medium.
- **For oils and oily solutions:** For the oils and oily solutions the media is added with 0.1% w/v of polyethoxyethanol or 1% w/v of polysorbate 80 or other emulsifying agents in suitable concentration and the sterility test is performed.
- **For ointments:** Ointments are 10 fold diluted with sterile diluents like fluid-B.
- **For solids:** Required quantity of material is transferred to the sterile medium.
- **For sterile devices:** The device is immersed in 1000 ml of culture medium. If not immersed properly then 20 units of lumen flushed with thioglycolate medium and soyabean casein digest medium separately and recovered 15 ml of each medium and incubated 100 ml of the each medium.

Interpretation of the sterility testing results is tabulated in Table 3.16.

Table 3.16: Observation and interpretation of sterility testing result

After the incubation and during the incubation period



QUESTIONS

Long Essays:

1. Explain morphology, classification, reproduction and cultivation methods of Fungi.
2. Explain morphology, classification, reproduction and cultivation methods of Viruses.
3. Explain classification and mode of action of disinfectants.
4. Explain in detail about antiseptics.
5. Explain detail about bactericidal and bacteriostatic agents with their evaluation test.
6. Explain in detail about sterility testing for various categories of products as per IP, BP and USP.
7. Explain Phenol coefficient test in details.

Short Essays:

1. Explain reproduction of fungi.
2. Explain classification of viruses.

3. Explain cultivation of viruses.
4. Explain morphology of Fungi.
5. Explain physical method of disinfectant.
6. Explain chemical method of disinfectant.
7. Differentiate sterilization and disinfection and disinfectants and antiseptics.
8. Explain the factor influencing disinfection.
9. Explain in detail about the role of antimicrobial agents.
10. Explain antibiotic susceptibility test.
11. Explain evaluation test for bacteriostatic disinfectants.
12. Explain evaluation test for bactericidal disinfectants.
13. Explain Kelsey-Sykes Test.
14. Explain RW Test.
15. Explain counting method for bactericidal activity.
16. Explain sterility testing method by direct inoculation method.

MULTIPLE CHOICE QUESTIONS

1. Blastocondia is formation of ……
   (a) Filament (b) Nucleus
   (c) Cell wall (d) Bud
2. The main structural component of cell wall of yeast is ……
   (a) Glucan (b) Protein
   (c) Lipid (d) Chitin
3. Optimum temperature of growth for saprophytic fungi is ……
   (a) 30 – 37°C (b) 20 – 30°C
   (c) 40 –50°C (d) 10-20°C
4. Optimum temperature of growth for parasitic fungi is ……
   (a) 30 – 37°C (b) 20 – 30°C
   (c) 40 – 50°C (d) 10-20°C
5. Lumen is the component of ……
   (a) Cell wall (b) Hyphae
   (c) Cell components (d) Spores
6. Histoplasmosis is caused by ……
   (a) Molds (b) Septate fungi
   (c) Dismorphic fungi (d) Coenocytic fungi
7. Bisidiomycetes are ……
   (a) sac fungi (b) water molds
   (c) club fungi (d) conjugation fungi
8. *Phytophthora cinnamomi* causes ……
   (a) downy mildews (b) damping off
   (c) root rots (d) seedling blight
9. Chitin chitosan is the hyphal component of ……
   (a) Oomycetes (b) Ascomycetes
   (c) Bisidiomycetes (d) Zygomycetes

10. Ascospore present in ……
    (a) Deuteriomycetes (b) Basidiomycetes
    (c) Ascomycetes (d) Zygomycetes
11. Mucor is an example of ……
    (a) Deuteriomycetes (b) Basidiomycetes
    (c) Ascomycetes (d) Zygomycetes
12. Dimorphic fungi are initially differentiated using ……
    (a) Crystal violet (b) Safranin
    (c) Alcohol (d) Papanicolaou stain
13. Telemoph is the type of ……
    (a) Sexual reproduction of fungi (b) Classification of fungi
    (c) Sexual reproduction of molds (d) Classification of molds
14. Zoosporangia is ……
    (a) Flagellated motile spores (b) Non-flagellated motile spores
    (c) Flagellated non-motile spores (d) Non-flagellated non-motile spores
15. Fungal sex organs are known as ……
    (a) sporangia (b) gametangia
    (c) spermatia (d) none of these
16. Parvo virus is ……
    (a) DNA virus (b) RNA virus
    (c) Both of these (d) None of these
17. Capsid is made up of ……
    (a) Lipid (b) Protein
    (c) Chitin (d) Glycogen
18. Structure of Pox viruses are ……
    (a) Complex symmetry (b) Icosahedral symmetry
    (c) Helical symmetry (d) Plain symmetry
19. Example of tadpole shaped virus is ……
    (a) TMV (b) Pox virus
    (c) Rabies virus (d) Bacteriophage
20. Peplomers are ……
    (a) Spike (b) Spores
    (c) Cell wall constituent (d) Glycoprotein
21. Reo virus is an example of ……
    (a) Double stranded RNA virus (b) Double stranded DNA virus
    (c) Single stranded DNA virus (d) Single stranded RNA virus
22. Adeno virus is an example of ……
    (a) Animal virus (b) Plant virus
    (c) Insect virus (d) Bacteriophage
23. Lytic cycle present in ……
    (a) Reproduction of molds (b) Reproduction of yeast
    (c) Reproduction of viruses (d) Reproduction of bacteria

24. Pore size of membrane filter is ……
    (a) 0.45 micron (b) 0.54 micron
    (c) 0.35 micron (d) 0.25 micron
25. 3% hydrogen peroxide solution for 10 minutes is used as ……
    (a) High level disinfectant (b) Intermediate disinfectant
    (c) Low level disinfectant (d) None of these
26. Mechanism of action of Glutaraldehyde as disinfectant is ……
    (a) DNA strand breakage (b) cross linking of protein
    (c) membrane leakage (d) membrane damage
27. Mechanism of action of phenol is ……
    (a) DNA strand breakage (b) cross linking of protein
    (c) membrane leakage (d) membrane damage
28. Idophor antiseptic is ……
    (a) Peridex (b) Betadine
    (c) Hi Phene (d) Ioprep
29. MIC is determined in ……
    (a) Dextrose medium (b) Hinton Agar medium
    (c) Mueller Hinton Agar medium (d) B-5 medium
30. CFU stand for ……
    (a) colony forming unit (b) cell forming unit
    (c) colony per sq.feet unit (d) cell per sq.feet unit
31. Ditch test is used for ……
    (a) Bacteriostatic (b) Bactericidal
    (c) Both of these (d) None of these
32. Crown test is used for ……
    (a) Bacteriostatic (b) Bactericidal
    (c) Both of these (d) None of these
33. Sampling time for Chick Martin test is ……
    (a) 10 minutes (b) 20 minutes
    (c) 30 minutes (d) 5 minutes
34. Organism used in R-W test ……
    (a) *S. aureus* (b) *S. typhi*
    (c) *E. coli* (d) *Proteus vulgaris*
35. Medium volume of AOAC test is ……
    (a) 10 ml (b) 5 ml
    (c) 15 ml (d) 20 ml
36. Diluent for disinfectant use in Crown test ……
    (a) Yeast suspension (b) Water
    (c) Oil (d) Artificial sea water

Answers:

1. (a)	2. (a)	3. (b)	4. (a)	5. (b)	6. (c)	7. (c)	8. (c)	9. (d)	10. (c)
11. (d)	12. (d)	13. (a)	14. (a)	15. (b)	16. (a)	17. (b)	18. (a)	19.(d)	20. (d)
21. (a)	22. (a)	23. (c)	24. (a)	25. (c)	26. (b)	27. (c)	28. (b)	29. (c)	30. (a)
31. (a)	32. (b)	33. (c)	34. (b)	35. (a)	36.(d)				

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Unit ... 4

ASEPTIC ENVIRONMENT AND DIFFERENT MICROBIOLOGICAL ASSAY

♦ LEARNING OBJECTIVES ♦

After completing this chapter, reader should be able:

- To know about designing of aseptic area and what are the equipments are used in aseptic room.
- To know about various sources of contamination in aseptic room and how they are prevented.
- To know the classification of clean area.
- To know about principle and methods of various microbiological assay.
- To know the standardization methods for antibiotics, vitamins and amino acids.
- To know the method of assessment of a new antibiotic.

4.1 DESIGNING OF ASEPTIC AREA

Aseptic area is the area where strict control measures are adopted to avoid contamination of the preparations or any transfer of microorganisms. Hence, proper care should taken while designing the aseptic area. There are some differences between clean area, aseptic area and sterile area which are listed in Table 4.1.

Table 4.1: Differences between Clean Area, Aseptic Area and Sterile Area

	Clean	Aseptic	Sterile
Procedure space	On ward	Dedicated area	Dedicated room
Gloves	Clean	Sterile	Sterile surgical
Hand hygiene before the procedures	Routine	Antiseptic (Alcohol)	Surgical scrub, Iodophores
Skin antisepsis	No	Alcohol	Long acting agent
Sterile field	No	No	Yes
Sterile gown, mask, hand covers	No	No	Yes

Building Design, Construction and Production Facilities:

Production for sterile products is carried out in a clean environment. The environment should be freed from quality microbes and dust particle contamination. The limit of contamination is essential for reduce the product contamination. The production area is having many other small areas like clean up area, compounding area, aseptic area, quarantine area and packaging area (Fig. 4.1).

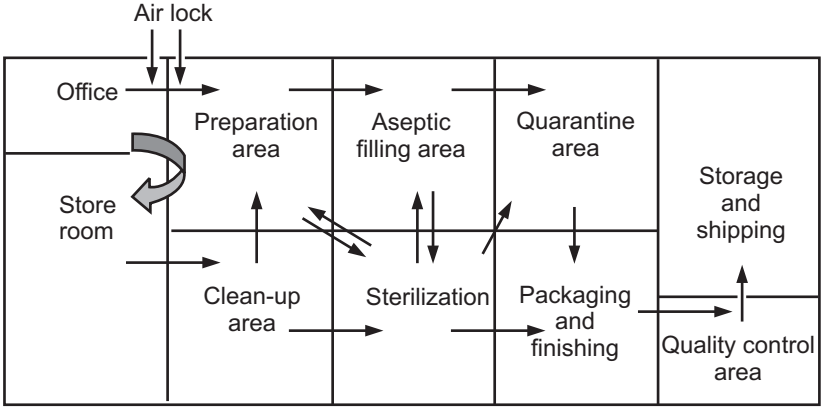


Fig. 4.1: Aseptic quality control area

Floors, Walls and Ceilings:

All the floors, walls and ceilings must be smooth, easy to clean, disinfected and be constructed to minimize microbial and particulate contamination. Flexing and non-flexing types of materials are used for construction of floor. Flexing materials are made up of synthetic elastromers like polyvinylchloride (PVC) are used for flooring which is easily repaired, cleaned, simple and cheap. Non-flexing flooring materials are made up of hard inorganic filler substances like concrete floor is properly sealed with chemical resistant materials, solvents and cleaning fluids.

Wall should be made up of non-inflammable materials like glass, stainless steel etc. For minimization of fungal growth, 1% 8-hydroxyquinolone or pentachlorophenol is added with the paint for painting the wall. Epoxy resin paint, polyurethane paints are applied on the wall that helps to reduce peeling of plaster and cracking of wall. The ceilings are sealed to prevent the entry of contaminants. Cupboards, drawers, shelves and other equipments are kept in aseptic room as minimum as possible otherwise there is a chance for contamination.

Doors, Windows and Other Services:

Doors and windows are fitted much closed together with the wall. Windows are kept at minimum only for providing illumination; not for ventilation to minimize openings. Doors must be minimal, and well fitted by maintaining positive pressure air flow and self-closing. All pipes passing through the wall should be sealed properly and should be closely fitted. They should be cleaned easily. Gas cylinder should be avoided in the aseptic room. The gas connection should be through pipeline where cylinder be kept outside area. Sinks and drains must be avoided inside the aseptic room.

All lights are fitted with the ceiling or inside the ceiling and covered with glass so that to avoid deposition of dust particles, other contaminants and also to avoid the disturbance of the air flow pattern inside the room. All the switches of the lights should be outside the aseptic room.

Personnel and Protective Clothing:

Contamination mainly comes from the skin surface of the operator of the room. Persons should be neat and clean and reliable to the selected work. They should be in good health, skilled for good manufacturing practices and free from any dermatological diseases otherwise it increases microbial load inside the aseptic area. Borne contamination can be controlled by limiting the more number of persons in clean area. They should also be skilled for aseptic techniques. They should wear sterile protective clothing like head wear, rubber or plastic gloves, non-fibre shedding face mask and foot wear. All the cloths should be designed to prevent the contamination from the body. Cloths should be sterilized by moist heat sterilization or ethylene oxide sterilization. Each time when the persons are entering inside the aseptic room they should wear freshly sterile dresses (Fig. 4.2).



Fig. 4.2: Clothing inside sterile room

Cleaning and Disinfection:

Disinfection and cleaning procedures are used for the removal of microbes and other contaminations. Alkaline detergents, ionic and non-ionic surfactants are used as cleaning agents. Various types of disinfectants are used to prevent the development of the resistant strains of microorganisms. Different concentration of quats, sodium hypochloride, ethanol and formaldehyde solutions are used as disinfectants inside the aseptic room. Chlorhexidine in 70% alcohol is mainly used as skin disinfectant.

Training is required for staff who works in clean room:

- They should know about microorganisms and controlling of microbial contamination.
- They should know the entry and exit rules inside the aseptic room.
- They should get personal hygiene training.
- They should know specific microbial risk associated with specific production tasks.
- Training must be effective and documented and regular wise review is required.

Air supply:

In the aseptic room, the air supply should be filtered through high efficiency particulate air (HEPA) filter. The HEPA filter must be fitted at the inlet of the clean room and prefilter also is fitted upstream of the HEPA filters to increase the life span of the original filters. HEPA filters are used in the construction of vertical and horizontal laminar air flow bench which filters 99.97% of the dust particles against 0.3 μm particles. HEPA filters are tested for efficiency by hot DOP (dioctylphalate) (efficiency test), cold DOP and air flow resistance test. It is oil, used by air filter manufacturers and various testing agencies to make an aerosol to test the effectiveness of air filters. The DOP aerosol used to challenge HEPA filters to test for efficiency. The hot DOP aerosol has a very narrow particle size distribution and is used to produce a high concentration of 0.3 micron particles that considered the most penetrating of filter media. Cold DOP is having a broad particle size distribution aerosol which is useful for field testing for leaks and ensuring the integrity of an installation but does not provide the ultimate test of filter efficiency. The penetration or efficiency of a filter is strongly affected by the particle size of the challenge aerosol. A small change in particle size has significant effect on penetration. The smaller the particle has the lower efficiency until the maximum penetrating particle size is reached (Fig. 4.3).

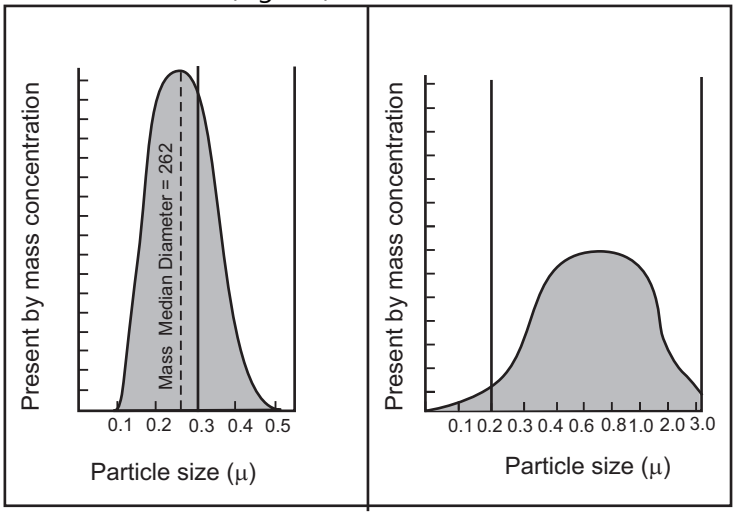


Fig. 4.3: Hot and cold DOP for testing HEPA filter

Air Flow Resistance Test:

This method is very fast and efficient measurement of HEPA filter.

HEPA filters are supported to provide 100 air and they should be certified every 6-12 months. Air quality is evaluated by settle plates, microbial air sampler and by particle counters.

One more advanced technology used recently is ULPA. ULPA is Ultra low penetration air. They are used where the smaller size particles are critical to their process mainly on semi-conductor industries. These filters are replaceable extended media dry filters that have minimum particle collection efficiency of 99.9997% efficient for particles more than 0.12 microns in size. The ULPA filters are more costly than HEPA filters.

4.2 LAMINAR FLOW EQUIPMENTS

Laminar airflow is an enclosed bench cabinet. It is designed to prevent contamination of biological samples or any particle sensitive materials.

Principle:

The air is passed through a HEPA filter which removes all airborne contamination to maintain sterile conditions.

