Shree H. N. Shukla Institute of Pharmaceutical Education and Research,Rajkot

B. Pharm
Semester-5

STUDY MATERIAL

Subject Name:Pharmaceutical Microbiology
Subject Code: BP504TP

CHAPTER 1 **Introduction and history of microbiology** :

CONTAINS:
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 & viable count). Study of different types of phase contrast microscopy, dark field microscopy and electron microscopy

introduction to microbiology :

Microbiology is the — 'scientific study of the microorganisms'.

In fact, microorganism invariably refers to the minute living body not perceptible to the naked eyes, especially a bacterium or protozoon. Importantly, microorganisms may be carried from one host to another as follows :

(a) Animal Sources. Certain organisms are pathogenic for humans as well as animals and may be communicated to humans via direct, indirect, or intermediary animal hosts.

(b) Airborne. Pathogenic microorganisms in the respiratory track may be discharged from the mouth or nose into the air and usually settle on food, dishes or clothing. They may carry infection if they resist drying.

(c) Contact Infections. Direct transmission of bacteria from one host to another viz., sexually transmitted diseases (STD).

(d) Foodborne. Food as well as water may contain pathogenic organisms usually acquired from the handling the food by infected persons or via fecal or insect contamination.

(e) Fomites. Inanimate objects e.g., books, cooking utensils, clothing or linens that can harbor microorganisms and could serve to transport them from one location to another.

(f) Human Carriers. Persons who have recovered from an infectious disease do remain carriers of the organism causing the infection and may transfer the organism to another host.

(g) Insects. Insects may be the physical carriers, for instance : housefly (Musca domestica), or act as intermediate hosts, such as : the Anopheles mosquito.

(h) Soilborne. Spore-forming organisms in the soil may enter the body via a cut or wound. Invariably fruits and vegetables, particularly root and tuber crops, need thorough cleansing before being eaten raw.

Microbiology is the specific branch of 'biology' that essentially deals with the elaborated investigation of 'microscopic organisms' termed as microbes, that are composed of only one cell. These are typically either unicellular or multicellular microscopic organisms that are distributed abundantly both in the living bodies of plants and animals and also in the air, water, soil, and marine kingdom.

Interestingly, each and every microbe essentially bear both specific and special characteristic features that enable it to survive adequately in a wide spectrum of environments, such as : streams, ponds, lakes, rivers, oceans, ice, water-borne pipes, hot-springs, gastro-intestinal tract (GIT), roots of plants, and even in oil wells. In general, the microorganisms are usually characterized by very typical and extremely high degree of adaptability. Microbes are invariably distributed over the entire biosphere*, lithosphere, hydrosphere, and above all the atmosphere.

One may also define microbiology as — 'the study of living organisms of microscopic size, that include essentially bacteria, fungi, algae, protozoa and the infectious agents at the very borderline of life which are broadly known as viruses. It is mainly concerned with a variety of vital and important aspects, such as : typical form, inherent structure, reproduction, physiological characteristics, metabolic pathways (viz., anabolism, and catabolism), and logical classification. Besides, it includes the study of their :

• Distribution in nature,

• Relationship to each other and to other living organisms,

• Specific effects on humans, plants, and animals, and

• Reactions to various physical and chemical agents.

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

Bacteriology : the study of organism (bacteria), **Mycology :** the study of fungi, **Phycology :** the study of algae, **Protozoology :** the study of protozoans, and **Virology :** the study of viruses.

Advantages : The advantageous fields of microbiology are essentially the ones enumerated below :

1. Aero-Microbiology — helps in the overall preservation and preparation of food, food-prone diseases, and their ultimate prevention.

2. Beverage Microbiology — making of beer, shandy, wine, and a variety of alcoholic beverages e.g., whisky, brandy, rum, gin, vodka. etc.

3. Exomicrobiology — to help in the exploration of life in the outerspace.

4. Food Microbiology — making of cheese, yogurt.

5. Geochemical Microbiology — to help in the study of coal, mineral deposits, and gas formation ; prospecting the deposits of gas and oil, coal, recovery of minerals from low-grade ores.

6. Industrial Microbiology — making of ethanol, acetic acid, lactic acid, citric acid, glucose syrup, high-fructose syrup.

7. Medical Microbiology — helps in the diagnostic protocol for identification of causative agents of various human ailments, and subsequent preventive measures.

8. Pharmaceutical Microbiology — making of life-saving drugs, 'antibiotics' e.g., penicillins, ampicillin, chloramphenicol, ciprofloxacin, tetracyclines, streptomycin.

9. Soil and Agricultural Microbiology — helps in the maintenance of a good farm land by keeping and sustaining a reasonable and regular presence of microbes in it.

10. Waste-Treatment Microbiology — treatment of domestic and industrial effluents or wastes by lowering the BOD*, and COD**.

Disadvantages : The apparently disadvantageous and detrimental manner whereby the microorganisms may exhibit their effects are, namely : disease-producing organisms viz., typhus fever caused by Rickettsia prowazekii, malaria caused by Plasmodium falciparum ; food-spoilage microbes ; and a host of organisms that essentially deteriorate materials like optical lenses (in microscopes and spectrophotometers), iron-pipes, and wood filing

history of microbiology

Discovering the "organisms".

1. 1676: A. Leeuwenhoek – first to observe and describe microbes accurately

2. 1884: C. Chamberland – constructed a bacterial filter that allowed the identification of viruses

3. 1898: Loeffler and Frosch – identified filterable infectious agent as cause of foot-and-mouth disease in cattle

4. 1898-1900: M. Beijerinck – identified tobacco mosaic virus

5. 1982: S. Prusiner – described prions (infectious protein that causes a particular normal protein to alter its shape and become a prion)

B. Disproving spontaneous generation (that living organisms could develop from nonliving matter)

1. 1688: F. Redi – first to challenge theory of spontaneous generation by showing that if raw meat was protected from flies, the formation of maggots was prevented

2. 1748: R. Needham – supported spontaneous generation of microbes by showing that even after boiling mutton broth and pouring into sealed containers, growth of microbes occurred

3. 1776: L. Spallanzani - challenged spontaneous generation as it pertained to microbes by showing that sealed containers that were boiled do not produce microbes

4. 1861: L. Pasteur – rigorously disproved spontaneous generation a) filtered air ‡ showed that air contained microbial organisms b) constructed flasks with curved neck that allowed air into the flasks while dust, etc. remained in the neck ‡ placed broth into the flasks and boiled ‡ showed that no microbial growth resulted unless flasks were tipped to allow the broth into the neck

C. The germ theory of disease

1. Previously, people thought that disease was punishment for an individual's crimes, due to poisonous vapors, and/or an imbalance of the "four humors".

2. First proponents of the idea that invisible organisms caused disease were Lucretius (B.C.) and Fracastoro (1546)

3. 1835: A. Bassi showed that silkworm disease was due to a fungus. 3

4. 1867: J. Lister showed that antiseptic surgical procedures reduced the frequency of wound infections.

5. 1876/1884: R. Koch definitively proved that Bacillus anthracis caused the disease anthrax in cows and Mycobacterium tuberculosis caused the disease tuberculosis using Koch's postulates.

a) The suspected pathogen should be present in ALL cases of the disease and NOT present in healthy animals.

b) The suspected pathogen should be grown in vitro in pure culture.

c) Cells from a pure culture of the putative pathogen should cause disease in healthy animals. d) The putative pathogen should be reisolated from the infected animal.

D. Preventing disease by vaccination

1. E. Jenner inoculated people with cowpox to protect against smallpox.

2. 1885 – Pasteur developed the rabies vaccine.

3. 1890: von Behring and Kitasato produced antibodies to purified toxins to protect against diphtheria and tetanus.

4. 1884: E. Metchnikoff described phagocytosis of bacteria.

E. Discovering the effect of microbes on organic and inorganic matter

1. 1856: Pasteur described lactic acid fermentation; contributions to wine industry.

2. 1887-1900: S. Winogradsky and M. Beijerinck studied soil microbes and their role in the biochemical cycles of sulfur, carbon, nitrogen V. Recent history of microbiology – the 20th century

A. Infectious diseases: The etiological agent of most infectious diseases has been ascertained. Current research focuses on understanding the molecular mechanisms by which disease is caused.
B. Chemotherapy: Discovery of antibiotics; antibiotic resistance

C. Immunology develops as a science.

D. Physiology and biochemistry: Using microbes as a model, many physiological and biochemical processes have been elucidated.