Construction:

It is low cost and does not require more space. The cabinet is made up of stainless steel without gaps or joints thereby preventing the buildup of bacteria from collecting anywhere in the working zone. They are also known as clean benches because the air for the working environment is thoroughly cleaned by the precision filtration process. They consist of a filter pad, a fan, light with 100 lux and UV tube to disinfect the bacterial growth and a HEPA filter. The fan sucks the air through the filter pad to trap the dust particles. After that the pre-filtered air has to pass the HEPA filter where all the microorganisms are removed 99.97% of airborne particles 0.3 μm in diameter. Finally, sterile air flows into the working area without risk of contamination. The air flows at a speed of 100 ft/min and sweep dust particles making entire room free of particulate matter. This helps to perform work under aseptic condition.

Air Flow Pattern:

Generally air flow pattern in the clean rooms are of three types viz. unidirectional air flow, non-unidirectional air flow and combined air flow.

Unidirectional air flow is an air flow pattern in which essentially the whole body of the air within a confined area moves with uniform velocity and in single direction with parallel air streams that means it is an airflow where a fluid flows in one direction with no interruptions from turbulence. It should have uniformed airflow at a speed of ± 15% and the speed of the air is measured as it exits the HEPA / ULPA filter (Fig. 4.4).

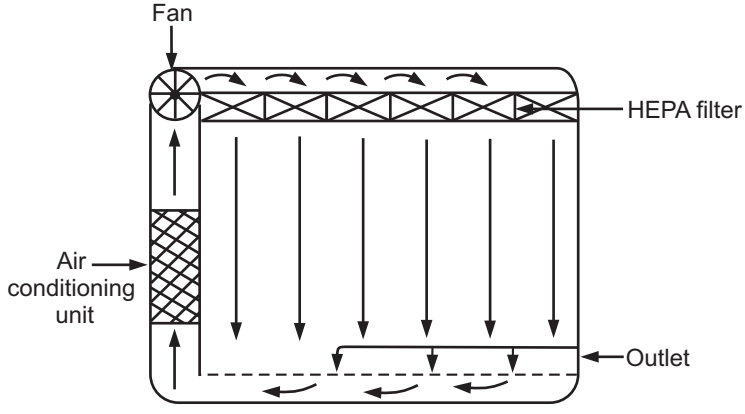


Fig. 4.4: Unidirectional air flow

Non-unidirectional air flow is an air distribution where the supply air entering the clean zone mixes with the internal air by means of induction. The room air change rate per hour is calculated by the total airflow for the room is divided by the room volume and a capture hood is used to measure the volume of air exiting the HEPA / ULPA filter (Fig. 4.5).

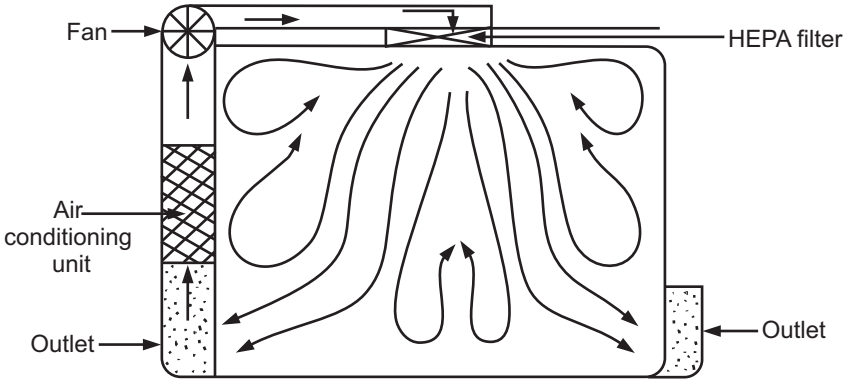


Fig. 4.5: Non-Unidirectional air flow

Combined air flow is a combination of both Unidirectional and Non-Unidirectional air flows (Fig. 4.6).

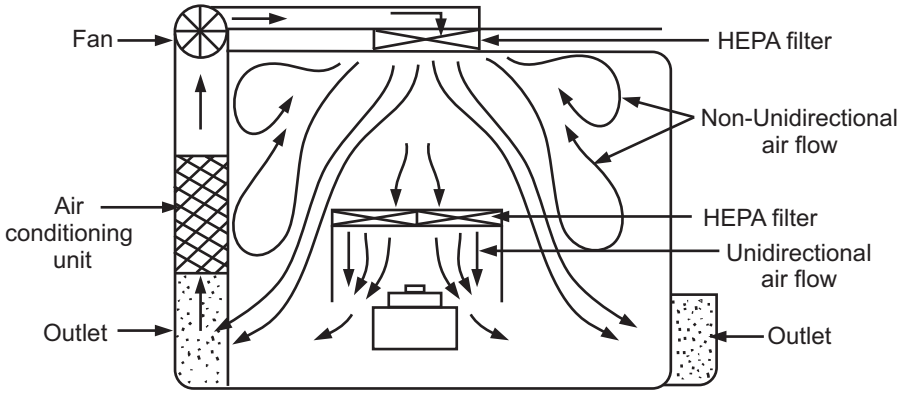


Fig. 4.6: Combined air flow

Advantages:

- The persons working in the area do not feel the movement of the air.
- The body of filters is made up of stainless steel which is not favorable for bacterial growth.
- The HEPA filter can reduce the contamination.

Types:

Mainly two types likely Vertical laminar air flow bench and Horizontal laminar air flow bench.

(a) Horizontal Laminar Flow Cabinets:

In this cabinet, the direction of air flow comes from above but then changes direction and is processed across the work in a horizontal direction. The constant flow of filtered air provides material and product protection (Fig. 4.7).

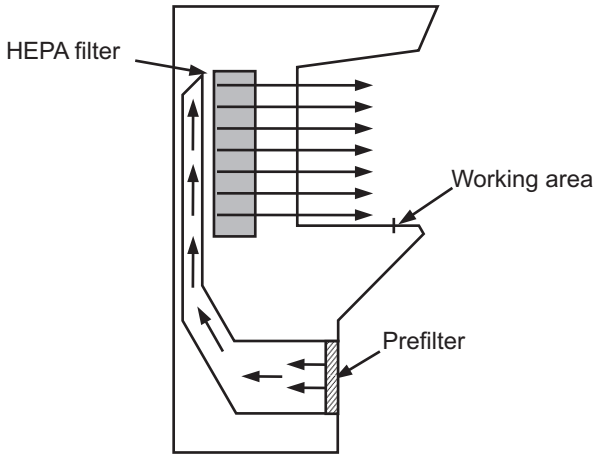


Fig. 4.7: Air flow in Horizontal Laminar equipment

(b) Vertical Laminar Flow Cabinets:

It is also same as well as horizontal Laminar Flow Cabinets with the laminar air directed vertically downwards onto the working area. The air can leave the working area via holes in the base. Vertical flow cabinets provides greater operator protection (Fig. 4.8).

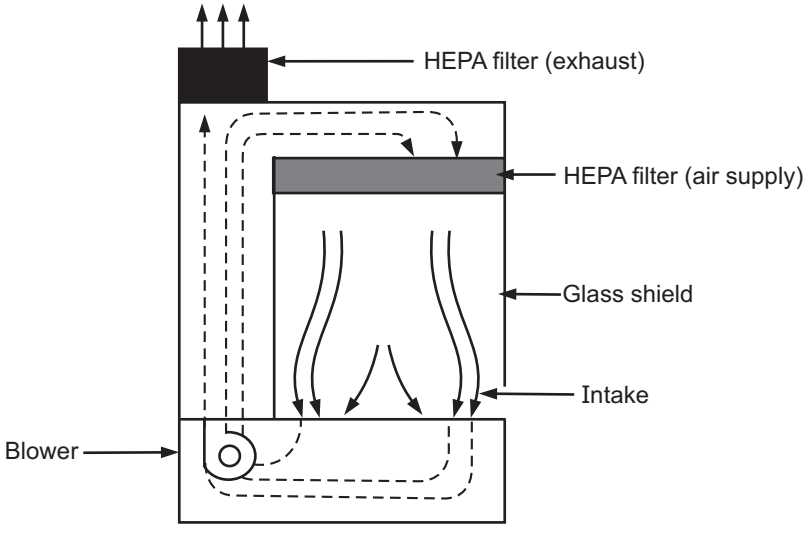


Fig. 4.8: Air flow in Vertical Laminar equipment

Biological safety cabinets come in several classes. For all classes, the air is HEPA filtered before being released as exhaust.

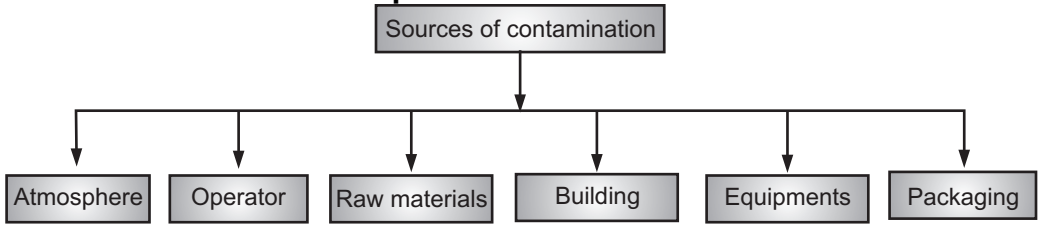
Class-I Laminar Flow Hoods: They are very simple laminar flow-hood styles. They provide adequate protection to both the user and the materials being worked with, but they do not protect the work substance from contamination. They are similar to chemical fume hoods, which contain hazardous fumes but do not disinfect.

Class-II Laminar Flow Hoods: They provide an aseptic environment in addition to protection from fumes and gases. They protect competently from the dangers of hazardous materials. They are safe and ideal for that application.

Class-III Laminar Flow Hoods: They provide the ultimate level of possible protection. They are gas-tight and so protect the worker from the danger of exposure to human pathogens. They are used for any applications involving potentially infectious substances.

4.3 STUDY OF DIFFERENT SOURCES OF CONTAMINATION IN AN ASEPTIC AREA AND METHODS OF PREVENTION

Microorganisms are easily spread everywhere. Asepsis is the method of practice to reduce or eliminate contaminants from the working area and to make the area sterile and clean. The contaminants are like bacteria, viruses, fungi and other parasites. The area free from contamination is known as **aseptic area**.



Flowchart 1: Sources of contamination

4.3.1 Atmosphere

Atmospheric gases namely oxygen and carbon dioxide are the main sources for microbial growth. Oxygen has more significant effect on microbial growth. Dust particles in the outside air are the main carriers for the soil organisms like Clostridium, yeast, molds etc. Indoor air also contains bacteria like streptococcus, staphylococcus species on cloths or human skins. These microorganisms are also present in droplets expelled out from the mouth and respiratory track of human while talking, sneezing and coughing. Microorganisms free from dust particles are found in indoor and outdoor air. Damp atmosphere contains less microorganisms than the dry atmosphere because the contaminants are carried down by the moisture droplets. Hence, the cold store air is usually free from microorganisms.

Method of Prevention:

1. Maintain the air humidity level inside the clean room.
2. Operators should move as much as less inside the clean room.
3. Aseptic rooms are maintained at higher differential air pressures than adjacent controlled areas.
4. Air filtration and air change rates should be set to ensure that the defined clean room class is attained.
5. Air flow over critical areas should be uni-directional (laminar flow) at a velocity sufficient to sweep particles away from filling/closing area.
6. Ultraviolet air locks and door barriers must be used to separate areas of unequal risk.
7. The air pressure of the changing room should be negative with regards to the manufacturing area corridor, but positive relative to external adjacent areas.
8. Possible ventilation dampers, filters and other services are designed and positioned such a way that they are accessible from outside the manufacturing areas for maintenance purposes.

9. All rooms and surfaces should be maintained and monitored for viable and non-viable particulates and the facility must be recertified on a semi-annual basis.
10. HEPA filtered air should be blown evenly to the entire clean room. The air flow must be uniform in velocity and direction throughout any given cross section of the area.
11. The air velocity should be 100 ± 20 ft/min.
12. The vertical flow from ceiling mounted HEPA filter units is utilized to protect critical sections of processing lines as well as working benches.

4.3.2 Operator

Operators are also another source for microbial contamination like the skin, hair and clothing of the operator. Skin mainly contains microorganisms like staphylococci, lipophilic yeast and dermatophytic fungi. Health and hygiene conditions also play an important role for microbial growth. The skin coliforms and other intestinal bacteria grown in poor health condition. Open wounds are also the sources of saprophytic and pathogenic microorganisms. Further the nasal passages also contain staphylococcus species. Some other reasons for contaminations are like lack of training, malpractices like eating foods, drinking beverages in the storage and processing areas.

Method of Prevention:

1. Access to production areas must be controlled to ensure entry is restricted to trained personnel only.
2. A high level of personnel hygiene must be observed.
3. All inexperienced workers whose activities may affect the quality of the product must receive regular training, including hygiene instructions.
4. Every person entering the manufacturing area should wear protective attire (over-garments, hair cover, beard or moustache cover, overshoes) appropriate to the operations to be carried out.
5. Direct contact should be avoided between the operator's hand and exposed products or any part of equipment that comes into contact with product.
6. Personnel access must only be made via changing rooms.
7. Materials access must be via specified routes – generally via air locks.

4.3.3 Raw Materials

The raw materials used for production can be a potential source of contamination. The main reasons for contamination from the raw materials due to storage and handling mistakes causing mix-ups or selection errors, contamination with microorganisms or other chemicals, degradation from exposure to excessive environmental conditions (such as heat, cold, sunlight, moisture), improper labeling, sampling and testing etc. Water also plays a major role for the microbial contamination. It acts as carrier of all kinds of microbes.

Method of Prevention:

1. Raw materials should be stored in proper dust proof area with proper labeling.
2. Water used in the manufacture of pharmaceutical products should be suitable for the intended use and should be of pharmaceutical grade, microbiologically controlled and monitored.
3. Steam used for cleaning and sanitization of production tools and equipment, supply for autoclaves and humidification must be clean and free of additives.

4.4.4 Building

The buildings and manufacturing facilities may also contribute to the contamination. The main reasons of contamination due to facility issues like insufficient size and inadequate organization of the space, cross contamination between consumables, raw materials, in-process materials, and finished products, inadequate filth and pest controls, rough floors, walls and ceilings, lack of air filtration systems, improper lighting and ventilation and inadequate washing, cleaning, toilet, and locker facilities, cleaning facilities and equipment.

Method of Prevention:

1. Interior surfaces, e.g. floors, walls, ceilings should be smooth, free from cracks and should permit easy and effective cleaning.
2. Windows/viewing panels should be non-opening, flush with the wall panels and properly sealed to prevent collection of dust and microbial material.
3. Pipe work, ventilation and light points should be designed to avoid creation of recesses which are difficult to clean.
4. Sinks within the production areas should be made of stainless steel.

4.4.5 Equipment

The equipments and other accessories that are used in processing, holding, transferring and packaging are the common sources of microbial contamination. The main reasons for contamination from the equipment are inappropriate design, size, material leading to corrosion and accumulation of static material and/or adulteration with lubricants, coolants, dirt, and sanitizing agents etc., improper cleaning and sanitization, design preventing proper cleaning and maintenance, irregular service, and deliberate use of defective equipments.

Method of Prevention:

1. Areas must be regularly cleaned and disinfected.
2. A high level of cleansing and hygiene should be practiced for the manufacture of drug product.
3. Suitable grade of cleaning agents should be used to minimize health risks.
4. Contact time, application, temperature, mechanical action and the chemistry of the cleaning agents should all be considered during the design of the cleaning process.
5. Materials used for cleaning should not come in direct contact with the product.
6. Validation of cleaning practices must be carried out to provide evidence that the process is effective in controlling microbial contamination.

4.4.6 Packaging and Manufacturing Process

Another way of contamination is the packaging materials throughout the manufacturing process. The main reasons for contamination during manufacturing process includes lack of dedicated facilities to manufacture a single product, inappropriate cleaning in-between batches to minimize the amount of product changeovers, use of an open manufacturing system exposing the product to the immediate room environment, inappropriate zoning, absence of an area line clearance according to approved procedures and lack of cleaning status labeling on all equipment and materials used within the manufacturing facilities.

Method of Prevention:

1. Flexible packaging used to inactivate microorganisms associated with foods include controlled atmosphere, vacuum, modified atmosphere, active, and edible packaging.
2. The combination of rigid packaging materials made from metal, glass, or plastic with heat provides the most effective and widely used method for inactivating microorganisms.
3. Manufacture process should maintain proper hygienic condition.

4.5 CLEAN AREA CLASSIFICATION

A clean room is a controlled environment that has a low level of pollutants such as dust, airborne microbes, aerosol particles, and chemical vapors. That means it has a controlled level of contamination, specified by the number of particles per cubic meter at a specified particle size. They are used in every industry for the safe manufacturing processes. They are constructed to minimize the introduction, generation and retention of air borne particles in the area.