E. Genetics: Many of the advances in molecular genetics were made using bacteria as models. A few of the many:

1. 1941: Beadle and Tatum – 1 gene = 1 enzyme

2. 1943: Luria and Delbruck – mutations are spontaneous in nature

3. 1944: Avery, MacLeod, and McCarty – DNA is the genetic material

4. 1961: Jacob and Monod – the operon and gene regulation

F. Molecular biology: Many of the advances in molecular biology were made using bacteria as models. A few of the many:

1. 1970: Restriction enzymes discovered

2. 1979: Insulin synthesized using recombinant techniques

3. 1990: Gene therapy trials begin

4. 1995: The nucleotide sequence of the first free-living organism

Branch and scope of microbiology

By Taxonomy

- **Bacteriology**: the study of bacteria.
- **Immunology**: the study of the immune system. It looks at the relationships between pathogens such as bacteria and viruses and their hosts.
- **Mycology**: the study of fungi, such as yeasts and molds.
- **Nematology**: the study of nematodes (roundworms).
- **Parasitology**: 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**: the study of algae.
- **Protozoology**: the study of protozoa, single-celled organisms like amoebae.
- **Virology**: the study of viruses.

By Type of Research

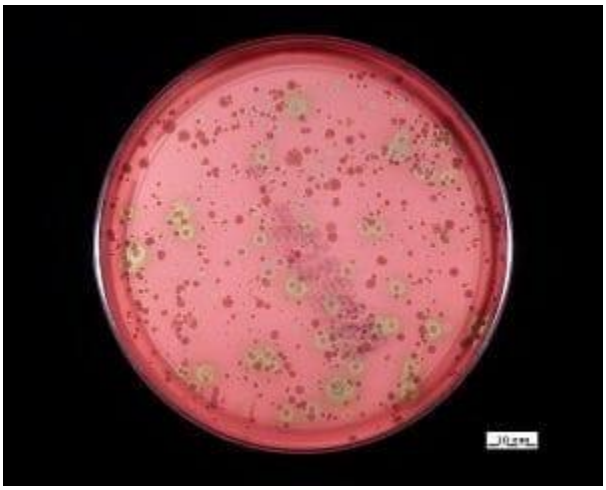
Microbiology research, like other fields of scientific research, can be subdivided into the categories of pure and applied. Pure (or basic) research is exploratory and conducted in order to better understand a scientific phenomenon, while applied research is based on information gleaned from pure research and used to answer specific questions or solve problems.

Pure microbiology research includes:

- **Astromicrobiology**: the study of the origin of life on Earth, and the search for extraterrestrial life.
- **Evolutionary microbiology**: the evolution of microorganisms.
- **Cellular microbiology**: the study of the structure and function of microbial cells.
- **Microbial ecology**
- **Microbial genetics**
- **Microbial physiology**
- **Systems microbiology**: mathematical/computational modeling of the activities of microbiological systems.

While applied microbiology research includes:

- **Agricultural microbiology**: the study of microorganisms that interact with plants and soils.
- **Food microbiology**: the study of microorganisms that spoil food or cause foodborne illnesses. Can also study how microorganisms are used in food production, such as fermentation of beer.
- **Medical microbiology**: the study of microorganisms responsible for human disease.
- **Microbial biotechnology**: using microbes in industrial or consumer products.
- **Pharmaceutical microbiology**: the study of microorganisms used in pharmaceutical products, such as vaccines and antibiotics.



This is an image of bacteria colonies growing on an agar plate.

Scope of microbiology

Most jobs in microbiology require at least a bachelor's degree. An individual who is interested in microbiology may obtain a bachelor's degree in biology or microbiology. The courseload is very similar for each of these majors; while a microbiology major may be more specific to the interests of someone who wants to study microbiology, it is also possible to achieve a similar level of specificity in the biology major by taking upper-level microbiology courses. The biology major may be preferred if one has interests in other subfields of biology, or if he or she is double majoring in biology and in another field. In both the microbiology and biology majors, students must take numerous biology courses and laboratories, and usually they must also take courses in chemistry (including organic), physics, mathematics, and statistics.

With a bachelor's degree, one can become employed as a research technician in an academic or industry laboratory and provide technical support. One could also become a quality assurance technician in the food, environmental, pharmaceutical, or biotechnology industries, or with some additional training, become a medical technologist. However, many individuals with bachelor's degrees in microbiology or biology go on to do further schooling. With a master's degree in microbiology, an individual may go on to become a laboratory manager/coordinator or a biosafety officer. Further schooling leading to a PhD opens up opportunities in teaching

and doing research at a university. Being a professor requires a PhD. Most heads of research laboratories in industry have PhDs as well. Other high-level careers involving microbiology include becoming a consultant/adviser, administrator, or lab director.

Introduction to Prokaryotes and Eukaryotes:

There are two fundamentally different types of cells. These are:

- The prokaryotic cell
- The eukaryotic cell

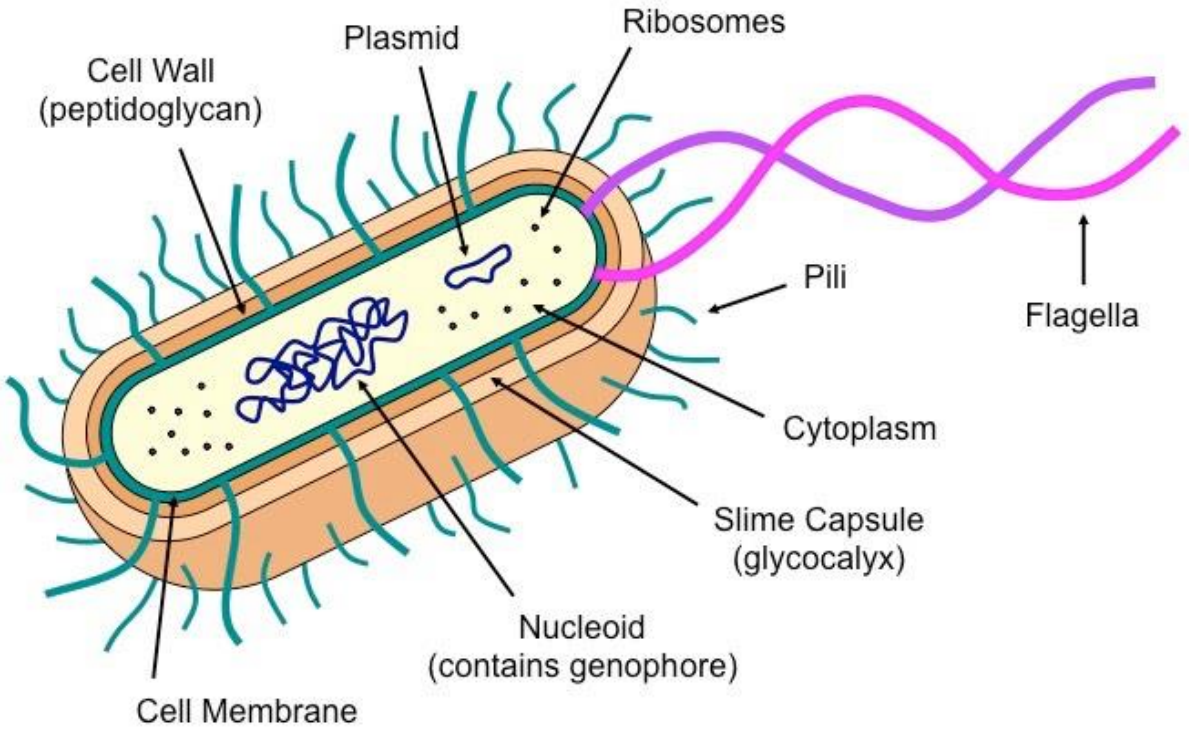
Organisms whose cells have a nucleus are called eucaryotes (from the Greek words *eu*, meaning —well‖ or —truly,‖ and *karyon*, a —kernel‖ or —nucleus‖). Organisms whose cells do not have a nucleus are called procaryotes (from *pro*, meaning —before‖).