4.5.1 Principle of Clean Room Air Flow

Clean rooms are maintained particulate-free air through the use of either HEPA or ULPA filters based on laminar or turbulent air flow principles. Laminar or unidirectional, air flow systems direct filtered air downward in a constant stream. Laminar air flow systems are typically employed across 100% of the ceiling to maintain constant and unidirectional flow. The maximum permitted concentration of particles C_n for each considered particle size D is determined from equation:

$$C_n = (0.1/D)^{2.08} \times 10^N$$

where, C_n = The maximum permitted concentration (particles/m^3) of airborne particles that are equal to or larger than the considered particle size.

N = The ISO classification number in the range from 1 to 9.

D = The considered particles size (μm).

4.5.2 Classification

The clean room classification standards FS 209E and ISO 14644-1 require specific particle count measurements and calculations to classify the cleanliness level. In the UK, British Standard 5295 is used to classify clean rooms. Clean area is classified into several grades which are listed in Table 4.2.

Table 4.2: Classification of clean area as per WHO

Grade	Maximum permitted number of particles/m^3			
	At rest		In Operation	
	0.5 μm	5 μm	0.5 μm	5 μm
A	3520	20	3520	20
B	3520	29	352000	2900
C	352000	2900	3520000	29000
D	3520000	29000	---	----

Table 4.2 indicates that for Grade A the airborne particle classification is ISO 4.8 dictated by the limit for particles ≥ 5.0 µm. For Grade B the airborne particle classification is ISO 5 for both particle sizes considered. For Grade C the airborne particle classification is ISO 7 and ISO 8, respectively and for Grade D the airborne particle classification is ISO 8.

Table 4.2 also summarizes as per the clean area air classifications and recommended action levels of microbiological quality in Table 4.3.

Table 4.3: Air classification[a]

Clean Area Classification (0.5 µm particles/ft^3)	ISO Designation[b]	≥ 0.5 µm particles/m^3	Microbiological Active Air Action Levels[c] (cfu/m^3)	Microbiological Settling Plates Action Levels[c,d] (diam. 90 mm; cfu/4 hours)
100	5	3,520	1[e]	1[e]
1,000	6	35,200	7	3
10,000	7	352,000	10	5
100,000	8	3520,000	100	50

- *a: All classifications are based on data measured in the vicinity of exposed materials/articles during periods of activity.*
- *b: ISO 14644-1 designations provide uniform particle concentration values for clean rooms in multiple industries. An ISO 5 particle concentration is equal to Class 100 and approximately equals EU Grade A.*
- *c: Values represent recommended levels of environmental quality. You may find it appropriate to establish alternate microbiological action levels due to the nature of the operation or method of analysis.*
- *d: The additional use of settling plates is optional.*
- *e: Samples from Class 100 (ISO 5) environments should normally yield no microbiological contaminants.*

Two clean areas are important to quality of sterile drug products namely the critical area and the supporting clean area. Critical area is the area where the sterilized drug product, containers, and closures are exposed to environmental conditions that must be designed to maintain product sterility. The activities conducted in this area are aseptic connections, sterile ingredient additions of sterile materials prior to and during filling and closing operations. This area is critical because an exposed product is vulnerable to contamination and will not be subsequently sterilized in its immediate container. To maintain product sterility, it is essential to maintain proper quality of the environment in which aseptic operations are conducted.

Supporting clean areas contain many support areas function as zones in which non-sterile components, formulated products, in-process materials, equipment, and container/closures are prepared, stored and transferred. These environments are designed to control the microbiological content of articles and sterilized components.

As per ISO 14644-1 and ISO 14644-2, there are eight other clean room standards documents exist and their meanings are tabulated in Table 4.4 and Table 4.5.

Table 4.4: Classification of eight other clean rooms

ISO Document	Title
ISO 14644-1	Classification of Air Cleanliness
ISO 14644-2	Clean room Testing for Compliance
ISO 14644-3	Methods for Evaluating and Measuring Clean rooms and Associated Controlled Environments
ISO 14644-4	Clean room Design and Construction
ISO 14644-5	Clean room Operations
ISO 14644-6	Terms, Definitions and Units
ISO 14644-7	Enhanced Clean Devices
ISO 14644-8	Molecular Contamination
ISO 14644-9	Surface Cleanliness by Particle Concentration
ISO 14644-10	Surface Cleanliness by Chemical Concentration
ISO 14698-1	Biocontamination: Control General Principles
ISO 14698-2	Biocontamination: Evaluation and Interpretation of Data
ISO 14698-3	Biocontamination: Methodology for Measuring Efficiency of Cleaning Inert Surfaces

Table 4.5: Meaning of the clean classroom classifications

Class 1:	This clean rooms class is used within the microelectronic industry when manufacturing integrated circuits which requires submicron resolution.
Class 10:	This clean room class is used within the semiconductor industry using bandwidths below 2 µm.
Class 100:	This clean room class is most useful critical cleanliness class. Clean rooms class 100 are known as sterile rooms and are used when > bacterial free < and /or > particle free < environments are required. Clean room class 100 is used in aseptic manufacturing within the pharmaceutical industry. This clean room class is frequently used during isolation and treatment of patients who are especially sensitive to bacterial infections, e.g. after bone marrow transplantation.
Class 1000:	This clean room class is used when producing high quality optics and also when mounting high quality miniature bearings.
Class 10000:	Clean rooms of class 10000 are used for mounting procedures in hydraulic or pneumatic equipment and on some occasions are also used within the food and beverage industry. They are also commonly used within the pharmaceutical industry.
Class 100000:	This classroom class is used by many industries, for example when working with optical products, when building large electronic systems based on smaller components, when building hydraulic and pneumatic systems and also within the food and beverage industry. The pharmaceutical industry also makes frequent use of this class of clean rooms.

4.6 PRINCIPLES AND METHODS OF DIFFERENT MICROBIOLOGICAL ASSAY

Microbiological assay is the technique in which the potency or concentration of a compound is assessed by determines its effect on microorganism. It is a legal quality control requirement for the assay of a number of antibiotics in both the British Pharmacopoeia and United States Pharmacopoeia. Many therapeutic agents that are either inhibit the growth of microorganisms (antibiotics) or essential for their growth (vitamins and amino acids) is standardized by microbiological assays.

The microbiological assay of an antibiotic is based upon a comparison of the inhibition of growth of microorganisms by measured concentrations of the antibiotics under examination with that produced by known concentration of a standard preparation of the antibiotic having a known activity.

Microbiological assay of vitamins is a type of biological assay performed with the help of microorganisms.

Applications:

1. This method determines the potency.
2. The method controls antimicrobial chemotherapy.
3. Good *in-vitro* and *in-vivo* correlations are provided by this method.
4. This method is accurate, in expensive and convenient.
5. This method is easy to interpret results.

Two methods are used for the microbiological assay namely cylinder plate or cup plate method and tube assay method or turbidity method.

Method A: Cylinder Plate Method (Fig. 4.9)

Principle:

This method depends on the diffusion of an antibiotic from a vertical cavity through the solidified agar layer in the petri plate. The growth of test microorganism is inhibited entirely in a zone around the cylinder containing antibiotic solution.

Procedure:

- The nutrient agar is melted, cooled and poured into petri dish.
- 0.2 ml of known concentration of inoculum is spread on the surface of solidified agar by spread plate technique.
- Holes or cavities are made by sterile borer.
- 0.2, 0.4, 0.6, 0.8 and 1 ml of antibiotic is poured into the cup of agar plate and then incubated in 37°C for 24 hours.
- Zone of inhibition is observed for antibiotic that has antimicrobial activity.
- Zone of inhibition is measured by scale from the back side of the plate from the center of the cavity or hole.

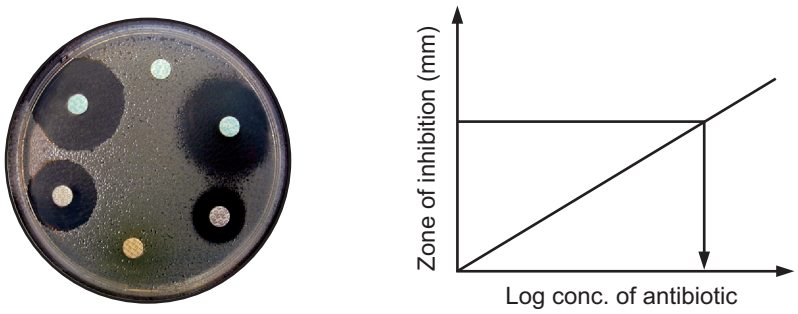


Fig. 4.9: Cup plate method and zone of inhibition to find out unknown sample

One Level Assay:

$$L = \frac{3a + 2b + c - e}{5} \qquad H = \frac{3e + 2d + c - a}{5}$$

where,

L = The calculated zone diameter for the lowest concentration of the standard curve response line.

H = The calculated zone diameter for the highest concentration of the standard curve response line.

c = Average zone diameter of 36 readings of the reference point standard solution.

a, b, d and e = Corrected average values for the other standard solutions, lowest to highest concentrations, respectively.

Two Level Factorial Assay:

Parallel dilutions containing two levels of both the standard (S_1, and S_2) and unknown (U_1 and U_2) are prepared. Cavities of each of plate are poured with diluted test samples along with standard. Finally, they are kept in the incubator and zone of inhibition is measured. The potency is calculated by the following formula

$$\% \text{ potency} = \text{Antilog } (2.0 + a \log I)$$

Where,

$$a = \frac{(U_1 + U_2) - (S_1 + S_2)}{(U_1 + U_2) + (S_1 - S_2)}$$

Where,

U_1 and U_2 = The sums of the zone diameters with solutions of the unknown of high and low levels.

S_1 and S_2 = The sums of the zone diameters with solutions of the standard of high and low levels.

I = Ratio of dilutions.

If the potency of the sample is less than 60 per cent or more than 150 per cent of the standard, the assay is invalid and should be repeated using higher or lower dilutions of the same solution. The potency of the sample may be calculated from the expression.

$$\text{Potency of sample} = \frac{\% \text{ Potency} \times \text{Assumed potency of the sample}}{100}$$

Method-B: Tube Assay Method or Turbidity Method:

The method is based on the correlation between turbidity and changes in the microbial cell number. The turbidity is measured in suitable liquid medium inoculated with microorganisms and finally standard optical density or turbidimetric curves are used to estimate the number of microbial cell (Fig. 4.10).

Principle:

This method depends on the inhibition of growth of microbial culture in a uniform solution of the antibiotic in the fluid medium that is favorable to its rapid growth in the absence of antibiotic.

Procedure:

- Five different concentrations of the standard solutions are prepared from the stock solution of standard as per step wise increased ratio 4 : 5.
- The concentration of media is selected and unknown substance solution is diluted to that concentration.
- 1 ml of each concentration of the standard solution as well as test sample solution in each of the tube is placed in duplicate.
- 9 ml of nutrient medium is added to each of the tube.
- Side by side three control samples are prepared. One contained the inoculated culture medium, another is blank (0.5 ml of dilute formaldehyde solution) and the third one contains uninoculated culture medium.
- All the tubes are placed in the incubator for 3-4 hours in 37°C and after incubation 0.5 ml of dilute formaldehyde is added in each tube.
- The growth of test microorganism is measured by determined the absorbance at about 530 nm of each of the solutions in the tubes against the blank.

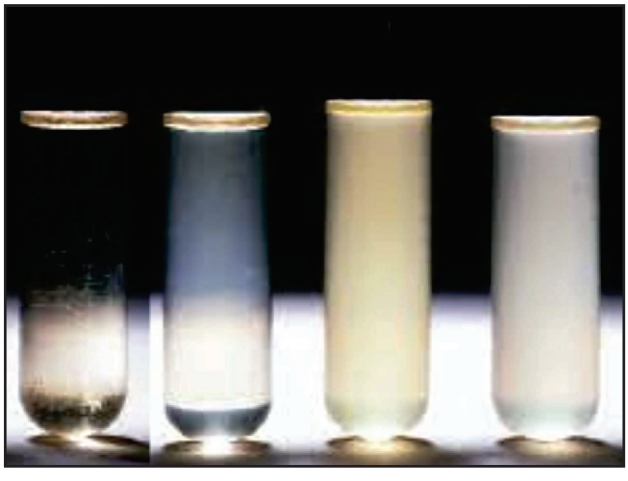


Fig. 4.10: Test tube containing turbidity

Calculation:

$$L = \frac{3a + 2b + c - e}{5} \qquad H = \frac{3e + 2d + c - a}{5}$$

where,

L = The calculated absorbance for the lowest concentration of the standard response line.

H = The calculated absorbance for the highest concentration of the standard response line.

a, b, c, d and e = Average absorbance values for the each concentration of standard response line, lowest to highest concentrations, respectively.

Advantage:

Shorter incubation period (3-4 hours) for the growth of the test organism.

Disadvantage:

The presence of solvent residues inhibitory substances affects more in this assay. Further this method is not applicable for the turbid or cloudy preparation.

4.7 METHODS FOR STANDARDIZATION OF ANTIBIOTICS

Antibiotic is a medical preparation, containing sufficient amount of chemical entity which is caused to produce naturally (by a microorganism) or by artificially (semi-synthetic way) and that possesses the inherent ability to either destroy (bactericidal) or inhibit (bacteriostatic) microorganisms in relatively dilute solutions.

There are mainly three important points are for standardization of antibiotics:

- FDA (Food Drug Administration) regulations governing all aspects of antibiotics testing are completely detailed and are subject to periodic amendment.
- FDA regulations need to be referred with regard to prescribed method for the assay of individual antibiotics and their preparations.
- During the evaluation of potency of antibiotic substances, the actual and apparent measured effect is the degree of inhibition of the growth of a suitable strain of microorganism i.e. the prevention of the multiplication of the test organisms.

Two methods are usually applied, the cylinder-plate (or cup-plate) method and the turbidimetric method. The cylinder-plate method depends upon diffusion of the antibiotic from a vertical cylinder through a solidified agar layer in a Petri dish. The growth of the added micro-organism is prevented entirely in a zone around the cylinder containing a solution of the antibiotic. The turbidimetric method depends upon the inhibition of growth of a microbial culture in a uniform solution of the antibiotic in a fluid medium that is favorable to its rapid growth in the absence of the antibiotic.

Media Preparation:

All the required ingredients are dissolved in sufficient water to produce 1000 ml and added sufficient amount of 1 M Sodium hydroxide or 1 M Hydrochloride acid, as required so that after sterilization the pH is between 6.5 to 7.5.

Buffer Solution Preparation:

Buffer solutions are prepared by dissolving the required quantities of dipotassium hydrogen phosphate and potassium dihydrogen phosphate in sufficient water to produce 1000 ml and pH adjusted with 8 M phosphoric acid or 10 M potassium hydroxide.

Standard Preparation:

Standard preparation is an authentic sample of the appropriate antibiotic for which the potency has been determined by reference to the appropriate international standard. The potency of the standard preparation is expressed in µg per mg of an international unit of the pure antibiotic.

A stock solution is prepared by dissolved a required amount of weighed quantity of the standard preparation of a given antibiotic.

Preparation of the Sample Solution:

Dilutions of sample or unknown samples are prepared at the range of dilutions of standard prepared dilutions.

Test Organisms:

Some of the test organisms for each antibiotic are listed in Table 4.6.

Table 4.6: Test organisms for microbial assay of antibiotics

Test organism	Antibiotics	*ATCC no.
Amikacin	*Staphylococcus aureus*	29737
Amphotericin B	*Saccharomyces cerevisiae*	9763
Gentamicin	*Styaphylococcus epidermidis*	12228
Kanamycin	*Bacillus pumilus*	14884
Neomycin	*Styaphylococcus epidermidis*	12228
Streptomycin	*Bacillus subtilis*	6633
Tetracycline	*Bacillus cereus*	11778

**American Type Culture Collection, 21301 Park Lawn Drive, Rockville, MD20852, USA*

Assay Method of Streptomycin:

Streptomycin is bactericidal antibiotic belongs to class aminoglycosides. It is derived from actinobacterium *Streptomyces griseus*. It is used against gram negative bacteria, mainly against tuberculosis by inhibiting protein synthesis of microorganisms. It is water soluble. *Bacillus subtilis* is used for the microbiological assay of Streptomycin.

4.8 METHOD FOR STANDARDIZATION OF VITAMINS

Microbiological assay of vitamins is a biological assay method performed with the help of microorganisms. Vitamins and amino acids are the essential components for the growth of microorganisms. The basis of this assay is to measure the ability of test organism to utilize the substance being assayed under a proper nutritional condition. Some examples of microorganisms used for bioassay of vitamins are listed in Table 4.7.

Table 4.7: Microorganisms for vitamin bioassay

Microorganism	Vitamin
Lactobacillus casei	Biotin, Folic acid, riboflavin
L. leichmannii	Cyanocobalamin
L. arabinosus	Nicotinic acid
L. viridans	Thiamine

Materials required:

A stock solution, inoculum media and assay medium are required. Finally a standard curve is obtained.

Assay of Vitamin B_{12}:

Vitamin B_{12} is also known as cyanocobalamin. It is a water soluble vitamin. Its main sources are liver, eggs, milk, meat and fish. Vitamin B_{12} deficiency causes Macrolytic anemia, Pernicious anemia.