Prokaryotes

Prokaryotes are organisms made up of cells that lack a cell nucleus or any membrane encased organelles. This means the genetic material DNA in prokaryotes is not bound within a nucleus. Additionally, the DNA is less structured in prokaryotes than in eukaryotes. In prokaryotes, DNA is a single loop. In Eukaryotes, DNA is organized into chromosomes. Most prokaryotes are made up of just a single cell (unicellular) but there are a few that are made of collections of cells (multicellular). Scientists have divided the prokaryotes into two groups, the Bacteria and the Archaea.

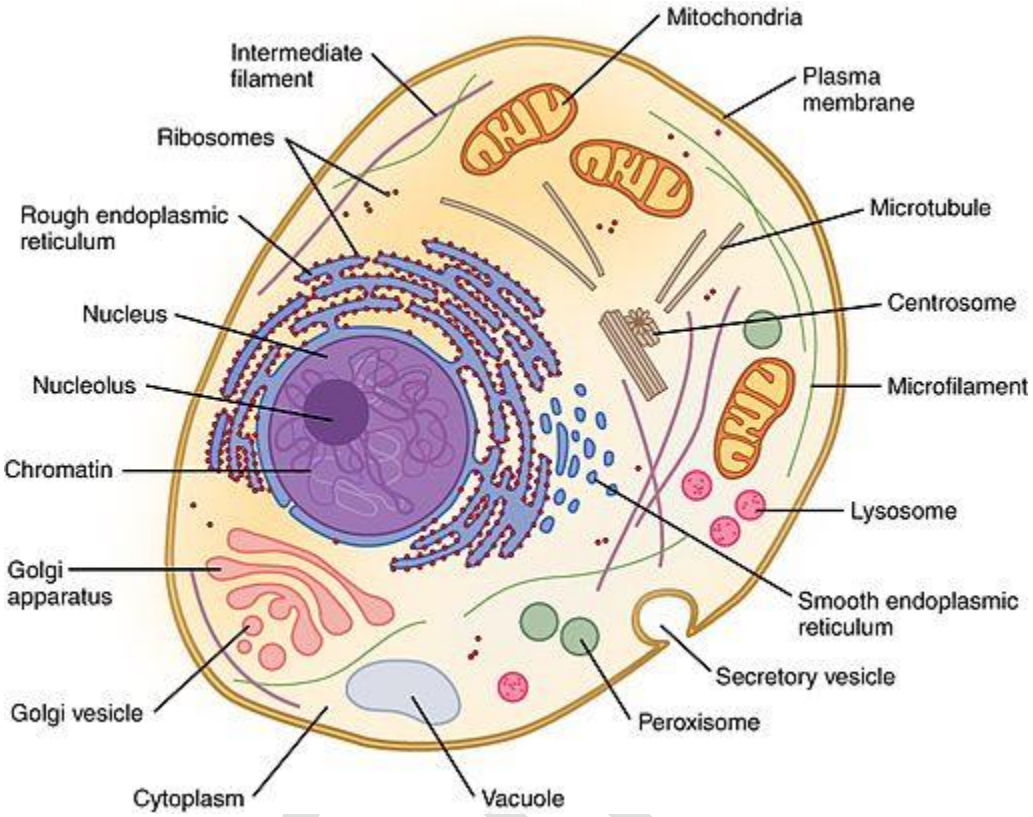
Procaryotes are typically spherical, rod like, or corkscrew-shaped, and small—just a few micrometers long, although there are some giant species as much as 100 times longer than this. They often have a tough protective coat, called a cell wall, surrounding the plasma membrane, which encloses a single compartment containing the cytoplasm and the DNA.

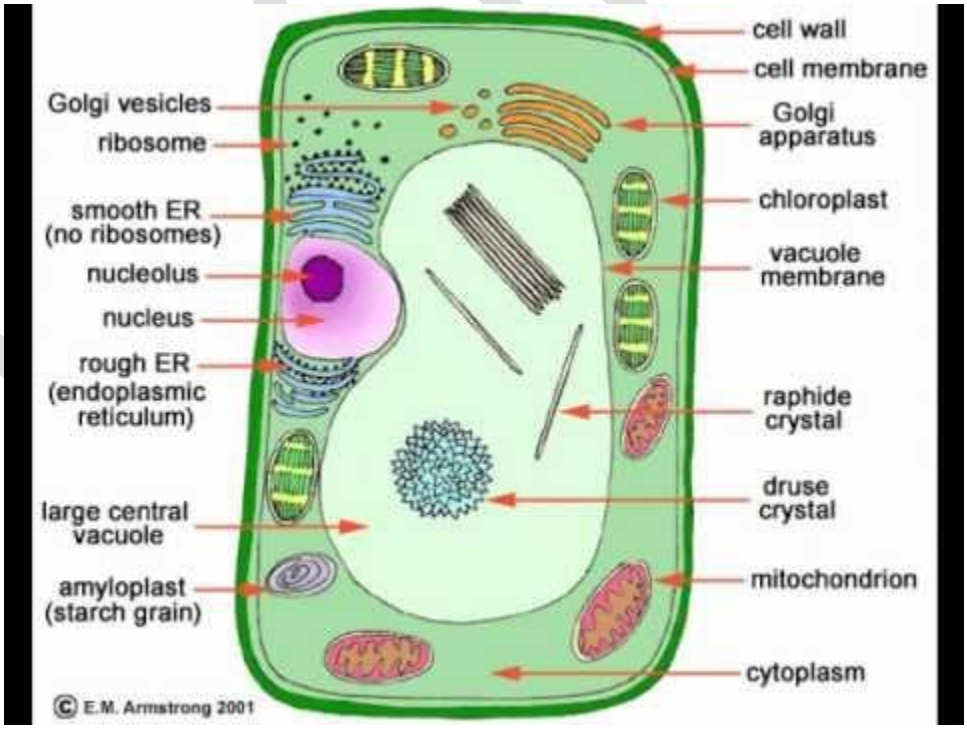
Spherical cells, e.g., *Streptococcus*	Rod-shaped cells, e.g.,*Escherichia coli, Salmonella*	Spiral cells, e.g., *Treponema pallidum*



Eukaryotes

Eukaryotes are organisms made up of cells that possess a membrane-bound nucleus as well as membrane-bound organelles. Genetic material in eukaryotes is contained within a nucleus within the cell and DNA is organized into chromosomes. Eukaryotic organisms may be multicellular or single-celled organisms. Eukaryotic cell membrane contains sterols, whereas no prokaryotes except the wall of *Mycoplasma,* has sterol in its membrane. All animals are eukaryotes. Other eukaryotes include plants, fungi, and protists.
Eukaryotic cells, in general, are bigger and more elaborate than bacteria and archaea. Some live independent lives as single-celled organisms, such as amoebae and yeasts; others live in multicellular assemblies. All of the more complex multicellular organisms—including plants, animals, and fungi—are formed from eukaryotic cells.


Mitochondria
Intermediate filament
Plasma membrane
Ribosomes
Microtubule
Rough endoplasmic reticulum
Centrosome
Nucleus
Microfilament
Nucleolus
Chromatin
Lysosome
Golgi apparatus
Smooth endoplasmic reticulum
Secretory vesicle
Golgi vesicle
Peroxisome
Cytoplasm
Vacuole


cell wall
cell membrane
Golgi vesicles
Golgi apparatus
ribosome
chloroplast
smooth ER (no ribosomes)
vacuole membrane
nucleolus
nucleus
rough ER (endoplasmic reticulum)
raphide crystal
druse crystal
large central vacuole
mitochondrion
amyloplast (starch grain)
cytoplasm
© E.M. Armstrong 2001

	Eukaryotic cell	Prokaryotic cell
Nucleus	Present	Absent
Number of chromosomes	More than One	One but not true chromosomes: plasmids
Cell type	Multicellular	Unicellular
True membrane bound molecules	Present	Absent
Examples:	Animals and plants	Bacteria and archaea
Lysosomes and Peroxisomes	Present	Absent
Microtubules	Present	Absent or rare
Endoplasmic reticulam	Present	Absent
Mitochondria	Present	Absent
Ribosomes	Larger 80S	Smaller 70S
Vesicles	Present	Present
Golgi Apparatus	Present	Absent
Chloroplasts	Present (in plants)	Absent
Plasma membrane with steroid	Yes	Usually no
Permeability of nuclear membrane	Selective	Not present
Vacuoles	Present	Absent
Cell Size	1-1000Um	1-10um
Flagella	Microscopic in size; membrane bound; usually arranged as nine doublets surrounding two singlets	Submicroscopic in size, composed of only one fiber

Nutritional requirement of bacteria

Every organism must find in its environment all of the substances required for energy generation and cellular biosynthesis. The chemicals and elements of this environment that are utilized for bacterial growth are referred to as **nutrients** or **nutritional requirements**. Many bacteria can be grown the laboratory in **culture media** which are designed to provide all the essential nutrients in solution for bacterial growth. Bacteria that are symbionts or obligate intracellular parasites of other cells, usually eucaryotic cells, are (not unexpectedly) difficult to grow outside of their natural host cells. Whether the microbe is a mutualist or parasite, the host cell must ultimately provide the nutritional requirements of its resident.

Many bacteria can be identified in the environment by inspection or using genetic techniques, but attempts to isolate and grow them in artificial culture has been unsuccessful. This, in part, is the basis of the estimate that we may know less than one percent of all procaryotes that exist.

The Major Elements

At an elementary level, the nutritional requirements of a bacterium such as *E. coli* are revealed by the cell's elemental composition, which consists of C, H, O, N, S. P, K, Mg, Fe, Ca, Mn, and traces of Zn, Co, Cu, and Mo. These elements are found in the form of water, inorganic ions, small molecules, and macromolecules which serve either a structural or functional role in the cells. The general physiological functions of the elements are outlined in Table 1.

Table 1. Major elements, their sources and functions in bacterial cells.

Element	% of dry weight	Source	Function
Carbon	50	organic compounds or CO_2	Main constituent of cellular material
Oxygen	20	H_2O, organic compounds, CO_2, and O_2	Constituent of cell material and cell water; O_2 is electron acceptor in aerobic respiration
Nitrogen	14	NH_3, NO_3, organic compounds, N_2	Constituent of amino acids, nucleic acids nucleotides, and coenzymes
Hydrogen	8	H_2O, organic compounds, H_2	Main constituent of organic compounds and cell water
Phosphorus	3	inorganic phosphates (PO_4)	Constituent of nucleic acids, nucleotides, phospholipids, LPS, teichoic acids
Sulfur	1	SO_4, H_2S, S^o,	Constituent of

		organic sulfur compounds	cysteine, methionine, glutathione, several coenzymes
Potassium	1	Potassium salts	Main cellular inorganic cation and cofactor for certain enzymes
Magnesium	0.5	Magnesium salts	Inorganic cellular cation, cofactor for certain enzymatic reactions
Calcium	0.5	Calcium salts	Inorganic cellular cation, cofactor for certain enzymes and a component of endospores
Iron	0.2	Iron salts	Component of cytochromes and certain nonheme iron-proteins and a cofactor for some enzymatic reactions

Trace Elements

Table 1 ignores the occurrence of trace elements in bacterial nutrition. **Trace elements** are metal ions required by certain cells in such small amounts that it is difficult to detect (measure) them, and it is not necessary to add them to culture media as nutrients. Trace elements are required in such small amounts that they are present as "contaminants" of the water or other media components. As metal ions, the trace elements usually act as cofactors for essential enzymatic reactions in the cell. One organism's trace element may be another's required element and vice-versa, but the usual cations that qualify as trace elements in bacterial nutrition are Mn, Co, Zn, Cu, and Mo.

Carbon and Energy Sources for Bacterial Growth

In order to grow in nature or in the laboratory, a bacterium must have an energy source, a source of carbon and other required nutrients, and a permissive range of physical conditions such as O_2 concentration, temperature, and pH. Sometimes bacteria are referred to as individuals or groups based on their patterns of growth under various chemical (nutritional) 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 a source of energy. Organisms that use radiant energy (light) are called **phototrophs**. Organisms that use (oxidize) an organic form of carbon are called **heterotrophs** or **(chemo)heterotrophs**. Organisms that oxidize inorganic compounds are called **lithotrophs**.

The carbon requirements of organisms must be met by organic carbon (a chemical compound with a carbon-hydrogen bond) or by CO_2. Organisms that use organic carbon are **heterotrophs** and organisms that use CO_2 as a sole source of carbon for growth are called **autotrophs**.

Thus, on the basis of carbon and energy sources for growth four major nutritional types of procaryotes may be defined (Table 2).

Table 2. Major nutritional types of procaryotes

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 (Lithoautotrophs)	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

Almost all eucaryotes are either photoautotrophic (e.g. plants and algae) or heterotrophic (e.g. animals, protozoa, fungi). Lithotrophy is unique to procaryotes and photoheterotrophy, common in the Purple and Green Bacteria, occurs only in a very few eucaryotic algae. Phototrophy has not been found in the Archaea, except for nonphotosynthetic light-driven ATP synthesis in the extreme halophiles.

FORMULATION OF CULTURE MEDIA

DEVELOPMENT AND MANUFACTURE

The formulation of all Oxoid culture media and the components can be divided into different roles or functions:

1 Nutrients: proteins/peptides/amino-acids.

2 Energy: carbohydrates.

3 Essential metals and minerals: calcium, magnesium, iron, trace metals: phosphates, sulphates etc.

4 Buffering agents: phosphates, acetates etc.

5 Indicators for pH change: phenol red, bromo-cresol purple etc.

6 Selective agents: chemicals, antimicrobial agents.

7 Gelling agent: usually agar.

There is often an overlap of functions of some media components, thus protein hydrolysates will supply amino-nitrogen, energy, some metals/minerals and act as buffering agents. Phosphate buffers are important suppliers of minerals and agar contributes metals.

1 Nutrients
Naegeli is credited with the earliest publications (1880/82) describing the requirements of micro-organisms for a protein component which he called `peptone'.

Later work showed that the group of bacteria, now defined as chemo-organotrophs, required amino-nitrogen compounds as essential growth factors in their culture media.

Meat infusions contain water-soluble fractions of protein (amino-acids and small peptides) along with other water-soluble products such as vitamins, trace metals, minerals and carbohydrates (glycogen). Such infusions or extracts may have been regarded as `peptones' but their amino-nitrogen content was usually too low to sustain the growth of large numbers of bacteria.

It was not until deliberate attempts were made to hydrolyse proteins with acids or enzymes that sufficiently high concentrations of water-soluble protein fractions (peptides) were made available for bacterial growth. Many nutrient media usually contain a mixture of protein hydrolysate (peptone) and meat infusion (meat extract/Lab-Lemco).

The difficulties associated with the production of protein hydrolysates were soon recognised and commercial suppliers of peptones became established by the 1920s. The commercial supply of dried peptone eventually led to complete culture media being produced in the form of dehydrated media.

Although meat was the first and most obvious protein to hydrolyse, other proteins were tried later and some showed specific advantages which ensured their retention in culture media to this day. Casein hydrolysate with its pale colour and high tryptophan content and soya peptone with its high energy carbohydrate content are popular examples of non-meat peptones.

A detailed description of these products is given in "Peptones-Hydrolysates" section.

The nutrient components of culture media are carefully selected to recover the required spectrum of organisms in the sample e.g. coliforms or anaerobes. General purpose media such as blood agar in its various forms will often contain mixtures of peptones to ensure that peptides of sufficient variety are available for the great majority of organisms likely to be present. However, more demanding organisms will require supplemental growth factors to be added and examples of such requirements can be seen in media for *Legionella* species.

Most of the components used for the nutrition of micro-organisms are undefined and require extensive testing with careful selection to ensure a reasonable degree of uniformity. Would it not be better to use wholly defined peptides and amino-acids to produce a totally defined medium? Whilst such media would improve uniformity, experience has shown that they lack good performance as general purpose media. They would also be very expensive compared with undefined media. The use of totally defined culture media is an understandable goal of most microbiologists but defined media have yet to prove themselves equal in performance to currently used complex mixtures of meat and plant protein hydrolysates.

2 Energy

The most common substance added to culture media as a source of energy to increase the rate of growth of organisms is glucose. Other carbohydrates may be used as required.

Carbohydrates added to media at 5-10 grammes per litre are usually present as biochemical substrates to detect the production of specific enzymes in the identification of organisms. It is usual to add pH indicators to such formulations.