Principle:

The test organism selected is *Lactobacillus liechmannii* that is capable of utilizing free cyanocobalamin. Assay is performed by using either titrimetric or turbidimetric method.

Preparation of Standard Stock Solution:

An accurately weighed amount of Cyanocobalamin reference standard is added to sufficient 25% ethanol (resulting in a solution containing 1.0 µ g of cyanocobalamin per ml) and stored in refrigerator. Further dilutions of this stock solution (1 µg/ ml) are made by added 1 ml stock solution to 99 ml purified water (1 ml = 10 ng) and further added 1 ml of the above solution to 199 ml purified water (1 ml = 0.05 ng).

Test Solution:

Accurate amount of vitamin to be assayed is taken and dissolved in water, dilute HCl or NaOH is added to adjust pH at 6.0 and make up the volume with water up to the mark.

Inoculum Preparation:

Transfer a loop full of *Lactobacillus liechmannii* from a recent sub-culture into two tubes each containing 10 ml of sterile culture medium.

Composition of culture media:

Yeast extract - 0.75 gm

Peptone - 0.75 gm

Dextrose - 1 gm

Potassium dihydrogen phosphate - 0.2 gm

Tomato juice filtrate - 10 ml

Sorbitan mono oleate solution - 1 ml

Water up to - 100 ml

Media is incubated for 18 to 24 hours at 37°C. The culture is centrifuged and decanted the supernatant fluid, under aseptic condition. These cultured cells are suspended into 10 ml of sterile suspension of Basal medium stock solution and again centrifuged and decanted off supernatant fluid. The cells are uniformly suspended in 10 ml of sterile medium and then aseptically transferred 1 ml of the cell suspension to 10 ml of sterile medium and mixed. Finally, this resulting cell suspension is taken as inoculum.

Assay Procedure:

In clean ten test tubes, added 0, 0.5, 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5 ml of standard cyanocobalamin solution. To each tube added 5 ml of Basal medium solution and the volume of each is adjusted to 10 ml by water. In another four test tubes added 1, 2, 3, 4 ml of test solution which is to be assayed. To each of this also added 5 ml of Basal medium stock solution and adjusted volume to 10 ml with water. All test tubes are sterilized in autoclave at 121°C for 15 minutes after that cooled the test tubes at room temperature. Inoculate a drop of inoculum prepared of *Lactobacillus liechmannii* and incubated the test tubes for 64 to 72 hours at temperature range of 30 to 37°C. After incubation period titrated contents of each test tube with 0.05 N NaOH using bromothymol blue as indicator until green colour end point. Calculate the readings.

The average of titration values of each level of both standard and test solutions are determined and plotted a graph considered average titration values (in ml) of 0.05 N NaOH against concentration of standard cyanocobalamin solution. A linear graph is obtained by interpolating the standard curve determine the concentration as activity per ml of vitamin B_{12}. From the graph the concentration of test solution of cyanocobalamin is calculated (Fig. 4.11).

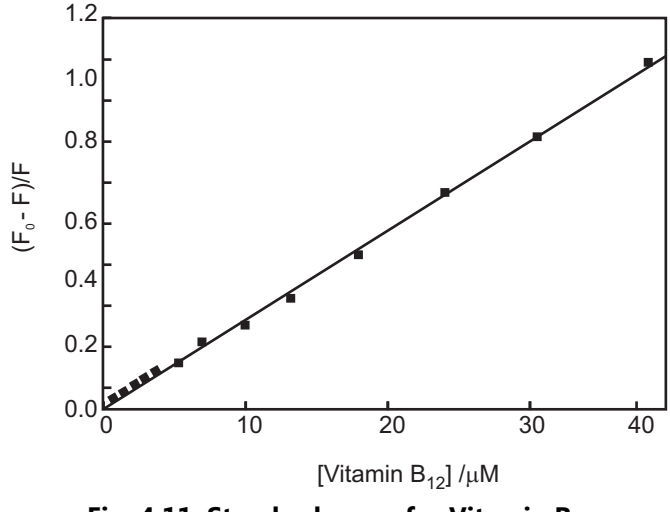


Fig. 4.11: Standard curve for Vitamin B_{12}

Assay of Calcium Pantothenate:

It refers to the B complex vitamins (or vitamin B complex).

Standardized Stock Solution:

Each ml of the stock solution consists of 50 mcg of calcium panthothenate. It is prepared by dissolving 50 mg of calcium pantothenate in 500 ml of double-distilled water as per

British Pharmacopeal Chemical Reference Standard; 10 ml of 0.2 M acetic acid, 100 ml of 1.6% (w/v) sodium acetate; and volume made upto 1 litre with distilled water.

Standard Solution:

The standard solution should contain approximately 0.04 mcg of calcium pantothenate in 1 ml, and is duly prepared by diluted the Standard Stock Solution.

Test Solution:

The test solution contains nearly the same equivalent amount of calcium pantothenate as present in the Standard Solution i.e., 0.4 mcg/ml prepared in double-distilled water.

Culture Medium:

The culture medium is composed of the following solutions and ingredients:

(i) Casein hydrolysate solution: 25 ml
(ii) Cysteine-tryptophane solution : 25 ml
(iii) Polysorbate-80 solution: 0.25 ml
(iv) Dextrose (anhydrous): 10 g
(v) Sodium acetate (anhydrous) : 5 g
(vi) Adenine-guanine-uracil solution: 5 ml
(vii) Riboflavin-Thiamine hydrochloride-Biotin Solution: 5 ml
(viii) PABA-Niacin-Pyridoxine hydrochloride solution: 5 ml
(ix) Calcium pantothenate solution A : 5 ml
(x) Calcium pantothenate solution B : 5 ml

The culture medium is usually prepared by dissolving both anhydrous dextrose and sodium acetate in previously mixed solutions and the pH is carefully adjusted to 6.8 with 1 M NaOH solution. The final volume is duly made upto 250 ml with distilled water and mixed thoroughly.

Stock Culture of Organism:

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

Preparation of Inoculum:

The cells consequently obtained from the stock culture, organism (Lactobacillus Plantarum) are transferred to a sterile tube containing 10 ml of the culture medium and is incubated at 30 to 37°C for a duration of 16–24 hours. Methodology. The various steps involved are namely:

(i) Standard Solution is added to five test tubes in varying amounts viz., 1, 2, 3, 4 and 5 ml in duplicate.
(ii) To each of the five above test tubes plus another four similar tubes without any standard solution is added 5 ml of culture medium, and the final volume made upto 10 ml with distilled water.

(iii) Now, volumes of test solution corresponding to either three or more of the levels as taken above, are incorporated carefully to similar test tubes, in duplicate.
(iv) To each test tube 5 ml of the medium solution and volume is made upto 10 ml with distilled water.
(v) Tubes of both the series are duly heated in an autoclave at 121°C for 5 minutes, cooled to ambient temperature and added 1 drop of inoculum to each tube except two of the four tubes that specifically has no 'standard solution' (i.e., the uninoculated tubes), and mixed thoroughly. The tubes are adequately incubated at 121°C at 30–37°C for 16– 24 hours.
(vi) Absorbance of the various tubes is measured with a spectrophotometer at wavelength ranging between 540–660 nm. A standard concentration response curve is plotted between the transmittance Vs log ml (volume) of the standard solution in each tube. Finally, the exact concentration of the calcium pantothenate in the 'test sample' is determined with the help of the standard concentration-response curve.

4.9 STANDARDIZATION OF AMINO ACIDS

Amino acids are the basic building blocks of proteins. They constitute all proteinaceous material of the cell including the cytoskeleton and the protein component of enzymes, receptors, and signaling molecules and also used for the growth and maintenance of cells. The amino acids are standardized by HPLC method. HPLC with pre-column derivatization is commonly used for the analysis of amino acids. Amino acid analysis is an important approach for the characterization of protein and peptide-based products.

Assessment of a New Antibiotic:

Antibiotic production: For production of new antibiotics there are few steps to be followed.

(i) Isolation or collection of culture.
(ii) Screening of cultures to detect antimicrobial activity.
(iii) Development of methods for submerged culture production.
(iv) Development of methods for isolation and purification of antibiotic.
(v) Determination of antibiotic properties like physical (adsorption, absorption) and chemical (reactions, solubility stability etc.)
(vi) Evaluation of antibiotics:
  (a) Pharmacological test: To check the toxicity, activity at lower concentration, bacterial sensitivity etc.
  (b) Antimicrobial activity: To check whether bacteriostatic or bactericidal.
  (c) Comparison with existing antibiotic: To check the potency.
(vii) Development of pilot plant production method.
(viii) Submission of license for clinical trials.
(ix) Testing of purified antibiotics.
(x) Development of plant scale production method.
(xi) Obtaining a product license for clinical use.
(xii) Other considerations.
  (a) Development of methods to control production of antibiotics.
  (b) Development of new applications.
  (c) Development of marketing and distribution system.
  (d) Financing of business.

QUESTIONS

Long Assays:

1. Explain designing of aseptic area.
2. Explain the role of laminar flow equipments in aseptic room.
3. Explain classification of clean area.
4. Explain various sources of contamination in an aseptic area and the method of prevention.
5. Explain various methods of microbiological assay.
6. Explain the method of standardization of antibiotics, vitamins and amino acids.

Short Assays:

1. Explain types of laminar flow cabinets.
2. Explain the sources of contamination in aseptic area.
3. Explain air classification and classification of clean classroom.
4. Explain cylinder plate assay method.
5. Explain turbidity assay method.
6. Explain standardization of vitamin B_{12}.
7. Explain standardization of calcium pantothenate.
8. Explain assessment of new antibiotics.

MULTIPLE CHOICE QUESTIONS

1. PVC is used as ……
   (a) Non-flexing material (b) Flexing material
   (c) Both (d) None of these
2. Disinfectant used in aseptic room is ……
   (a) Sodium hypochloride (b) Sulphuric acid
   (c) Nitric acid (d) None of these
3. Example of skin disinfectant is ……
   (a) Chlorhexidine in sulphuric acid (b) Chlorhexidine in ethanol
   (c) Quats in ethanol (d) Quats in sulphuric acid
4. Dust particle removed by HEPA filter is ……
   (a) 0.5 µm (b) 0.2 µm
   (c) 0.03 µm (d) 0.3 µm
5. ULPA is used to remove particle size ……
   (a) 0.03 µm (b) 0.12 µm
   (c) 0.21 µm (d) 0.3 µm
6. In vertical Laminar flow cabinet, air leave the working area via. ……
   (a) HEPA filter (b) Holes from the top
   (c) Holes in the base (d) None of these
7. Type of Laminar flow Hoods used for protection from fumes and gases ……
   (a) Class-I (b) Class-II
   (c) Class-III (d) Class-IV
8. Air velocity inside aseptic room is ……
   (a) 100 ± 10 ft/min (b) 200 ± 10 ft/min
   (c) 100 ± 20 ft/min (d) 200 ± 20 ft/min

9. Grade B air borne particle is based on classification of ……
   (a) ISO 9 (b) ISO 3
   (c) ISO 5 (d) ISO 7 and 8
10. Grade D air borne particle is based on classification of ……
   (a) ISO 8 (b) ISO 3
   (c) ISO 5 (d) ISO 7 and 8
11. As per ISO 7 microbiological active air action levels are ……
   (a) 100 cfu/m^3 (b) 7 cfu/m^3
   (c) 10 cfu/m^3 (d) 1000 cfu/m^3
12. Class 100 is stand for ……
   (a) Clean room is used in semiconductor industry
   (b) Clean room is used in microelectronic industry
   (c) Clean room is used in aseptic manufacturing in pharma industry
   (d) Clean room is used for hydraulic equipment
13. Diffusion of antibiotic takes place in ……
   (a) Turbidity method (b) Cylinder plate method
   (c) Both (d) None of these
14. Inhibition of growth of microbial culture takes place in ……
   (a) Turbidity method (b) Cylinder plate method
   (c) Both (d) None of these
15. Organism used for Streptomycin assay is ……
   (a) *Bacillus cereus* ATCC 11778 (b) *Bacillus subtilis* ATCC 6633
   (c) *Bacillus subtilis* ATCC 3366 (d) *Bacillus cereus* ATCC 6633
16. Thiamine vitamin assayed with organism ……
   (a) *Lactobacillus casei* (b) *L. arabinosus*
   (c) *L. viridians* (d) *L. leichmannii*
17. Cyanocobalamin is assayed with organism ……
   (a) *Lactobacillus casei* (b) *L. arabinosus*
   (c) *L. viridians* (d) *L. leichmannii*
18. Nicotinic acid is assayed with organism ……
   (a) *Lactobacillus casei* (b) *L. arabinosus*
   (c) *L. viridians* (d) *L. leichmannii*
19. Vitamin B_{12} assay is carried by ……
   (a) Turbidity method (b) Cylinder plate method
   (c) Both (d) None of these
20. In assay of vitamin B_{12} indicator is used as ……
   (a) Thymol blue (b) Bromocresol blue
   (c) Bromophenol blue (d) Bromothymol blue

Answers:

1. (b)	2. (a)	3. (b)	4. (d)	5. (b)	6. (c)	7. (b)	8. (c)	9. (c)	10. (a)
11. (c)	12. (c)	13. (b)	14. (a)	15. (b)	16. (c)	17. (d)	18. (b)	19. (a)	20. (d)

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Unit ... 5

MICROBIAL SPOILAGE, STABILITY OF PRODUCTS AND ANIMAL CELL CULTURE

♦ LEARNING OBJECTIVES ♦

After completing this chapter, reader should be able to understand:

- ❖ Definition of spoilage and its various types.
- ❖ Various factor affecting microbial spoilage of pharmaceutical products.
- ❖ Microbial contaminations and various types of contaminants.
- ❖ Various evaluation methods for microbial contamination and spoilage.
- ❖ Preservation method for pharmaceutical products by using antimicrobial agents.
- ❖ Various evaluation methods for microbial stability of formulations.
- ❖ Detail study on animal cell culture with respect to general procedure.
- ❖ Establishment of cell culture.
- ❖ Applications of animal cell culture in pharmaceutical industry and research.

5.1 INTRODUCTION

Spoilage is the term that indicates the goods that do not meet production standard. It is the method by which material or the amount of material is wasted or scrapped during the manufacturing process. In another word, it is a form of substandard drug or food in which the quality or value or usefulness of the article is destroyed or impaired by the action of microorganisms like fungi or bacteria as to render the article unfit for human consumption. Hence, spoilage is a complex reaction in which a combination of microbial and biochemical activities are interact. Legally they are known as **spoilage**. These substandard drugs when proceed further in order to be saleable as goods units are known as defectives. Examples: Rancid meat, sour milk, moldy cheese etc. The spoilage is mainly determined based on microbial colonization form which is depends on the characteristics of products, method of processing and the storage condition. These factors are characterized in to four types viz. intrinsic parameters (physical, chemical, structural properties of foods, water activity, acidity, redox potential), extrinsic parameters (temperature, humidity, atmosphere conditions), modes of preservation and processing (physical and chemical treatments, change characteristics of food products, determination of microbial flora in food products) and

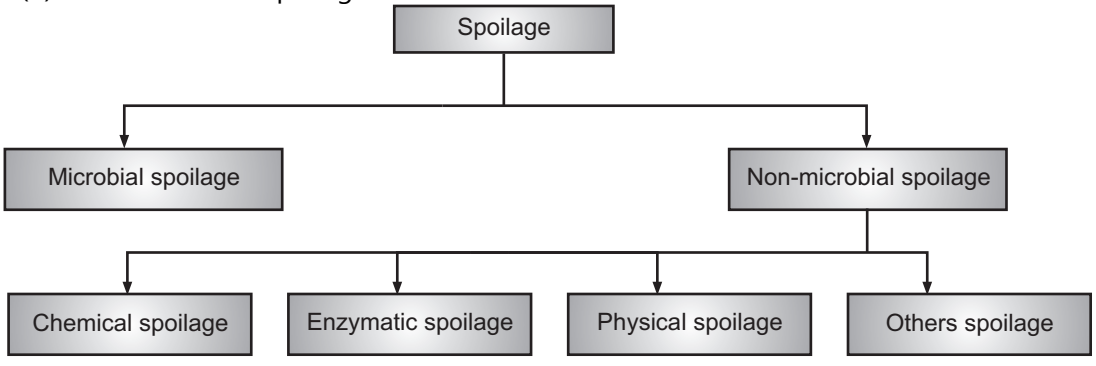
implicit parameters (microbial interferences either by synergistic or antagonistic action, destruction of one organism by another species).

5.2 TYPES OF SPOILAGE

Spoilages are mainly two types.