3 Essential Metals and Minerals

The inorganic essential components of culture media are many and can be divided on a semi-quantitative basis:
Typical macro-components (gm/litre): Na, K, Cl , P, S, Ca, Mg, Fe.
Typical micro-components (mgm-microgm/litre): Zn, Mn, Br, B, Cu, Co, Mo, V, Sr, etc.
As previously mentioned, a formulation may not have specific metals and minerals listed in its formulation. In such cases it is assumed that all the factors required are present in the hydrolysates, buffers and agar components.

4 Buffering Agents

It is important that 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.

Phosphates, acetates, citrates, zwitterion compounds and specific amino-acids are examples of buffering agents that may be added to culture media.

A side effect of such compounds is their ability to chelate (or bind) divalent cations (Ca ++ and Mg ++). Polyphosphate salts, sometimes present in sodium phosphate, are compounds which can bind essential cations so firmly that they are made inaccessible to the micro-organisms.

The effect of these binding or chelating agents will be seen in diminished growth or failure to grow at all, unless care has been taken to supplement the essential cations in the formulation. Opacity forming in a medium, after heating or on standing at 50°C for several hours, is commonly caused by phosphate interaction with metals. Such phosphate precipitates can very effectively bind Fe and lower the available amount of this essential metal in the medium.

5 Indicator Substances

The addition of coloured indicator substances is a very effective way of detecting fermentation of specific carbohydrates in a culture medium. Such compounds should change colour distinctly and rapidly at critical pH values.

Most of the compounds used e.g. phenol red, bromo-cresol purple, fuchsin, etc., are toxic and it is essential to use low concentrations of pre-screened batches/lots. Known sensitive strains of micro-organisms are used in the screening tests.

6 Selective Agents

Chemicals or antimicrobials are added to culture media to make them selective for certain micro-organisms. The selective agents are chosen and added at specific concentrations to suppress the growth of unwanted organisms in a polymicrobial sample. It is, of course, essential to have established that the selective agents, at the appropriate concentration, will allow uninhibited growth of the desired organisms.

Common chemical selective agents are: bile salts, dye-stuffs, selenite, tetrathionate, tellurite and azide. Antimicrobial agents are commonly used in mixtures when suppressing polymicrobial contaminating flora. Antimicrobials are more specific in their selective action than the chemical agents shown above. However, the critical weighing and heat-lability of most antimicrobials demand special care and post-sterilisation addition.

The wide variety of organisms and their almost infinite ability to adapt to changing conditions makes a truly selective medium unlikely. Selective media can be said to suppress most of the unwanted organisms and allow most of the desired organisms to grow. The final formulation is usually a compromise which achieves the best of these criteria.

7 Gelling Agents

Although gelatin is still used for a few specific media and carrageenans, alginates, silica gel and polyacrylamides are sometimes used as gelling agents, the outstanding gel-forming substance used in culture media is agar.

Hesse, a worker in Robert Koch's laboratory, is credited with its first use in culture media, although Frau Hesse gave him the idea from its use in table-jellies in hot climates.

Its inertness to microbial action, the unique setting and melting temperatures (38°C and 84°C respectively) the high gel strength which allows low concentrations of agar to be used, its clarity and low toxicity have contributed to its wide popularity with microbiologists. Its ability to retain its gel structure at 60°C makes agar of special value to culture media which have to be incubated at this temperature to isolate thermophilic organisms.

Agar is obtained from agarophyte sea-weeds mainly *Gelidium, Gracilaria* and *Pterocladia* species. It is extracted as an aqueous solution at greater than 100°C, decolourised, filtered, dried and milled to a powder.

Agar is not an inert gelling agent; it contributes nutrients and/or toxic agents to culture media, depending on the chemical processing carried out by the suppliers.

Microbiological agar is specially processed to yield a low toxicity, high clarity, low mineral and high diffusion gel.

Other Components
There are many other substances added to culture media for specific purposes e.g. growth factors for fastidious organisms, eH-reducing compounds for anaerobic organisms (thioglycollate and cysteine), whole blood to detect haemolytic enzymes and encourage the growth of organisms which are vulnerable to oxidation products.

Physical requirements

a. Temperature

Bacteria have a minimum, optimum, and maximum temperature for growth and can be divided into 3 groups based on their optimum growth temperature:

1. Psychrophiles are cold-loving bacteria. Their optimum growth temperature is between -5C and 15C. They are usually found in the Arctic and Antarctic regions and in streams fed by glaciers.

2. Mesophiles are bacteria that grow best at moderate temperatures. Their optimum growth temperature is between 25C and 45C. Most bacteria are mesophilic and include common soil bacteria and bacteria that live in and on the body.

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

4. Hyperthermophiles are bacteria that grow at very high temperatures. Their optimum growth temperature is between 70C and 110C. They are usually members of the Archaea and are found growing near hydrothermal vents at great depths in the ocean.

b. Oxygen requirements

Bacteria show a great deal of variation in their requirements for gaseous oxygen. Most can be placed in one of the following groups:

1. Obligate aerobes are organisms that grow only in the presence of oxygen. They obtain their energy through aerobic respiration .

2. Microaerophils are organisms that require a low concentration of oxygen (2% to 10%) for growth, but higher concentrations are inhibitory. They obtain their energy through aerobic respiration .

3. Obligate anaerobes are organisms that grow only in the absence of oxygen and, in fact, are often inhibited or killed by its presence. They obtain their energy through anaerobic respiration or fermentation .

4. Aerotolerant anaerobes , like obligate anaerobes, cannot use oxygen to transform energy but can grow in its presence. They obtain energy only by fermentation and are known as obligate fermenters.

5. Facultative anaerobes are organisms that grow with or without oxygen, but generally better with oxygen. They obtain their energy through aerobic respiration if oxygen is present, but use fermentation or anaerobic respiration if it is absent. Most bacteria are facultative anaerobes.

c. pH

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.

d. Osmosis

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). Osmosis is powered by the potential energy of a concentration gradient and does not require the expenditure 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 . The aquaporins form channels that span the cytoplasmic membrane and transport water in and out of the cytoplasm.

To understand osmosis, one must understand what is meant by a solution . A solution consists of a solute dissolved in a solvent . In terms of osmosis, solute refers to all the molecules or ions dissolved in the water (the solvent). When a solute such as sugar dissolves in water, it forms weak hydrogen bonds with water molecules. While free, unbound water molecules are small enough to pass through membrane pores, water molecules bound to solute are not Therefore, the higher the solute concentration, the lower the concentration of free water molecules capable of passing through the membrane.

A cell can find itself in one of three environments: isotonic , hypertonic , or hypotonic .

Growth curve of bacteria

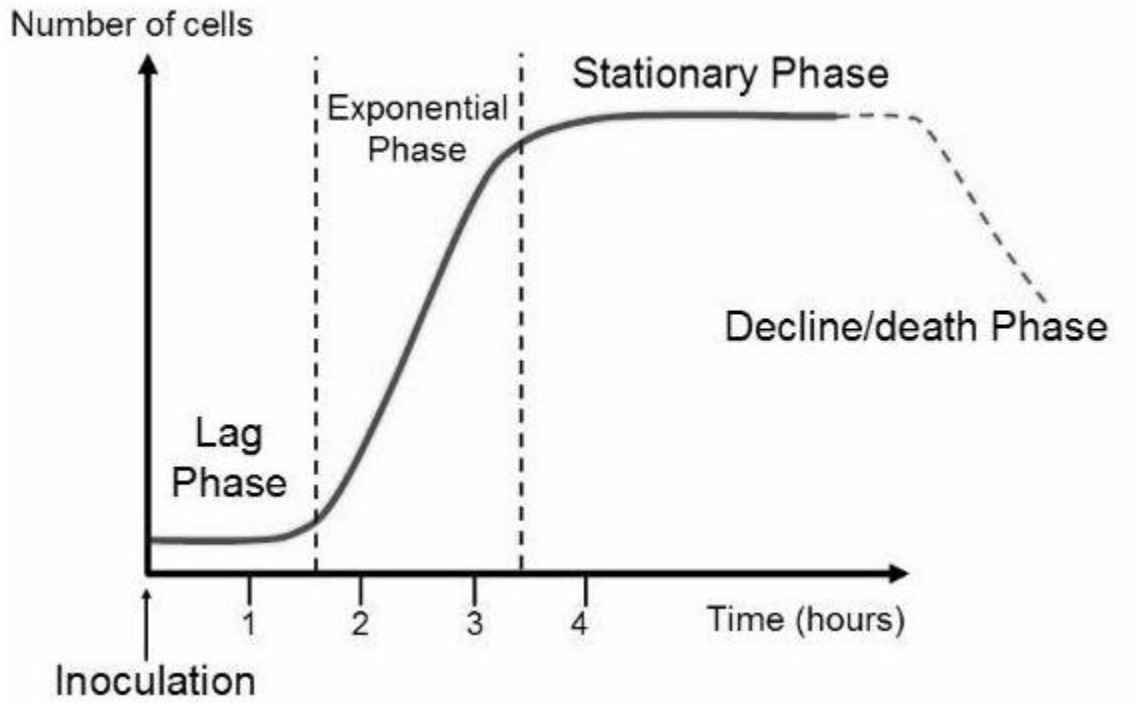
Bacteria are prokaryotic organisms that most commonly replicate by the asexual process of **binary fission**. These microbes reproduce rapidly at an exponential rate under favorable conditions. When grown in culture, a predictable pattern of growth in a bacterial population occurs. This pattern can be graphically represented as the number of living cells in a population over time and is known as a **bacterial growth curve**. Bacterial growth cycles in a growth curve consist of four phases: lag, exponential (log), stationary, and death.