(A) Microbial spoilage and

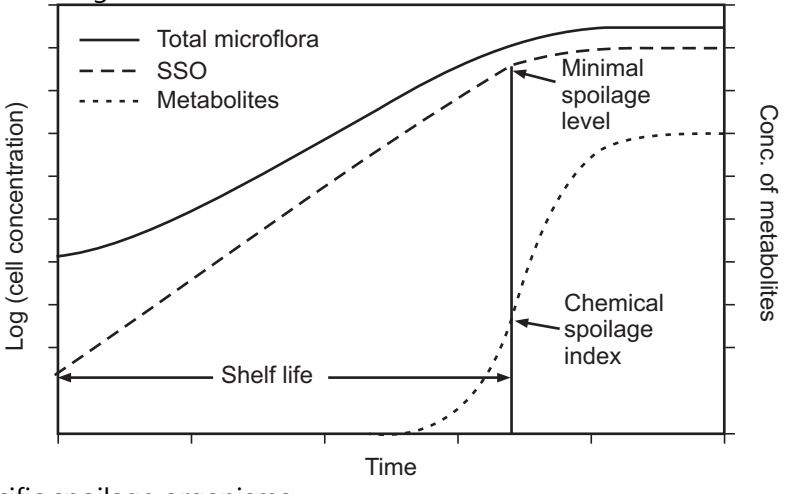
(B) Non-microbial spoilage.



Flowchart 1: Types of spoilage

5.2.1 Microbial Spoilage

It includes the contamination of Pharmaceutical products with the microbes that lead to spoilage of the product. It affects drug safety and quality, and is not intended for use. Hence, microbial spoilage is the deterioration of pharmaceutical products by the contaminant microbes such as bacteria, moulds, fungi and yeasts. The growth pattern of microbial spoilage is shown in Fig. 5.1.



- SSO = Specific spoilage organisms

Fig. 5.1: Growth pattern of microbial spoilage

5.2.2 Non-Microbial Spoilage

These are the chemical reactions caused by foreign material in the foodstuff or by enzymes that occur in the foodstuff naturally resulting in rancidity, staling, discolouration, drying out or damage by insect.

Chemical spoilage: It is based on non-enzymatic chemical reaction occurred within the foods and resulted change in flavor. Some examples are given in Table 5.1.

Table 5.1: Use of Chemical agents for changes of flavors in various foods

Chemical agents	Foods	Off flavor
Trimethylamine	Meat, egg, fish	Fishy
Dimethyl trisulphide	Meat, fish	Garlic
Ethanol	Fruit juices	Alcoholic
Trichloroanisol	Bread	Musty
Acetoin, Diacetyl	Meat	Cheesy
Acetic acid, lactic acid	Dairy, wine	Souring
2-Methoxy phenol	Juice, wine	Medicinal odour

Enzymatic spoilage: It is based on enzymatic reactions occurred in foods and changes in chemical nature and resulted rancidity. This rancidity occurred by two types of chemical reactions viz. hydrolytic and oxidative rancidity. For example: Triglyceride upon hydrolytic reaction in presence of lipase enzyme forms glycerol and free fatty acid. Further, Linolenic acid with various oxidative reactions forms 3-(E)-hexenal and 12-oxo-9 (Z) dodecenoic acid with the help of lipoxygenase and hydroperoxide lyase. Some examples are given in Table 5.2.

Table 5.2: Use of enzymes for changes of flavours in various foods

Enzymes	Foods	Spoilage action
Lipase	Milk, oils	Hydrolytic rancidity
Thiaminase	Meat, fish	Thiamine destruction
Peroxidases	Fruits	Browning
Proteases	Egg, crab	Reduction of shelf life
Lipoxygenases	Vegetables	Destruction of vitamin A

Physical spoilage: It occurs due to temperature, light, relative humidity and results in mechanical damage of the food components. For example: Oxidation of food occurs due to light and changes colour, flavour and chemical nature, like greening of potatoes, sunlight flavour in milk, loss of vitamin D, E etc.

Others spoilage: It occurs due to insects, rodents, birds, and other animals and resulting in changes in colour, odour, and chemical nature.

Based on rate of spoilage, they are classified into three types like high perishable, semi-perishable and stable or non-perishable spoilage.

(i) High perishable: Meat, fish, poultry, eggs, milk, fruits and vegetables etc.

(ii) Semi-perishable: Potatoes, some apple varieties, nutmeats etc.

(iii) Stable or non-perishable: Sugar, flour, dry beans etc.

- **Spoilage of fruits and vegetables:**

  Acid tolerant bacteria are responsible for the spoilage of fruits and vegetables such as Lactobacillus species. Not only that deterioration is also caused by animals and birds, but wounding, cutting, freezing, environmental conditions, contact with spoiled foods etc. also causes food spoilage. Mainly two types of spoilages are observed likely bacterial soft rot and rhizopus soft rot.

Bacterial soft rot is caused by *Erwinia carotovora*, ferment pectins, *Pseudomonas marginalis* etc. *Bacillus* and *Clostridium* are causes for water soaked appearance and bad odour of vegetables, black mold rot causes spotting of leaves and fruits.

Rhizopus soft rot is caused by Rhizopus. Cottony growth of mold forms black spots of sporangia covering the foods. Alternaria rot is causes by *Alternaria tenuis*, resulting in green brown spot. Rots of juicy fruits results in leakage.

- **Spoilage of cereals:**
  Moisture content above 12 to 13 per cent causes spoilage of cereals due to the growth of molds, bacteria and yeasts. Molds grown in little moisture whereas in high moisture condition yeasts and bacteria grows. *Aspergillus, Penicillium, Mucor, Rhizopus, Fusarium* are some common molds that produces mycotoxins. Acetobacter spp., lactics and *Coli forms*, *Micrococci* and *Bacillus* are some species causing spoilage of cereals.
- **Spoilage of milk:**
  Souring of milk is caused by *Streptococcus lactis, Enterococci, Lactobacilli* etc. Proteolysis causes bitter taste of milk which is caused by *Bacillus, Serratia, Pseudomonas* etc.
- **Spoilage of meat:**
  Raw meat is spoiled by microbial as well as enzymatic action. Excessive autolysis causes souring of meat.
- **Spoilage of fish:**
  Fish is spoiled by autolysis, oxidation or by bacterial activity. Under anaerobic conditions, surface slime of fishes is caused by *Pseudomonas, Streptococcus*, *Micrococci* etc. Surface colour changes due to pigmentation. For example: Red spot is caused by *Serratia marcescens*, Pseudomonas gives blue colour.
- **Spoilage of eggs:**
  Spoilage of eggs is promoted by cracking, improper washing and storage. Egg yolk is good growth medium for microorganism due to the high nutrient. Molds that cause the spoilage of eggs are *Penicillium, Mucor, Sporotrichum* etc. Spoilage of eggs is also occurred by fungi. There are two stages of egg spoilage like pin spot molding and fungal rotting.
- **Spoilage of canned foods:**
  Canned foods are chemically spoiled due to hydrogen swells as a result discolouration of inside the can, cloudiness of liquors and that affects the nutritive value of the canned foods. Due to bacterial spoilage loss of nutritive value occurs by thermophilic and mesophilic bacteria. Thermophilic anaerobes forms acid and gas whereas sulfide spoilage occurs by *Desulfotomaculum nigrificans* that resulted black colonies. Sometimes ends of can flatten due to souring caused by *Bacillus stearothermophilus*.

Some of the important food spoilages along with their causative microorganisms are tabulated in Table 5.3.

Table 5.3: Causative microorganisms for foods spoilage

Foods	Type of spoilage	Spoilage Microorganisms
Fresh Meat	Putrefaction	*Clostridium, Pseudomonas, Proteus*
	Souring	*Chromobacterium, Lactobacillus*
Cured Meat	Mouldy	*Penicillium, Aspergillus, Rhizopus*
	Souring	*Pseudomonas, Micrococcus, Bacillus*
	Slimy	*Leuconostoc*

Foods	Type of spoilage	Spoilage Microorganisms
Vacuum packed Meat	Souring Greening	*Lactobacillus, Carnobacterium*
Poultry	Odour, Slime	*Pseudomonas, Alcaligenes, Xanthomonas*
Milk	Bitterness	*Pseudomonas spp.*
	Souring	*Lactobacillus thermophilus*
	Sweet curdling	*Bacillus cereus*
Cheese	Green discolouration	*Penicillium*
	Green to black discolouration	*Cladosporium*
	Black discolouration	*Candida*
	Sliminess	*Pseudomonas spp.*
Fish	Discolouration	*Pseudomonas*
	Putrefaction	*Chromobacterium, Halobacterium*
Egg	Green rot	*Pseudomonas*
	Black rot	*Coliforms*
	Fungal rot	*Proteus, Penicillium, Mucor*
Fresh vegetables and fruits	Bacterial soft rot	*Pseudomonas spp.*
	Rhizopus soft rot	*Rhizopus nigrican*
	Gray mould rot	*Botryitis cinerea*
	Blue mould rot	*Penecillium italicum*
Canned food	Thermophilic acid	*Clostridium thermosaccrolyticum*
	Sulfide stinker	*Clostridium nigrificans*
	Softening of fruits	*Byssochlamys fulva*
	Sliminess	*Yeast, molds*

5.3 FACTORS AFFECTING THE MICROBIAL SPOILAGE OF PHARMACEUTICAL PRODUCTS

Pharmaceutical products contain wide range of excipients in addition to active pharmaceutical ingredients for making the formula easy to manufacture, stable, effective and convenient to the patient. These products in pharmaceutical industry must meet high microbiological specs that means if they are not sterile, they should have not more than a minimal microbial population at the time of product release as well as afterwards. If there is microbial spoilage of products then products are unsuitable for use, resulting in financial problem to the manufacturers and also potential health hazard to the patients. Hence microbial spoilage is required to be minimized which depends on many factors that are enlisted as:

- Types and size of contaminant inoculum.
- Nutritional factors.
- Water.
- Storage temperature.
- pH.
- Oxidation-reduction balance (Redox potential).
- Package design.

5.3.1 Types and Size of Contaminant Inoculum

Very low levels of contaminants which are unable to replicate in a product might not cause appreciable spoilage but, if a higher contaminant bio-burden occurs, the built in protection could be insufficient and spoilage occurs.

This higher contamination arises if:

1. Raw materials were unusually contaminated;
2. A problem of the plant-cleaning protocol occurs;
3. Biofilm detached itself from within supplying pipework;
4. There was demolition or maintenance work in the vicinity of the manufacturing site
5. Misuse of the product occuring during administration.

5.3.2 Nutritional Factors

Many spoilage microorganisms have simple nutritional requirements and metabolic adaptability which enables them to utilize many formulation components as substrates for biosynthesis, growth and also trace materials contained in them. The use of animal products and crude vegetable materials in a formulation provides an additionally nutritious environment. Demineralized water prepared by ion-exchange methods, contains sufficient nutrients to allow significant growth of many water-borne Gram-negative bacteria like Pseudomonas spp.

5.3.3 Water

It is the most important cause of the survival and growth of micro-organisms. Some solute-rich medicines such as syrups appear to be 'wet', microbial growth in them may be difficult since the microbes have to compete for water molecules with the large numbers of sugar and other molecules of the formulation which also interact with water via hydrogen bonding. An estimate of the proportion of the non-complexed water in a formulation available to equilibrate with any microbial contaminants and facilitate growth can be obtained by measuring its water activity (A_w).

$$A_w = \frac{\text{Vapour pressure of formulation}}{\text{Vapour pressure of water under similar conditions}}$$

The greater the solute concentration, the lower is the water activity. Most microorganisms grow best in dilute solutions (high A_w) and, as solute concentration rises (lowering A_w), growth rates decline until a minimal, growth-inhibitory A_w is reached. The Aw of aqueous formulations are lowered to increase resistance to microbial attack by the addition of high concentrations of sugars or polyethylene glycols. Condensed water films are accumulate on the surface of 'dry' products such as tablets or bulk oils following storage in damp atmospheres with fluctuating temperatures, resulting in high localized A_w to initiate fungal growth.

5.3.4 Storage Temperature

The actual storage temperature determines the spoilage by particular types of microorganisms. Spoilage of pharmaceuticals occurs potentially over the range of about 20°C to 60°C. Storage in a deep freeze at −20°C or lower is used for long-term storage of foodstuffs and some pharmaceutical raw materials, and dispensed total parenteral nutrition feeds are stored in hospitals for short periods at −20°C to even further minimize the risk of

growth of any contaminants that are introduced during their aseptic compounding. Reconstituted suspensions and multi-dose eye drop packs are sometimes dispensed with the instruction to 'store in a cool place' such as a domestic fridge (8°-12°C), or "store in refrigerator " partly to reduce the risk of in-use contamination growing before the expiry date. Water for Injections is recommended to be held at 80°C or above after distillation and prior to packing and sterilization to prevent possible regrowth of Gram-negative bacteria, and the release of endotoxins.

5.3.5 pH

Extremes of pH prevent microbial attack. They grow at neutral pH, therefore acidic or alkaline formulations are less susceptible to spoilage. Around neutrality bacterial spoilage is more likely, with reports of pseudomonas and related Gram-negative bacteria growing in antacid mixtures, flavoured mouth washes and in distilled or demineralized water. Above pH 8, the spoilage is rare for soap-based emulsions. Products with low pH levels such as the fruit juice-flavoured syrups are attacked by mould or yeast. Yeasts are metabolizes of organic acids and raise the pH to levels where secondary bacterial growth occurs. In food industry, low pH adjustment is made to preserve foodstuffs only.

5.3.6 Redox Potential

The ability of microbes to grow in an environment is influenced by its oxidation-reduction balance since they require compatible terminal electron acceptors to permit function of their respiratory pathways. The redox potential in viscous emulsion is high due to the high solubility of oxygen in most fats and oils.

5.3.7 Packaging Design

It has a major influence on microbial stability of some formulations in controlling the access of contaminants during both storage and use. The most important dosage form such as parenteral drugs is protected because of the high risks of infection by this route. Self-sealing rubber closures are used to prevent microbial entry into multi-dose injection containers following withdrawals with a hypodermic needle. Wide-mouthed cream jars are replaced with narrow nozzle and flexible screw capped tubes to remove the likelihood of operator-introduced contamination during use. For medicines which rely on their low A_w to prevent spoilage, packaging such as strip foils are must be of water vapor-proof materials with fully efficient seals. Sacking, cardboard, card liners, corks and paper are unsuitable for packaging pharmaceuticals because they are heavily contaminated with bacterial or fungal spore. These are replaced by non-biodegradable plastic materials. In the past, packaging in hospitals is frequently re-used for economic reasons.

Other factors affecting microbial spoilage of pharmaceutical products include:

- Relative Humidity
- Oxygen Availability
- Osmotic Pressure
- Surface Tension

5.4 SOURCES AND TYPES OF MICROBIAL CONTAMINANTS

Most of the raw materials for pharmaceutical products support microbial growth based on the nutritive properties and moisture contents. Microbiological contaminants are infectious materials, such as bacteria, mold, fungi and viruses. These contaminants are often found in food and water and cause serious illnesses. Deterioration of pharmaceutical products by the contaminant microbes are known as **microbial contamination**. These

microorganisms are cause spoilage of the product and results in loss of therapeutic properties. Various sources through which microbial contaminants gain entry are: atmosphere, operators, raw materials from which products are manufactured and equipments.

Atmosphere: Microbial growth in heating ventilation and air-conditioning (HVAC) systems and their contamination of the indoor air environment is a growing concern. A poor HVAC system is a potential source of growth of microbes and a transportation mode for dispersing contaminants throughout the manufacturing facility. The main reasons of contamination due to HVAC issues includes, accumulations of organic material in or near HVAC air intakes, ineffective filtration of the supply air, insufficient magnitude of pressure differentials causing flow of reversal, erroneous ratio of fresh air to recirculated air, inability to access ventilation dampers and filters from outside the manufacturing areas and non-directional airflow within production or primary packing areas.

Operators: The main reasons for the contamination from the operators includes mainly lack of training, Direct contact between the operator's hands and starting materials, primary packaging materials and intermediate or bulk product, Inadequate cleanliness, access of unauthorized operators into production, storage, and product control areas, malpractices like eating food, drinking beverages, or using tobacco in the storage and processing areas.

Raw materials: The raw materials used for production is a potential source of contamination. The main reasons for contamination from the raw materials includes storage and handling mistakes causing mix-ups or selection errors, Contamination with microorganisms or other chemicals, degradation from exposure to excessive environmental conditions such as heat, cold, sunlight, moisture, etc., improper labeling, sampling and testing.

Equipments: The equipment used in processing, holding, transferring and packaging are the common source of pharmaceutical contamination. The main reasons for contamination includes, inappropriate design, size, material leading to corrosion and accumulation of static material, coolants, dirt, and sanitizing agents, improper cleaning and sanitization, design preventing proper cleaning and maintenance, improper calibration and irregular service, and, Deliberate use of defective equipment etc.

5.4.1 Sources of Contaminants

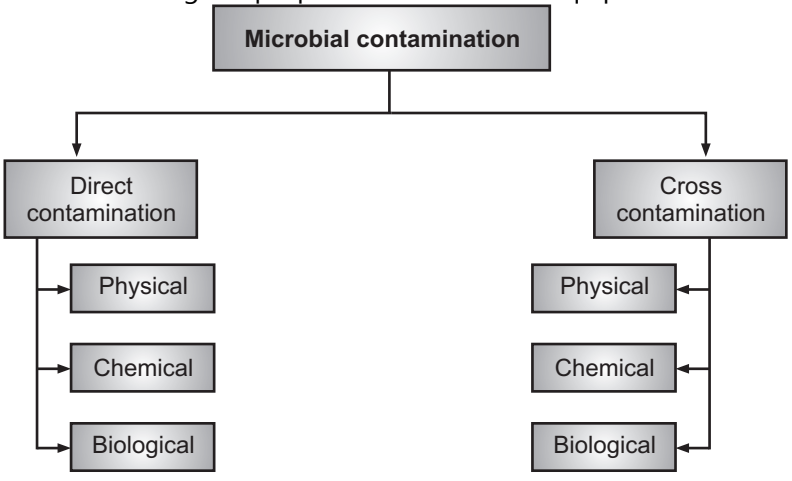
- **Air carrying dust:** Dust is everywhere. Air is the carrier of the dust particles. Very small dust particles that present inside the aseptic room are the source of contamination. Hence, HEPA filter is used for reduction of dust particle inside the room.
- **Skin of the operators:** Outer skin of the operators should smooth and covered properly. Any skin infections or rashes are the sources of microorganism growth inside the room.
- **Movement of persons inside the aseptic room:** It is advisable that movement of persons inside the aseptic room should be restricted. It is evident that person walking inside the room liberate 5000 bacteria/minute.
- **Operators health:** Persons those who are inside the aseptic room, should be healthy and free from any infections. A single sneeze will produce up to 1 million bacteria.
- **Manufacturing process:** Manufacturing process generates contaminants. The main reasons for contamination during manufacturing process are lack of dedicated facilities to manufacture a single product, inappropriate cleaning in-between batches to minimize the amount of product changeovers, use of an open manufacturing system exposing the product to the immediate room environment, inappropriate zoning, lack of cleaning status labeling on all equipment and materials used within the manufacturing facility etc.

5.4.2 Types of Microbial Contaminant

Microbial contamination is broadly classified in to direct contamination and cross contamination (Flowchart 2).

(a) Direct contamination: Contamination occurred by microbial components and poorly maintained heating, ventilation and air conditioning system.

(b) Cross contamination: It is the process by which microbes are spread indirectly from one to another through improper and unsterilized equipment.



Flowchart 2: Types of microbial contamination

Direct contamination is classified as follows:

(a) Direct Physical contamination: Examples: Particles, fibres, metal parts etc.

(b) Direct chemical contamination: Examples: Moisture, gases, vapors etc.

(c) Direct biological contamination: Example: Microorganisms like bacteria, viruses, molds, fungi etc.

Cross contamination is classified as follows:

(a) Physical cross contamination: Example: Leakage of oil seal from the reactor.

(b) Chemical cross contamination: Examples: Moisture content is increased when a product exposed to high relative humidity.

(c) Biological cross contamination: Example: Improper cleaning of equipment, unclean equipments used for manufacturing process.

Types of Food Contaminants:

(a) Biological contaminants: Microbial contaminants are responsible for food borne diseases that are caused by bacteria, viruses, fungi, parasites, biological toxins etc.
e.g.: Sea food toxins, Mushroom toxins.

(b) Chemical contaminants: Chemical substances that are responsible for food borne illness.
e.g.: Toxic metals, Pesticides etc.

(c) Physical contaminants: Any foreign substances that are accidentally observed in the food.
e.g.: Hair, wire, dust, sand particles etc.

Some of the examples of contaminants for the pharmaceutical products are given in Table 5.4.

Table 5.4: Contaminants for pharmaceutical products

Pharmaceutical products	Contaminant
Plague vaccine	*Clostridium tetani*
Serum vaccine	*Staphylococcus aureus*
Thyroid tablets	*Salmonella muenchen*
Antibiotic eye ointments	*Pseudomonas aeruginosa*
Talcum powder	*Clostridium tetani*
Saline solution	*Serratia marcescens*
Antiseptic mouth wash	*Coliforms*
Surgical dressings	*Clostridium species*
Hand cream	*Klebsiella pneumoniae*

5.5 ASSESSMENT OF MICROBIAL CONTAMINATION AND SPOILAGE

Early indications for microbial spoilage are gas production, pH changes, organoleptic changes like smell, sour, fishy amines, bad eggs, earthy taste, bitter etc. Microbial polymerization of sugars and surfactant molecules can produce slimy, viscous, masses in syrups, shampoos and creams, and fungal growth in creams produce 'gritty' textures. Acidic or basic microbial metabolites change pH of the drug and also enhance microbial growth which is inhibited by the initial pH. This contamination and spoilage is assessed by quality control tests. As per the Pharmacopoeial and regulatory limits for the maximum permissible numbers of microorganisms in manufactured products or raw materials are not more than 100–1000 colony forming units (cfu) per ml or gram. The risk of microbial infection and spoilage arises from microbial contamination during manufacture, storage and use and that is eliminated by presenting all medicines in sterile, impervious, single-dosage units. Beyond these, some pathogens initiate infections resulting in detectable changes in chemical composition, physical appearance or stability.

Assessment of microbial contamination and spoilage is also known as **microbial quality control**. This includes laboratory applications like sterility testing, bioburden determination, air monitoring and product testing. These methods are conducted to:

- monitor microbial contamination of raw materials.
- monitor and confirm the efficacy of sterilization.
- control the risk from pathogenic microorganisms by confirming their absence.

Quality means meeting the predetermined requirements of users for a particular substance or service. Quality includes: Total Quality management (TQM), Continuous Quality Improvement (CQI) which are under Good Pharmaceutical Manufacturing Practice (GPMP), and Quality Assurance (QA).

It is possible that presence of the microorganisms can be detectable by:

- Their physical presence (cloudiness in liquid medicines, moulds on, or in, creams and syrups, or as discolouration of tablets stored in a damp environment).

- Changes in colour (pigment production).
- Smell (due, for example, to amines, acetic or other organic acids, or sulfides from protein breakdown).
- Gas accumulation without any obvious odour (bubbles of carbon dioxide following sugar fermentation).

Quality Control:

The methods for counting and detecting some microbes in non-sterile products have poor accuracy and precision. For example, low number microorganisms sometimes damage the product and cannot be isolated, or products with active spoilage yield very low microbial count, or microorganism in high number but it is not the primary spoilage agent nor pathogenic. Uneven distribution of microorganism in viscous formulations provides serious sampling problem. The type of culture medium and conditions of recovery and incubation may influence viable counts obtained. Pharmacopoeias (Ph Eur, BP and USP) have included quantitative and qualitative standards for non-sterile products. The British Pharmacopoeia and the European Pharmacopoeia describe a single challenge preservative test that routinely uses four test organisms (two bacteria, a yeast and a mould) that are used for some specific situations. There are limits of maximum total microbial levels, for example: Oral product total viable count: 103 aerobic bacteria, 102 fungi/g or ml and exclusion of specific microorganism depending on route of administration, e.g. *E. coli* and *Salmonella*. Higher levels are permissible if the product contains raw material of natural origin. There is more interest in rapid methods: e.g. fluorescent dyes and epifluorescence, use of vital signs (e.g. detection of ATP using luciferase), Endotoxin (pyrogen) levels in parenterals must be very low to prevent endotoxic shock. Formerly this was tested by injecting rabbits and noting any febrile response. Nowadays the test is performed using Limulus Amoebocyte Lysate (LAL) test (gel clot method) where an amoebocyte lysate from the horseshoe crab *Limulus polyphemus* reacts specifically with microbial lipopolysaccharides to give a gel and opacity even at very high dilutions. Tissue culture tests are under development where the ability of endotoxins to induce cytokine release is measured directly.

Sterility Testing: Sterility testing of pharmaceutical articles is required during the sterilization validation process as well as for routine release testing. It is a very tedious and artful process that must be performed by trained and qualified laboratory personnel. Sterility testing is an essential part of sterilization validation. It is designed and executed to eliminate false positive results. False positive results are generally due to laboratory contamination from the testing environment or technician error. These tests are suitable to reveal the presence of viable forms of bacteria, fungi and yeast in a pharmaceutical products or devices. It is a testing procedure to ensure the batch of products is sterile or has been sterilized, meaning free of viable microorganisms like bacteria, yeast or molds.

Good Pharmaceutical Manufacturing Practice (GPMP):

Quality Control (QC) is that part of GPMP dealing with specification, documentation and assessing conformance to specification. A high assurance of overall product quality is raised only from a detailed specification, control and monitoring of all the stages that contribute to the manufacturing process. Parametric release is accepted as an operational alternative to

routine sterility testing for batch release of some finished sterile products where the manufacturer can provide assurance that the product is of the stipulated quality, based on the evidence of successful validation of the manufacturing process and review of the documentation on process monitoring carried out during manufacturing.

Quality Assurance (QA):

It is a combined scheme of management which embraces all the procedures necessary to provide a high probability that a medicine will conform consistently to a specified description of quality. It includes formulation design and development (R&D), good pharmaceutical manufacturing practice (GPMP), quality control (QC) and post-marketing surveillance. The risk of microbial infection and spoilage are raised from microbial contamination during manufacture and storage and hence preservatives are recommended further protection against environmental microbial contaminants but it is relatively non-specific in their reactivity. Laboratory tests are devised to challenge the product by used 'preservative challenge tests' where relatively large inocula of various laboratory cultures are added to aliquots of the product and determine their rate of inactivation by viable counting methods (single challenge tests).

The Preservative Challenge Test (Antimicrobial Effectiveness Test):

It is required for assessment of the microbial preservation of multiple used cosmetics and pharmaceutical products. They are added to products to prevent or limit microbial contamination, which occurs during normal conditions of storage and use. The efficacy of an antimicrobial preservative may be enhanced or diminished by the active constituent of the preparation, or by the formulation in which it is incorporated, or by the container and/or closure being used as the final packaging material. The test method is qualified for the product under evaluation to ensure that the correct diluents are used in assays for surviving microorganisms. The product is inoculated with specified number of each challenge organism. The inoculated product is held at room temperature for 28 days and examined by the duplicate plate count method to determine the number of viable microorganisms that survive at each specified time interval.

Results are evaluated with the tabulated acceptance criteria of the relevant Pharmacopoeias or test protocols.

Post-market Surveillance:

It is most important stage to follow up a medicine that is smooth floating in the market without any complain by the customers. A proper quality assurance system is included for monitoring in-use performance and for responding to customer complaints. These are constantly followed up in great detail in order to decide carefully constructed and implemented schemes for product safety.

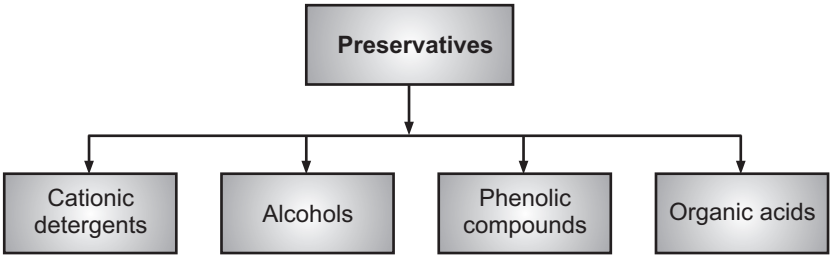
5.6 PRESERVATION OF PHARMACEUTICAL PRODUCTS USING ANTIMICROBIAL AGENTS

Pharmaceutical products are defined as any substance or mixture of substances for use in humans for the diagnosis, treatment or prevention of a disease, or abnormal physical statements, or altering or modifying any organic function. When these pharmaceutical products deteriorate due to contaminant microbes, is known as microbial spoilage. Hence,

products are required to preserve to minimize or inhibit spoilage during the storage and multi-dose applications by the risk of microbial contamination. Prescription drugs are the main pharmaceutical industry products like flumadine, acyclovir, amantidine (essential medicines) whereas biological products are composed of different types of products such as vaccines, antibodies, blood, therapeutic proteins, tissues etc. Some medical devices are like needle, syringe, hand gloves, some OTC drugs like aspirin, paracetamol are also considered as pharmaceutical products. These products are contaminated by used of various microorganisms such as *E. coli, Clostridium botulinum, Clostridium perfingens, Pseudomonas aeruginosa, Salmonella cavaban* etc. Antimicrobial preservatives are considered exceptions to such categorization, being added to help to improve antimicrobial stability and hence required for antimicrobial activity. Therefore, preservatives play a vital role for the storage of pharmaceutical products using antimicrobial agents. Some ideal characters of preservatives are:

- They should be free from toxic or irritant effects at the specified concentration.
- They should be effective in preventing the growth of microorganism.
- They should be soluble in water so that they should achieve required concentration for the activity.
- They should be heat stable and prolong storage capability.
- They should be chemically compatible with all other formulations.
- They should be not adversely affected by container or closure of the products.
- They should be cheap.

There are various types of preservatives used in the preservation of pharmaceutical products that are listed in Flowchart 3.



Flowchart 3: Types of preservatives

1. **Cationic detergents:** Examples: Benzalkolium chloride, alkyl trimethyl ammonium chloride.
2. **Alcohols:** Examples: Chlorbutanol, bronopol, phenyl and phenoxy ethanol.
3. **Phenolic compounds:** Examples: chlorinated and isopropyl derivatives of meta cresol.
4. **Organic acids:** Examples: Salicylic acid, benzoic acid, acetic acid, lactic acid, hydroxyl benzoic acid.

Factor affecting the efficacy and availability of preservatives:

- Temperature.
- Chemical structure of preservatives.
- Capacity of preservatives.
- Inoculum size.
- Effect of pH.
- Effect of containers and packaging.
- Changes of concentration.

Methods of Preservation:

1. **Physical protection:** It is used for proper packaging of the pharmaceutical products under aseptic condition or else there is a chance for microbial growth. Operating persons are also an important factor for proper processing of the products under aseptic environment.
2. **Preservative coating:** Aqueous raw materials used in the formulation of paints and coatings create the perfect environment for the growth of bacteria, fungi and yeast. They can destroy valuable pharmaceutical formulations. Controlling these microorganisms helps increase efficiency, helps deliver a better end-use product and helps employees and consumers avoid contact with spoilage microorganisms. Biocides are necessary for protecting the integrity and functionality of water-based paints and coatings from destruction by microbial contamination as a result lengthening of product's shelf life and protect dry film from algae, mold and mildew.
3. **Water proof protection:** Packaging of the pharmaceutical products should be under water proof protection because water favours the growth of microorganisms.
4. **Water vapour proof protection:** This method is applicable for certain pharmaceutical products when they are packing under proper care for minimizing microbial activity. For 'dry' dosage forms with very low water activity (A_w) provides protection against microbial attack. The moisture vapour properties of packaging materials require careful examination.
5. **Water vapour proof protection with desiccant:** This method is also used for dry products that absorbed moisture from the environment and is spoiled due to growth of microorganisms. Packing should be proper with this method to minimize the microbial growth and spoilage of the products.

Classification of Preservatives based on Mechanism of Action:

1. **Antioxidants:** The agents which prevent oxidation of active pharmaceutical ingredient which otherwise undergo degradation due to oxidation as they are sensitive to oxygen.
   For example: Vitamin E, Vitamin C, Butylatedhydroxyanisole (BHA), Butylated-hydroxytoluene (BHT).
2. **Antimicrobial agents:** They are the agents that are active against gram positive and gram negative micro-organism which causes degradation of pharmaceutical preparation, active in small inclusion level. For example: Benzoates, Sodium benzoate, Sorbates.
3. **Chelating agents:** They are the agents which form the complex with pharmaceutical ingredient and prevent the degradation of pharmaceutical formulation.
   For example: Disodium ethylenediaminetetraacetic acid (EDTA), Polyphosphates, Citric acid.

Modes of Action of Preservatives:

Preservatives provide limited protection against viral contamination. Bactericides and fungicides may evince their effects on a variety of microbial cellular targets, for example; the cell wall, the cytoplasmic membrane or the cytoplasm. It is sometimes difficult to assign a precise target for a specific class of preservative; the target does change with preservative concentration. Hence, they should have:

- A wide spectrum of antimicrobial activity at low inclusion levels.
- Maintain activity throughout product manufacture, shelf life and usage.
- No compromise on the quality or performance of product, pack or delivery system.
- Adverse influence on patient safety or tolerance of the product.

With these concepts the preservative system protects the product against microbial proliferation.

Mode of action of antimicrobial agents as preservative:

Antimicrobial agents act by cell wall inhibition, protein synthesis inhibition, DNA and RNA synthesis inhibition. For example: Benzoates, Sodium benzoate, Sorbates, Propionates, Nitrites.

Emulsions contain water which will support microbial growth. Microbes produce unpleasant odour, colour changes and gases and may affect the emulsifying agents. Other ingredient of emulsions support the growth medium for microbes. Examples: Arachis oil supports *Aspergillus* species and liquid paraffin supports *Penicillium* species.