A bacterial population's **generation time**, or time it takes for a population to double, varies between species and depends on how well growth requirements are met.

Phases of the Bacterial Growth Cycle

In nature, bacteria do not experience perfect environmental conditions for growth. As such, the species that populate an environment change over time. In a laboratory, however, optimal conditions can be met by growing bacteria in a closed culture environment. It is under these conditions that the curve pattern of bacterial growth can be observed.

The **bacterial growth curve** represents the number of live cells in a bacterial population over a period of time.



- **Lag Phase:** This initial phase is characterized by cellular activity but not growth. A small group of cells are placed in a nutrient rich medium that allows them to

synthesize proteins and other molecules necessary for replication. These cells increase in size, but no cell division occurs in the phase.

- **Exponential (Log) Phase:** After the lag phase, bacterial cells enter the exponential or log phase. This is the time when the cells are dividing by binary fission and doubling in numbers after each generation time. Metabolic activity is high as DNA, RNA, cell wall components, and other substances necessary for growth are generated for division. It is in this growth phase that antibiotics and disinfectants are most effective as these substances typically target bacteria cell walls or the protein synthesis processes of DNA transcription and RNA translation.
- **Stationary Phase:** Eventually, the population growth experienced in the log phase begins to decline as the available nutrients become depleted and waste products start to accumulate. Bacterial cell growth reaches a plateau, or stationary phase, where the number of dividing cells equal the number of dying cells. This results in no overall population growth. Under the less favorable conditions, competition for nutrients increases and the cells become less metabolically active. Spore forming bacteria produce endospores in this phase and pathogenic bacteria begin to generate substances (virulence factors) that help them survive harsh conditions and consequently cause disease.
- **Death Phase:** As nutrients become less available and waste products increase, the number of dying cells continues to rise. In the death phase, the number of living cells decreases exponentially and population growth experiences a sharp decline. As dying cells lyse or break open, they spill their contents into the environment making these nutrients available to other bacteria. This helps spore producing bacteria to survive long enough for spore production. Spores are able to survive the harsh conditions of the death phase and become growing bacteria when placed in an environment that supports life.

Obtaining Pure Culture of Microorganisms: 4 Methods

1. Streak Plate Method:

This method is used most commonly to isolate pure cultures of bacteria. A small amount of mixed culture is placed on the tip of an inoculation loop/needle and is streaked across the surface of the agar medium The successive streaks “thin out” the inoculum sufficiently and the micro-organisms are separated from each other.

It is usually advisable to streak out a second plate by the same loop/needle without reinoculation. These plates are incubated to allow the growth of colonies. The key principle of this method is that, by streaking, a dilution gradient is established across the face of the Petri plate as bacterial cells are deposited on the agar surface.

Because of this dilution gradient, confluent growth does not take place on that part of the medium where few bacterial cells are deposited. Presumably, each colony is the progeny of a single microbial cell thus representing a clone of pure culture. Such isolated colonies are picked up separately using sterile inoculating loop/needle and re-streaked onto fresh media to ensure purity.

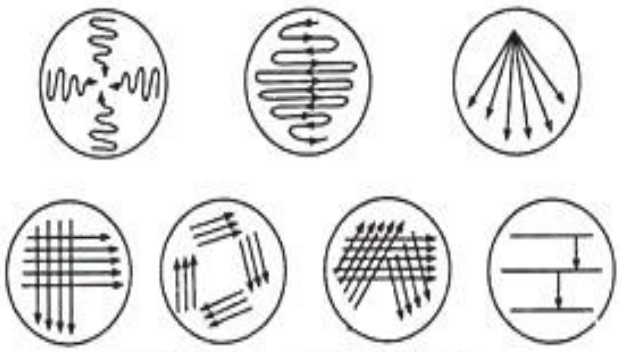


FIG. 16.13. Various methods of streaking.

2. Pour Plate Method:

This method involves plating of diluted samples mixed with melted agar medium The main principle is to dilute the inoculum in successive tubes containing liquefied agar medium so as to permit a thorough distribution of bacterial cells within the medium.

Here, the mixed culture of bacteria is diluted directly in tubes containing melted agar medium maintained in the liquid state at a temperature of 42-45°C (agar solidifies below 42°C). The bacteria and the melted medium are mixed well.

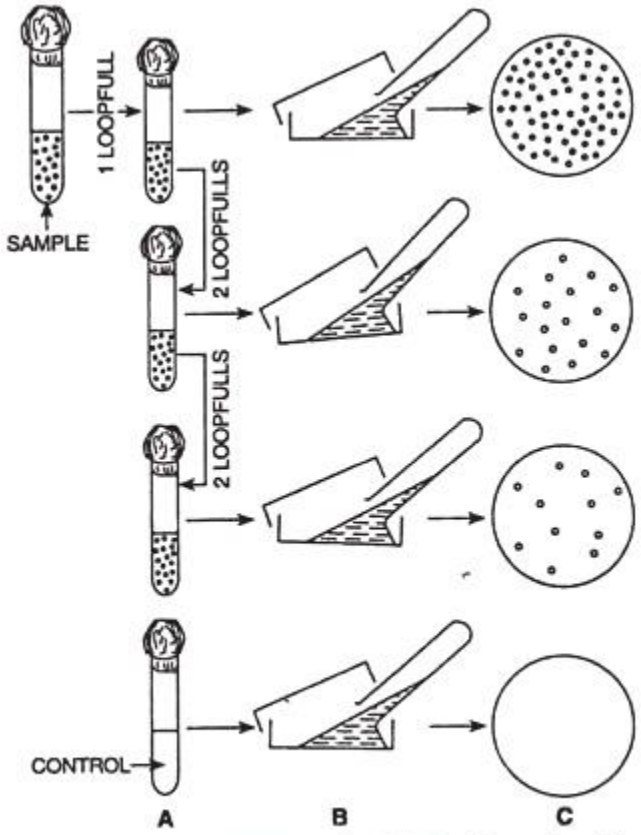


FIG. 16.14. Pour plate method. **A.** Media/dilution; **B.** pouring of the plate; and **C.** colony development after incubation. Control consists of the sterilized plating medium alone.

The contents of each tube are poured into separate Petri plates, allowed to solidify, and then incubated. When bacterial colonies develop, one finds that isolated colonies develop both within the agar medium (subsurface colonies) and on the medium (surface colonies). These isolated colonies are then picked up by inoculation loop and streaked onto another Petri plate to insure purity.

Pour plate method has certain disadvantages as follows:

(i) The picking up of subsurface colonies needs digging them out of the agar medium thus interfering with other colonies, and

(ii) The microbes being isolated must be able to withstand temporary exposure to the 42-45° temperature of the liquid agar medium; therefore this technique proves unsuitable for the isolation of psychrophilic microorganisms.