Antimicrobial preservatives are added to non-sterile dosage forms to protect from microbiological growth or from microorganisms that are introduced inadvertently during or subsequent to the manufacturing process. In the case of sterile articles packed in multiple-dose containers, antimicrobial preservatives are added to inhibit the growth of microorganisms that may be introduced from repeatedly withdrawing individual doses. Examples of antimicrobial preservatives include alcohol, formaldehydes and iodine.

An antimicrobial 'preservative' is included in a formulation to minimize the risk of spoilage and to kill low levels of contaminants introduced during storage or repeated use of a multidose container. The strong antimicrobial agents are often non-selective in action but interact significantly with formulation ingredients as well as with patients and microorganisms.

5.7 EVALUATION OF MICROBIAL STABILITY OF FORMULATIONS

Stability is defined as an extent to which a pharmaceutical product retains, within specified limit and throughout its storage period and use i.e. its shelf life, the same properties and characteristics that are possessed at the time of its manufacture.

Microbial stability of formulations is defined as the formulation which are not any microbiological attack and is meet the standard with respect to lack of contamination or sterility.

Microbial contamination in formulation mainly occurs from water (*Pseudomonas, Xanthamonas* etc.), air (*Penicillium, Aspergillus, Bacillus species, Yeasts* etc.) and raw materials (*Micrococci*).

Stability is used to determine:

- Quality of a drug substances or drug products.
- Shelf life of a drug products.
- Recommended storage conditions.

Hence, stability testing is recommended because chemical degradation sometimes leads to lower concentration of drugs in dosage form and also toxic product is formed due to degradation of active ingredients.

In other way,

Stability is the capacity of a drug product to remain within specifications established to ensure its identity, strength quality and purity.

There are three types of stability studies:

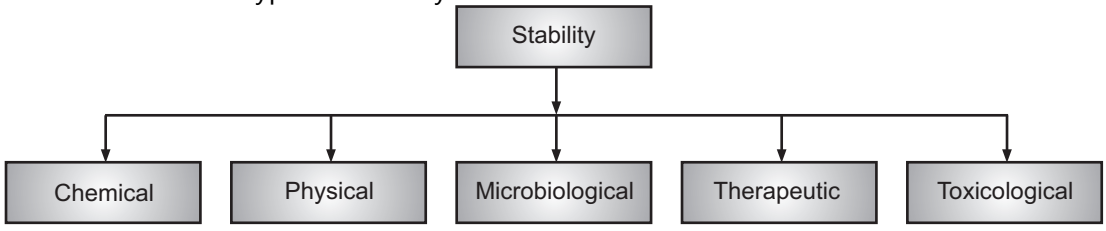
(a) Long term study (Minimum 1 year and maximum 5 years): Studies carried out at 25°C ± 2°C / 60% RH ± 5% RH. For 1 year every 3 months, for 2 years every 6 months and for 3 years every year once.

(b) Intermediate (Minimum 6 months and maximum 12 months): Studies carried out at 30°C ± 2°C / 65% RH ± 5% RH.

(c) Accelerated (6 Months): Studies carried out at 40°C ± 2°C/75% RH ± 5% RH.

Types:

There are various types of stability that are listed in Flowchart 4.



Flowchart 4: Types of stability

Stability evaluation for various formulations:

1. **Tablets:** Colour, odour, assay, degradation, dissolution, moisture content, hardness, friability.
2. **Capsules:** Appearance, brittleness, colour, odour, content uniformity, assay, degradation product, dissolution, moisture and microbial content.
3. **Emulsions:** Appearance, phase separation, colour, odour, assay, pH, viscosity, microbial assay, preservative content, mean size of globules, distribution of dispersed globules.
4. **Oral solutions and suspensions:** Mean size and distribution of particles, for suspensions redispersibility, rheological properties etc.
5. **Oral powders for reconstitution:** Moisture, Reconstitution time.
6. **Inhalations and nasal aerosols:** Appearances (content, container, valve, its components), dose content uniformity, labeled number of medication actuations per container meeting, aerodynamic particle size distribution, water content, microscopic evaluation, Leak rate, Microbial limit.
7. **Topical formulations (ointments, cream, lotions, gel, paste, solutions, non-metered aerosols):** Clarity, colour, odour, pH, resuspendability (for lotions), consistency, viscosity, preservatives, antioxidant content, microbial limit, sterility testing, weight loss.
8. **Ophthalmic preparations (cream, ointments, solutions, suspensions):** Sterility, particulate matters, non-metered aerosols, delivery rate, microbial limit, spary patterns, water content, particle size.
9. **Suppositories:** Softening range, dissolution at 37°C, microbial limits.

10. **Small and large volume parenterals:** Particulate matters, pH, sterility, endotoxins, pyrogens.
11. **Transdermal patches:** *In-vitro* release rate, leakage, microbial limits, sterility test, peel and adhesive force, drug release rate.
12. **Freeze drying products:** Appearance, reconstituted products, assay, degradation products, pH, water content, rate of solution.

Microbial Stability Test: Microbial contaminants usually originate from two different sources during production and filling and during the use of the cosmetics by the consumers (by consumer's hands and body). It is necessary to carry out routine microbiological analysis of each batch of the finished product. The main potential pathogens in cosmetics are *Pseudomonas aeruginosa, Candida albicans, and Staphylococcus aureus*. These pathogens must not be detectable more than 0.1 g or 0.1 ml in a cosmetic product. Microbial contamination in some cosmetic products is given in table 5.5.

Table 5.5: Microbial contamination in cosmetic products

Products	Microorganisms	CFU/g
Skin cream	*Enterobacter gergoviae*	12,0000
Eye cream	*Enterobacter gergoviae*	29,0000
Herbal tooth powder	*E. faecium, K. pneumonia*	54,0000
Hair shampoo	*P. aeruginosa*	57,0000
Sun lotion	*E. cloacae, E. faecium, Escherichia spp*	8,000,000

Some tests are as follows:

1. Screening test:

It is also known as plate count or dip slide method. This method is used to detect aerobic bacteria in aqueous sample. Dip slide is coated on both sides with a solid agar gel medium. A small quantity of TTC (2,3,5-triphenyl tetrazolium chloride) is added to detect aerobic bacteria in the sample. The slide is dipped in to the aqueous solution for 10 seconds and excess liquid is drained off from the slide. Then it is incubated at 35-37°C for 18-48 hours. The colour appeared is compared with calibration chart. Aerobic bacteria species grow on this medium and is detected by their ability to reduce TTC dye to a red coloured formozan dye. Developing bacterial colonies are alter the TTC dye and are appeared as red spots.

2. Quantitative test:

Quantitative tests determine the actual count level of bacteria, molds and yeasts in cosmetic products. This method is used for isolation of microorganisms from cosmetic products include direct colony counts and enrichment culturing.

Microbial stability studies are also carried out in Pharmaceutical products. Raw materials play vital role in the product formulation. Pharmaceutical raw material is defined as a substance that is used in the manufacturing of pharmaceutical products. During the manufacture of pharmaceuticals and cosmetics, untreated raw materials are contaminated with microorganisms beyond acceptable limits. Hence it is necessary to control microbiological contamination from raw materials under GMP regulations in the pharmaceutical industry. These microorganisms from plants (e.g., species of *Erwinia, Pseudomonas, Lactobacillus, Bacillus* or *Streptococcus*) such as gum acacia, tragacanth, agar,

powdered rhubarb, and starches contains bacteria, which causes disease. Water plays major role in product formulations because water is the good media for microbial growth. The water activity (A_w) of pharmaceutical and cosmetic products is the measure of free water in the formulation. The amount of water that is in free form is available to microorganisms for survival. The measurement of A_w in pharmaceutical and cosmetic products predicts the type and number of microorganisms responsible for product degradation and maintains chemical stability.

Water activity is determined by dew point method and using electric hygrometer (measure relative humidity).

Antimicrobial Effectiveness Test:

This test is used for estimating of preservative in a product. This test is mainly used during development of formulation and in stability studies. In this test, a product is inoculated with a controlled quantity of specific microorganisms. The test then compares the level of microorganisms found on a control sample versus the test sample over a period of 28 days. Test organisms are used for the purpose of challenging the preservative system in a product. The suitable media are Soyabean Casein digest or Sabouraud Dextrose Agar used for the test. The bacteria (likely, *Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus*), yeast (*Candida albicans*) and mold (*Aspergillus niger*) are used for this test. A product is inoculated or contaminated with a number of organisms between 1×10^5 (100,000) to 1×10^6 (1,000,000) colony forming units (CFU) per ml of product. At various intervals, the product is tested to determine its ability to control reproduction or destroy the microorganisms.

Test and standard are incubated at 32.5 ± 2.5°C and 22.5 ± 2.5°C for bacteria and yeast respectively and the concentration of the microorganisms in each of the standardized inoculum is determined by plate count method.

Pharmaceutical Products:

For testing purposes, the USP has divided test articles into four separate categories:

Category 1 – Injections, other parenterals including emulsions, sterile nasal products made with aqueous bases or vehicles.

Category 2 – Topically used products made with aqueous bases or vehicles, non-sterile nasal products, and emulsions, including those applied to mucous membranes.

Category 3 – Oral products other than antacids made with aqueous bases or vehicles.

Category 4 – Antacids made with an aqueous base.

5.8 GROWTH OF ANIMAL CELLS IN CULTURE

Removal of cells from animals and their subsequent growth in a favorable artificial environment is animal cell culture. Animal cells are more difficult to culture than microorganisms because animal cells require many more nutrients and typically grow only when attached to specially coated surfaces. The environment consists of a suitable glass or plastic culture vessel containing liquid or semisolid medium that supplies the nutrients essential for survival and extra proliferation. The cells are removed from the tissue directly and disaggregated by enzymatic or mechanical means and then cultured in artificial media

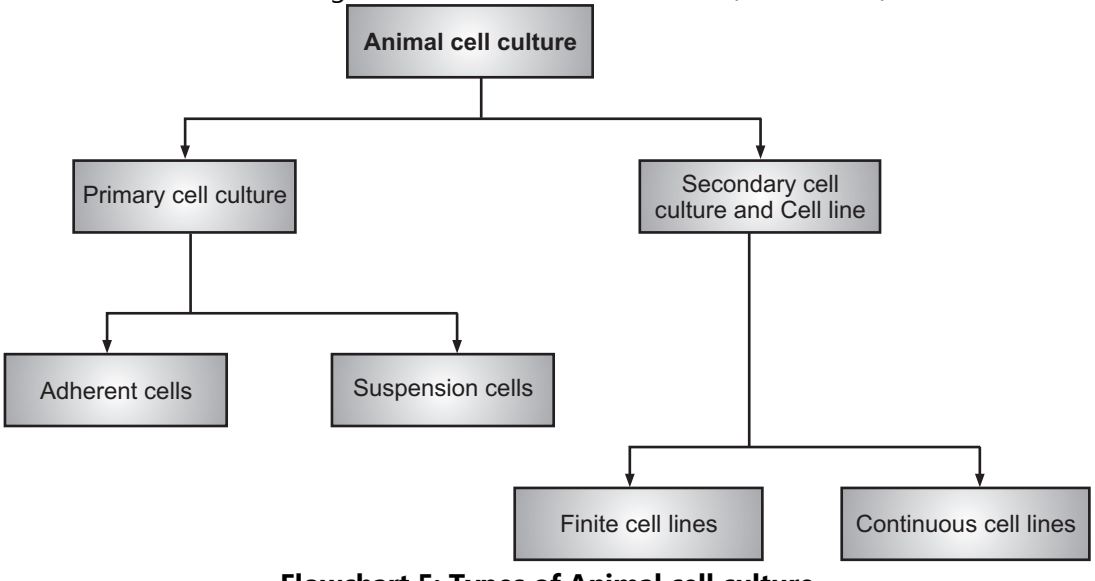
under aseptic environment similar to culture of microorganisms. This *in-vitro* cell culture system explores cell growth and differentiation, as well as perform genetic manipulations required to understand gene structure and function.

Animal cell cultures are initiated by the dispersion of a piece of tissue into a suspension of its component cells, which is then added to a culture dish containing nutrient media. In animal cells fibroblasts and epithelial cells are selected because these cells grow very fast. The plastic surface of dishes is used for cell culture. In 1907, Harrison cultivated frog nerve cells in a lymph clot and observed the growth of nerve fibres *in-vitro* for several weeks and hence he is recognized as the father of cell culture.

Animal cell culture is helpful to study the effect of new drugs, to investigate cancerous cells, to study the interactions between disease causing agents and cells, and are useful for gene therapy, genetic engineering etc.

Types of Animal Cell Culture:

Based on the number of cell divisions, cell culture is classified as primary cell culture and cell lines. Cell lines can undergo finite or infinite cell divisions (Flowchart 5).



Flowchart 5: Types of Animal cell culture

Primary Cell Culture:

This cell culture is obtained from the cells of a host tissue. The cells dissociated from the parental tissue are grown on a suitable container and the culture thus obtained is called primary cell culture. The culture is comprised of heterogeneous cells and most of the cells divide only for a limited time. Based on their origin, primary cells grow either as an adherent monolayer or in a suspension.

Adherent Cells:

These cells are propagated as a monolayer and are anchorage dependent. Monolayer cultures are defined as when the bottom of the culture vessel is covered with a continuous layer of cells of one cneed to be attached to a solid or semi-solid substrate for proliferation. They adhere to the culture vessel with the use of an extracellular matrix which is derived from tissues of organs that are immobile and embedded as connective tissue. For example: Fibroblasts and epithelial cells.

Majority of continuous cell lines grow as monolayers and such type of cells are transferred directly to a cover slip for examination under microscope.

Suspension Cells:

These types of cells do not attach to the surface of the culture vessels. Hence, they are also known as anchorage independent or non-adherent cells which are grown in liquid culture medium. Hematopoietic stem cells (derived from blood, spleen and bone marrow) and tumor cells are grown in suspension much faster which do not require frequent replacement of the medium. These cultures have short lag period.

Secondary Cell Culture and Cell Line (Figure 5.2)

Secondary culture is the culture when a primary culture is sub-cultured. It is also known as cell line or sub-clone. The process involves removing the growth media and disassociating the adhered cells by enzymatic treatment. Sub-culturing of primary cells to different divisions leads to the generation of cell lines. During growth period, cells with the highest growth capacity predominate and result in genotypic and phenotypic uniformity in the population. Based on the life span of the culture cell lines are two types viz. Finite cell lines and Continuous cell lines.

Finite cell lines:

It is defined as the cell line that undergoes limited number of cell division with a limited life span. The cells passage several times and then lose their ability to proliferate, which is a genetically determined event known as senescence. Cell lines derived from primary cultures of normal cells are finite cell lines.

Continuous cell lines:

It is defined as a finite cell line which undergoes transformation and acquires the ability to divide indefinitely. This transformation occurs spontaneously or chemically or virally induced or from the establishment of cell cultures from malignant tissue. Prepared cell cultures are then sub-cultured and grown indefinitely as permanent cell lines. These cells are less adherent and fast growing as a result the cell density becomes higher and different in phenotypes from the original tissue. They grow more in suspension medium.

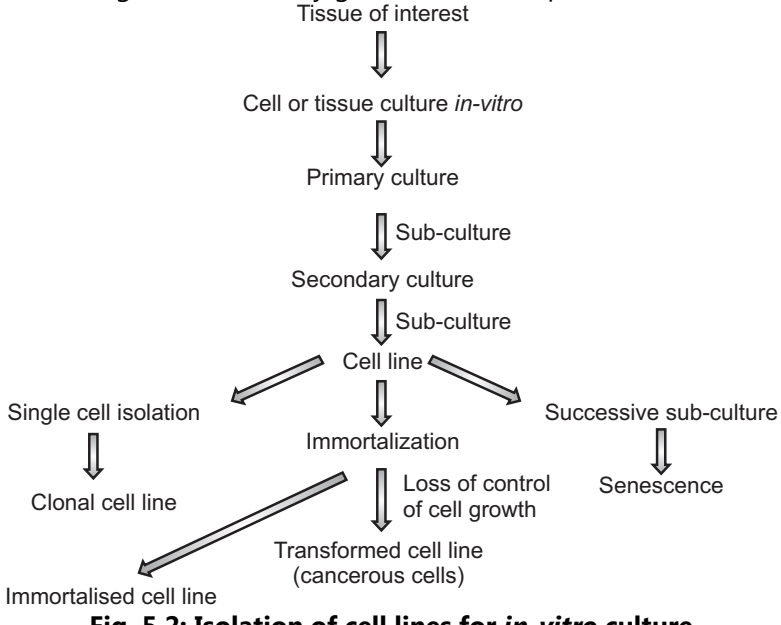


Fig. 5.2: Isolation of cell lines for *in-vitro* culture

5.9 GENERAL PROCEDURE FOR CELL CULTURE

Procedure (Figure 5.3)**:**

(a) Requirements: Vertical Laminar air flow, Incubator, Refrigerator, Microscope, Tissue culture ware.

(b) Temperature: The temperature sets at as the same as body temperature of the host from which cells are procured. Most animal cells required 36-37^{o}C.