However, the pour plate method, in addition to its use in isolating pure cultures, is also used for determining the number of viable bacterial cells present in a culture.

3. Spread Plate Method:

In this method, the mixed culture or microorganisms is not diluted in the melted agar medium (unlike the pour plate method); it is rather diluted in a series of tubes containing sterile liquid, usually, water or physiological saline.

A drop of so 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 now incubated.

When the colonies develop on the agar medium plates, it is found that there are some plates in which well-isolated colonies grow. This happens as a result of separation of individual microorganisms by spreading over the drop of diluted liquid on the medium of the plate.

The isolated colonies are picked up and transferred onto fresh medium to ensure purity. In contrast to pour plate method, only surface colonies develop in this method and the microorganisms are not required to withstand the temperature of the melted agar medium.

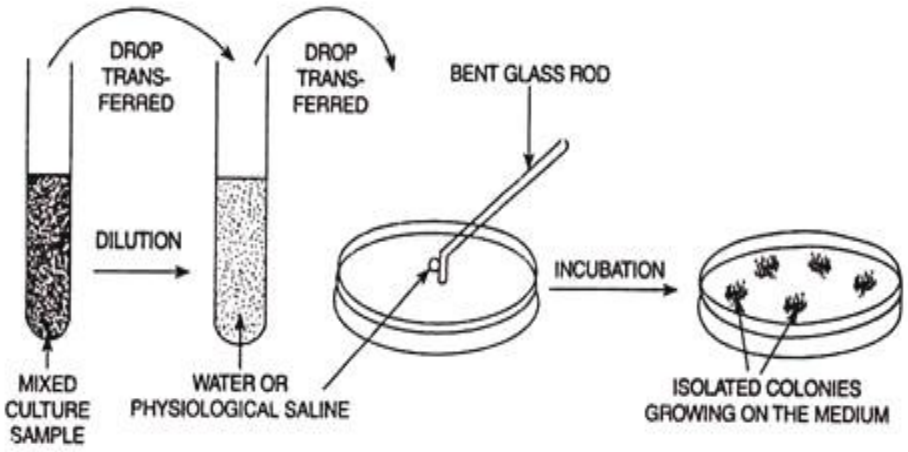


FIG. 16.15. Spread plate method.

4. Serial Dilution Method:

As stated earlier, this method is commonly used to obtain pure cultures of those microorganisms that have not yet been successfully cultivated on solid media and grow only in liquid media.

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. For convenience, suppose we have a culture containing 10 ml of liquid medium, containing 1,000 microorganisms i.e., 100 microorganisms/ml of the liquid medium.

If we take out 1 ml of this medium and mix it with 9 ml of fresh sterile liquid medium, we would then have 100 microorganisms in 10 ml or 10 microorganisms/ml. If we add 1 ml of this suspension to another 9 ml. of fresh sterile liquid medium, each ml would now contain a single microorganism.

The following points highlight the top four methods used for maintenance and preservation of pure cultures.
The methods are:
1. Refrigeration
2. Paraffin Method
3. Cryopreservation
4. Lyophilisation.

Method # 1. Refrigeration:

- Pure cultures can be successfully stored at 0-4°C either in refrigerators or in cold-rooms. This method is applied for short duration (2-3 weeks for bacteria and 3-4 months for fungi) because the metabolic activities of the microorganisms are greatly slowed down but not stopped.

- Thus their growth continues slowly, nutrients are utilized and waste products released in medium. This results in, finally, the death of the microbes after sometime.

Method # 2. 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 upright at room temperature.

- The layer of paraffin ensures anaerobic conditions and prevents dehydration of the medium. This condition helps microorganisms or pure culture to remain in a dormant state and, therefore, the culture is preserved for several years.

Method # 3. Cryopreservation:

- Cryopreservation (i.e., freezing in liquid nitrogen at-196°C) helps survival of pure cultures for long storage times. In this method, the microorganisms of culture are rapidly frozen in liquid nitrogen at -196°C in the presence of stabilizing agents such as glycerol, that prevent the formation of ice crystals and promote cell survival.

Method # 4. Lyophilisation (Freeze-Drying):

- 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.

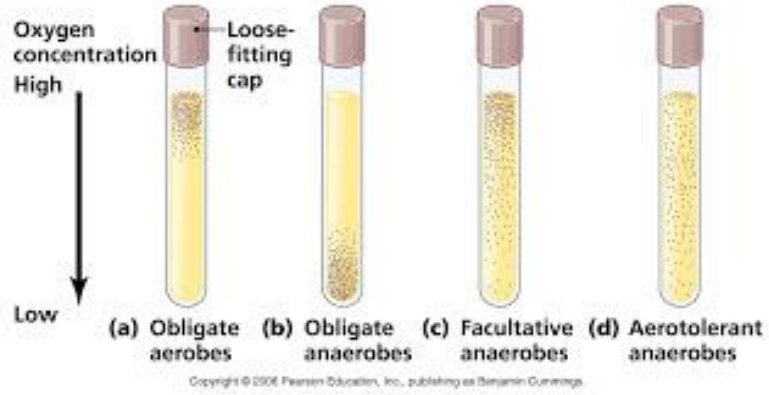
- Lyophilized or freeze-dried pure cultures and then sealed and stored in the dark at 4°C in refrigerators. Freeze- drying method is the most frequently used technique by culture collection centres.

Cultivation of Anaerobic Bacteria

Main 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 needed to culture anaerobic microorganisms. A number of procedure are available for **reducing the O2 content** of cultures; some simple but suitable mainly for less sensitive organisms, others more complex but necessary for growth of strict anaerobes.

- Bottles or tubes filled completely to the top with culture medium and provided with tightly fitting stopper. Suitable for organisms not too sensitive to small amounts of oxygen.
- Addition of a reducing agent that reacts with oxygen and reduces it to water e.g., Thioglycolate in thioglycolate broth. After thioglycolate reacts with oxygen throughout the tube, oxygen can penetrate only near the top of the tube where the medium contacts air.
    - Obligate aerobes grow only at the top of such tubes.
    - Facultative organisms grow throughout the tube but best near the top.
    - Microaerophiles grow near the top but not right at the top.
    - Anaerobes grow only near the bottom of the tube, where oxygen cannot penetrate.



A redox indicator dye called resazurin is added to the medium because the dye changes color in the presence of oxygen and thereby indicates the degree of penetration of oxygen into the medium.

Strict anaerobes, such as methanogenic bacteria can be killed by even a brief exposure to O_2. In these cases, a culture medium is first boiled to render it oxygen free, and then a reducing agent such as H_2S is added and the mixture is sealed under an oxygen- free gas. All manipulations are carried out under a tiny jet of oxygen free hydrogen or nitrogen gas that is directed into the culture vessel when it is open, thus driving out any O_2 that might enter. For extensive research on anaerobes, special boxes fitted with gloves, called anaerobic glove boxes, permit work with open cultures in completely anoxic atmospheres.