(c) Substrate: Good compatible substrate is required for attachment and optimum growth. Glass and specially treated plastics are commonly used as substrate. Thereafter attachment factor such as collagen, gelatin, laminin etc. are used as substrate coating to improve growth and function of normal cells derived from brain, blood vessels, kidney, liver, skin etc.

(d) Culture medium: It is an important and complex factor for the cell growth. The culture medium is supplemented with various growth factors, pH and osmolality regulator and provides essential gases like oxygen and carbon dioxide. The medium is also supplemented with various nutrients like amino acids, vitamins, minerals and carbohydrates which are essential for growth of cells and provided energy for metabolism. Choice of media is used based on the cells being cultured. Generally, media like Dulbecco's modified Eagle's medium (DMEM), Eagle's minimal essential medium (EMEM), Glasgow Minimum Essential Medium (GMEM) are used for cell culture. Prepared media is filtered and incubated at 4^{o}C.

(e) Media and growth requirement: Temperature should maintain at 37°C and optimum pH is 7.2 to 7.5. The humidity is required to be maintained properly in the media with proper gas phase ratio (Bicarbonate concentration and carbon dioxide in equilibrium). For growth of cultured cells light intensity also plays a vital role. Inside environment cells are cultured in dark because light induced production of toxic compound. Commonly used antibiotics are penicillin, streptomycin, Kanamycin etc. Commonly used fungicides are Nystatin, Amphotericin B. Bulk ions like sodium, potassium, calcium, magnesium, chlorine, phosphorus, carbon dioxide, oxygen etc. Trace elements like iron, zinc, selenium, sugar, amino acids, vitamins, choline, inositol etc.

(f) Selection of organ: Different type of cells are grown in cultures including connective tissue elements such as fibroblasts, skeletal tissue, cardiac, epithelial tissue (liver, breast, skin, and kidney) and many different types of tumor cells. On the basis of morphology (shape and appearance) or on the functional characteristics of cells, they are divided into three types.

- Epithelial like - attached to a substrate and appears flattened and polygonal in shape.
- Lymphoblast like - cells do not attach remain in suspension with a spherical shape.
- Fibroblast like - cells attached to an substrate appears elongated and bipolar.

(g) Culturing of cell: Cells are cultured as anchorage dependent or independent. Cell lines derived from normal tissues are considered as anchorage-dependent which grows only on a suitable substrate e.g. tissue cells. Suspension cells are anchorage-independent e.g. blood cells whereas Transformed cell lines either grows as monolayer or as suspension.

(h) Steps:

1. **Use sterile technique:** Tissue part is harvested and processed using sterile equipment, reagents and techniques. Personal protective equipments are used to avoid contamination. All enzymes and reagents are filtered sterile condition using a 0.22 micron membrane.

2. **Mince/cut tissue:** Mince the tissue specimen into small pieces (usually 2 × 4 mm) with sterile scissors or scalpel, and then placed the small pieces into selected buffer, media or salt solution.
3. **Wash and add enzyme:** Wash tissue two to three times to eliminate excess blood proteins and then add enzyme(s) of choice, likely, collagenase, protease, papain or trypsin. Usually about 0.5 to 1.5 mg/ml of selected enzyme is sufficient.
4. **Incubation:** Further tissue specimen is incubated in optimum temperature at 37°C for 30 to 90 minutes with periodical mixed the rock specimen.

5. Disperse and wash cells:

Cells are dispersed by gently pipetted them and then cell suspension is filtered using a fine mesh. The cells became settled and decanted excess liquid containing enzymes. Further cells are washed two to three times with Fetal Bovine Serum (FBS), Bovine Serum Albumin (BSA) or other inhibitors can also be used to halt enzyme digestion.

6. Resuspension and measure cells:

Cells are resuspended in the correct medium or buffer and then quantitatively determined the cell yield and viability. This is an important step in the cell isolation process to evaluate the result by dissociation technique. Most researchers are used a hemocytometer for determining cell yield and trypan blue diazo dye to measure cell viability.

$$\% \text{ of viable cells} = \frac{\text{Number of unstained cells} \times 100}{\text{Total number of cells}}$$

(i) Cell toxicity: Cytotoxicity causes inhibition of cell growth which observed effect on the morphological alteration in the cell layer or cell shape. It is characterized with abnormal morphology like giant cells, multinucleated cells, a granular bumpy appearance, vacuoles in the cytoplasm or nucleus etc. Cytotoxicity is determined by substituting materials such as medium, serum, supplements flasks etc.

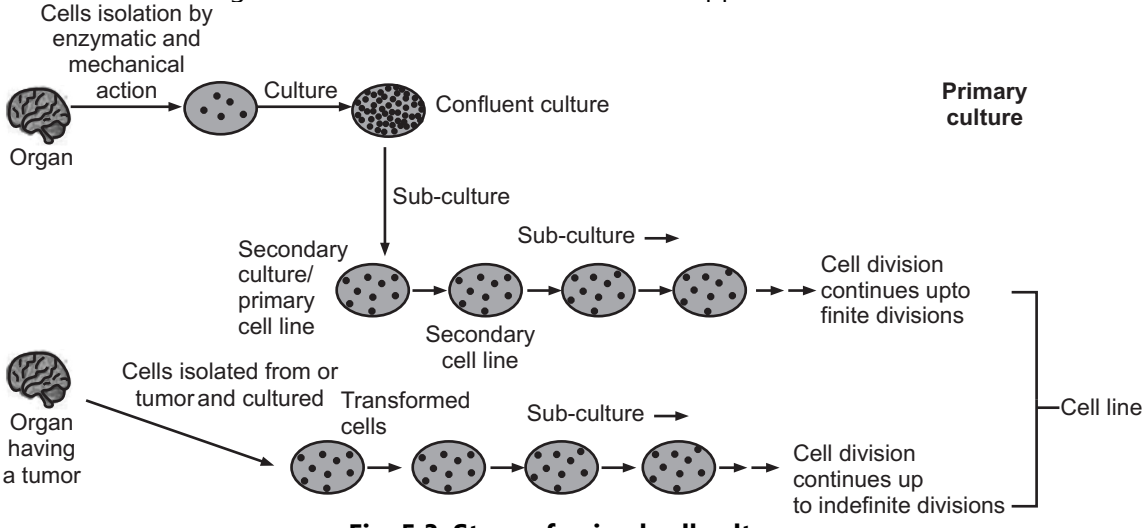


Fig. 5.3: Steps of animal cell culture

5.9.1 Primary Culture

Cells when surgically or enzymatically are removed from the organism and are placed in suitable culture environment and grown are called primary culture. They have finite life span and contain heterogeneous population of cells. These cells upon subculture lead to generate cell lines which have limited life span. Lineage of cells are originated from the primary culture

is known as cell strain. Primary cultures are morphologically similar to the parent tissues. They have limited number of cell division (Fig. 5.4).

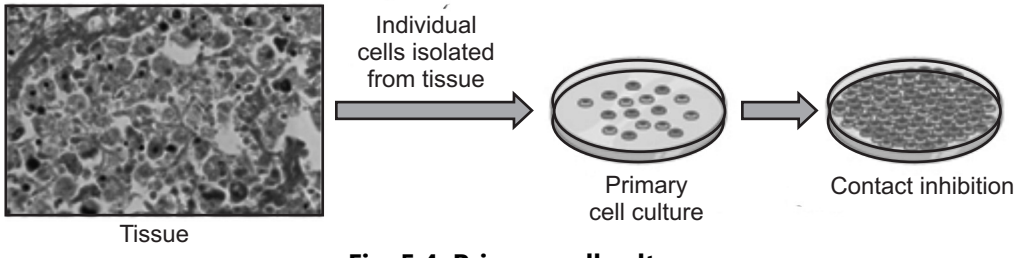


Fig. 5.4: Primary cell culture

5.9.2 Established Cell Culture

Primary cell culture when first subcultured is known as secondary cell culture. Established or immortalized cell line is the ability to proliferate indefinitely by random mutation and artificial modification such as artificial expression of the telomerase gene.

Advantages:

- Many kinds of cell lines.
- Generally easy to grow and manipulate.
- Proliferate indefinitely.
- Contact inhibition.

Example: HeLa, Sf-9, Cervical cancer.

5.9.3 Transformed Cell Culture

Transformation is a process of conversion of normal cell into a cell having some or many of the attributes of different cell. When cell is transformed, cells lose contact inhibition and become immortal. For example: NIH 3T3 mouse cells are partially transformed and became immortal but contact inhibited and grows in a mono layer. Transformed cells lack contact inhibition of movement due to change of cell surface property and loss of many receptors. They continue to grow and pile up on top of one another as they proliferate.

Transformed cells are also cultured through suspension medium where cells do not attach to the surface of the culture vessels and are grown liquid culture medium. Hematopoietic stem cells (derived from blood, spleen and bone marrow) and tumor cells are grown in suspension much faster which do not require the frequent replacement of the medium.

5.10 APPLICATION OF CELL CULTURES IN PHARMACEUTICAL INDUSTRY AND RESEARCH

Cell culture is used in cellular and molecular biology, providing excellent model systems for studying the normal physiology and biochemistry of cells (for example: metabolic studies, aging), the effects of drugs and toxic compounds on the cells and mutagenesis and carcinogenesis. It is also used in drug screening and large scale manufacturing of biological compounds (for example: vaccines, therapeutic proteins).

1. **Model System:** Cell culture is used as model system to study basic cell biology and biochemistry. It is also used to study the interaction between cell and disease causing agents like bacteria, virus. It helps to study the effect of drugs and to study triggers for ageing.
2. **Cancer Research:** Cell culture is one of the most important tools in cancer research. The basic difference between normal cell and cancer cell can be studied using animal cell culture technique. Normal cells are induced cancer cells by using radiation, chemicals, viruses and then cause of cancer is studied. Cell culture is also used to determine the effective drugs for selectively destroy only cancer cells.

3. **Virology:** Animal cell cultures are used to replicate the viruses instead of animals for the production of vaccine. Cell culture can also be used to detect and isolate viruses, and also to study growth and development cycle of viruses. It is also used to study the mode of infection. This technique is also used for production of human and veterinary viral vaccine against a variety of diseases.
4. **Toxicity Testing:** Animal cell culture is used to study the effects of new drugs, cosmetics and chemicals and growth of multiple cells, especially liver and kidney cells. This technique is also used to determine the maximum permissible dosage of new drugs.
5. **Vaccine Production:** Cultured animal cells are used for virus production and these viruses are used to produce vaccines. For example: Vaccines like polio, rabies, chicken pox, measles and hepatitis B are produced using animal cell culture. The polio vaccine produced in 1954 was first human vaccine is produced using large scale cell culture techniques.
6. **Genetically Engineered Protein:** Animal cell cultures are used to produce commercially important genetically engineered proteins such as monoclonal antibodies, insulin, hormones etc. Proteins extracted from biological sources are important for substitution therapy. Interferone is discovered in 1957 by cell cultured method by viral infection. Tissue plasminogen activator is produced in large scale from transferred CHO-K1 cells. It is used to prevent fibrin clots in the blood stream. Blood clotting factor VIII is produced in large scale by Bayer through transfection of the kidney cell line with an appropriate gene.
7. **Replacement Tissue or Organ:** Animal cell culture is used as replacement tissue or organs. For example: artificial skin is produced to treat patients with burns and ulcers. Recently artificial organ culture such as liver, kidney and pancreas are successfully carried out for transplantation. Organ culture techniques and research are also conducted on both embryonic and adult stem cell culture. These cells have the capacity to differentiate into many different types of cells and organs.
8. **Genetic Counseling:** Fetal cell culture extracted from pregnant women is used to study or examine the abnormalities of chromosomes, genes using karyotyping and these findings are used in early detection of fetal disorders.
9. **Genetic Engineering:** Cultured animal cells are used to introduce new genetic material like DNA or RNA into the cell. This method is used to study the expression of new genes and its effect on the health of the cell. Insect cells are used to produce commercially important proteins by infecting them with genetically altered baculoviruses. With this method mass produced insulin, human growth hormone, human albumin, vaccines etc.
10. **Gene Therapy:** Cultured animal cells are genetically altered and are used in gene therapy technique. First cells are removed from the patient lacking a functional gene or missing a functional gene. These genes are replaced by functional genes and altered cells are culture and grown in laboratory condition and are introduced into the patient. Functional gene is inserted into the genome of viral vector and then they are allowed to infect the patient and the missing gene will be expressed with the help of the viral vector.
11. **Drug Screening and Development:** Animal cell cultures are used to study the cytotoxicity of new drug. This is also used to find out the effective and safe dosage of new drugs. Cell-based assay plays an important role in pharmaceutical industry. Originally these cell culture tests were done in 96 well plates but recently these tests are conducted in 384 and 1536 well plates.

QUESTIONS

Long Essays:

1. Explain spoilage and types of spoilage with examples.
2. Explain the sources and types of microbial contaminants.
3. Explain preservation of Pharmaceutical products using antimicrobial agents.
4. Explain general procedure of cell culture.

Short Essays:

1. Explain factor affecting microbial spoilage of pharmaceutical products.
2. Explain the evaluation method of microbial contamination and spoilage.
3. Explain the type of microbial contamination.
4. Explain the evaluation of microbial stability of formulations.
5. Explain the growth of animal cell in culture and type of cell culture.
6. Explain the application of cell culture in Pharmaceutical industry and research.
7. Explain the evaluation of stability of various formulations.

MULTIPLE CHOICE QUESTIONS

1. Musty flavor of bread is due to ……
   (a) Ethanol (b) Acetoin
   (c) Trichloroanisol (d) Trimethylamine
2. Garlic flavor of fish is due to ……
   (a) Ethanol (b) Dimethyltrisulphide
   (c) Acetoin (d) Lactic acid
3. Hydrolytic rancidity of milk is due to ……
   (a) Lipase (b) Proteases
   (c) Peroxidases (d) Thiaminase
4. Thiamine destruction of meat is due to ……
   (a) Lipase (b) Proteases
   (c) Peroxidases (d) Thiaminase
5. Bad odour of vegetables is due to ……
   (a) Clostridium (b) Pseudomonas
   (c) *E. coli* (d) Rhizopus
6. Proteolysis of milk is caused by ……
   (a) Enterococci (b) Serratia
   (c) *E. coli* (d) Streptococcus
7. *Serratia marcescens* is the causative organism for fish spoilage of ……
   (a) Blue colour (b) Green colour
   (c) Red spot (d) Silver spot
8. Sulfide spoilage in canned food is due to ……
   (a) Bacillus (b) Penicillium
   (c) Desulfotomaculum (d) Mucor
9. Green discolouration of cheese is due to ……
   (a) *Candida* (b) *Pseudomonas*
   (c) *Proteus* (d) *Penicillium*
10. Black rot of eggs is due to ……
    (a) *Coliforms* (b) *Pseudomonas*
    (c) *Proteus* (d) *Rhizopus*

11. Redox potential in viscous emulsion is high because ……
    (a) High solubility of oxygen in fats
    (b) High solubility of oxygen in water
    (c) High solubility of carbon dioxide in fats
    (d) High solubility of carbon dioxide in water
12. Biological food contaminant is ……
    (a) Dust (b) Pesticides
    (c) Mushroom (d) Toxic metals
13. Contaminant of talcum powder is ……
    (a) *Coliforms* (b) *Clostridium tetani*
    (c) *Salmonella muenchen* (d) *Klebsiella*
14. Contaminant of thyroid tablet is ……
    (a) *Coliforms* (b) *Clostridium tetani*
    (c) *Salmonella muenchen* (d) *Klebsiella*
15. Limit of total microbial level of oral products is ……
    (a) 103 aerobic bacteria (b) 203 aerobic bacteria
    (c) 303 aerobic bacteria (d) 403 aerobic bacteria
16. LAL test is done for ……
    (a) Oral formulation (b) Parenteral formulation
    (c) Liposomes (d) Solid formulation
17. Microbial contamination from water occurs in formulation by ……
    (a) *Xanthamonas* (b) *Micrococci*
    (c) *E. coli* (d) *Bacillus*
18. Long term stability study is for ……
    (a) Minimum 2 years and maximum 5 years
    (b) Minimum 1 year and maximum 5 years
    (c) Minimum 5 years
    (d) Minimum 1 month
19. Accelerated stability study is for ……
    (a) 1 year (b) 6 years
    (c) 6 months (d) 1 month
20. The main potential pathogen in cosmetics is ……
    (a) *Bacillus* (b) *Candida*
    (c) *Enterobacter* (d) *E. coli*
21. Fungicide is used in cell culture medium ……
    (a) Kanamycin (b) Nystatin
    (c) Inositol (d) Minerals
22. Tissue plasminogen activator is produced from ……
    (a) Transferred COH-K1 cells (b) Transferred CHO-K2 cells
    (c) Transferred CHO-K1 cells (d) Transferred COH-K2 cells

Answers:

1. (c)	2. (b)	3. (a)	4. (d)	5. (a)	6. (b)	7. (c)	8. (c)	9. (a)	10. (a)
11. (a)	12. (c)	13. (b)	14. (c)	15. (a)	16. (b)	17. (a)	18.(b)	19. (c)	20. (b)
21. (b)	22. (c)								

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