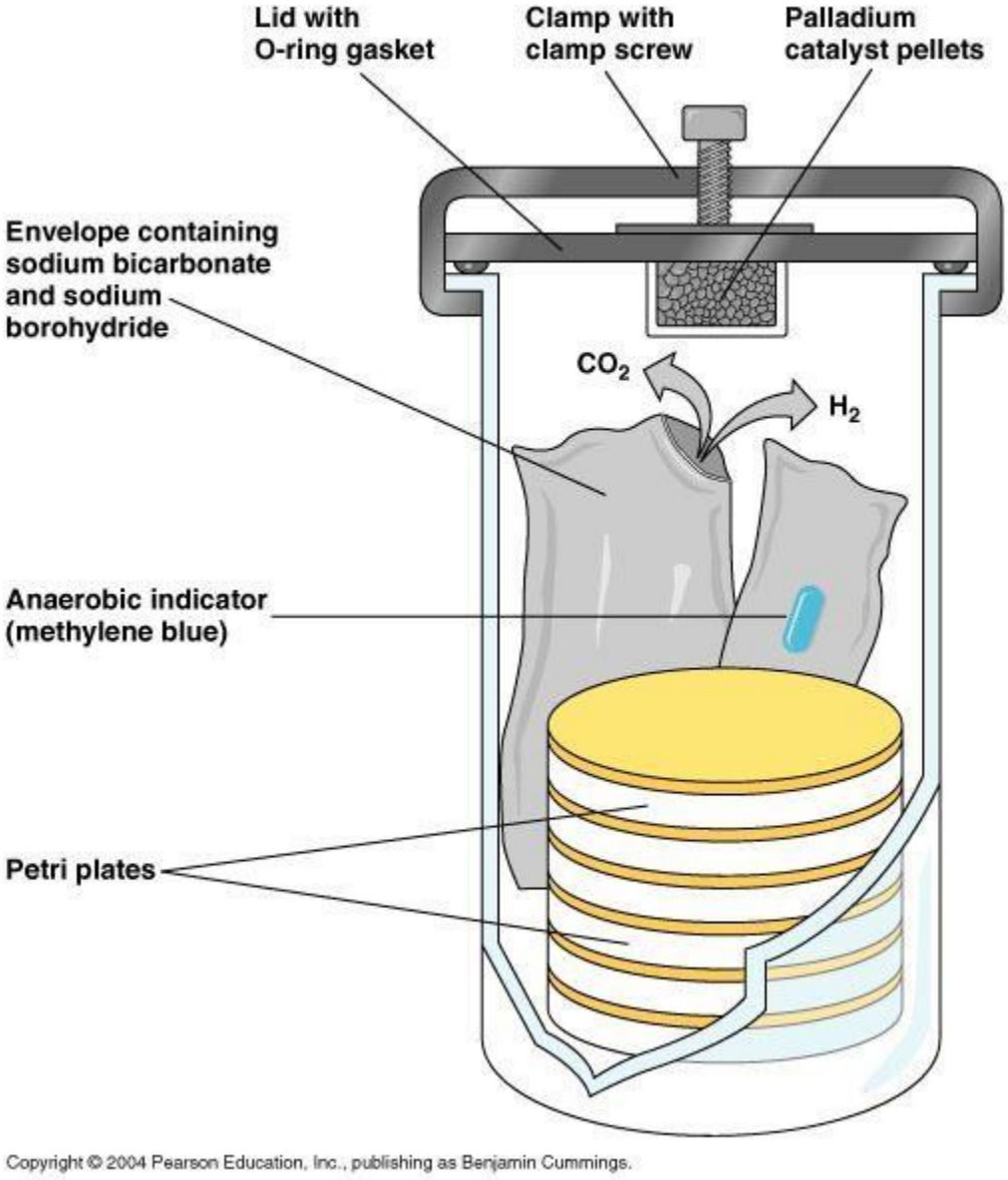
Stringent anaerobes can be grown only by taking special precautions to exclude all atmospheric oxygen from the medium. Such an environment can be established by using one of the following methods:

1. **Pre-reduced media**
   During preparation, the culture medium is **boiled** for several minutes to drive off most of the dissolved oxygen. A reducing agent e.g., cysteine, is added to further lower the oxygen content. Oxygen free N_2 is bubbled through the medium to keep it anaerobic. The medium is then dispensed into tubes which are being flushed with oxygen – free nitrogen, stoppered tightly, and **sterilized by autoclaving**. Such tubes are continuously flushed with oxygen free CO_2 by means of a cannula, restoppered, and incubated.
2. **Anaerobic Chambers**

Anaerobic Chamber

This refers to a plastic anaerobic glove box that contains an atmosphere of H_2, CO_2, and N_2. Culture media are placed within the chamber by means of an air lock which can be evacuated and refilled with N_2. Any oxygen in the media is slowly removed by reaction with hydrogren, forming water; this reaction is aided by a palladium catalyst. After being rendered oxygen free, the media are inoculated within the chamber (by means of the glove ports) and incubated (also within the chamber).

3. **Anaerobic Jar**



Anaerobic Jar: GasPak system

Anaerobic jar is a heavy- walled jar with a gas tight seal within which tubes, plates, or other containers to be incubated are placed along with H_2 and CO_2 generating system (GasPak system) . After the jar is sealed **oxygen** present in the atmosphere inside jar and dissolved in the culture medium, **is gradually used up** through reaction with the hydrogen in the presence of catalyst. The air in the jar is replaced with a mixture of H_2 and CO_2, thus leading to anoxic conditions.

ENUMERATION OF MICROORGANISMS

Direct Count of Cells

Cells are counted directly under the microscope or by an electronic particle counter. Two of the most common procedures used in microbiology are discussed below.

Direct Count Using a Counting Chamber

Direct microscopic counts are performed by spreading a measured volume of sample over a known area of a slide, counting representative microscopic fields, and relating the averages back to the appropriate volume-area factors. Specially constructed counting chambers, such as the Petroff-Hauser and Levy counting chambers, simplify the direct counting procedure because they are made with depressions in which a known volume overlies an area that is ruled into squares. The ability to count a defined area and convert the numbers observed directly to volume makes the direct enumeration procedure relatively easy.

Direct counting procedures are rapid but have the disadvantage that they do not discriminate between living and dead cells. This method is used to assess the sanitation level of a food product and in performing blood cell counts in hematology. The differential white blood cell count, which is used as an indication of the nature of a microbial infection, involves direct counting of blood cells that have been stained to differentiate different types of white blood cells.

Direct Count Using Fluorescent Dyes

Fluorescent dyes are becoming more used in recent years for a variety of procedures, one of which is bacterial counts. These dyes can be employed to stain all species, a particular species of interest in an environmental sample or even a specific component of cells.

The most widely used fluorescent dye for counting the number of bacterial cells is acridine orange which stains both living and dead cells by interacting with DNA and protein components of cells. The stained cells fluoresce orange when excited near ultraviolet light. This stain is particularly useful for determining the total number of microorganisms in samples, such as soil and water, where the co-existence of metabolically diverse populations precludes establishing conditions for the simultaneous enumeration of microbial populations by viable count procedures.

The procedure is widely used in marine microbiology where population levels are often low and where viable plate counts are known to severely underestimate total number of bacteria. Typically, the viable count is less than 1% of the direct count for marine samples. In this procedure the bacteria in a known volume of sample are stained with acridine orange and the sample is then filtered through a 0.22 µm filter. The bacteria are trapped on the filter that is then examined under a fluorescence microscope. The bacteria in a defined area of the filter are counted and the concentration in the original sample is then calculated.

Other fluorescent dyes that are also gaining popularity are cyanoditolyl tetrazolium chloride (CTC), auramine and rhodamine. CTC binds to respiration proteins in the cell and thus can demonstrate live

cells. Auramine and rhodamine bind to cell wall of Mycobacteria and emit bright yellow or orange color under a fluorescent microscope. These latter stains are gradually replacing the acid-fast stain.

2. Indirect Count of Cells

Microorganisms in a sample are diluted or concentrated and grown on a suitable medium; the development of growing microorganisms (for example, colony formation on agar plates) is then used to estimate the numbers of microorganisms in the original sample.

3 Viable Count

The most common procedure for the enumeration of bacteria is the viable plate count. In this method, serial dilutions of a sample containing viable microorganisms are plated onto a suitable growth medium. The suspension is either spread onto the surface of agar plates (spread plate method), or is mixed with molten agar, poured into plates, and allowed to solidify (pour plate method). The plates are then incubated under conditions that permit microbial reproduction so that colonies develop that can be seen without the aid of a microscope.

It is assumed that each bacterial colony arises from an individual cell that has undergone cell division. Therefore, by counting the number of colonies and accounting for the dilution factor, the number of bacteria in the original sample can be determined. There are several drawbacks to the viable count method. The major disadvantage is that it is selective and therefore biased. The nature of the growth conditions, including the composition and pH of the medium used as well as the conditions such as temperature, determines which bacteria in a mixed population can grow.

Since there is no universal set of conditions that permits the growth of all microorganisms, it is impossible to enumerate all microorganisms by viable plating. This same disadvantage, however, becomes advantageous when one is interested in only a specific microbial population. For example, we can design selective procedures for the enumeration of coliforms and other physiologically defined microbial groups.

The viable count is an estimate of the number of cells. Because some organisms exist as pairs or groups and because mixing and shaking of the sample does not always separate all the cells, we actually get a count of the "colony forming units". One cell or group of cells will produce one colony, therefore when we record results for a viable count, it is customary to record the results as colony forming units per ml (cfu/ml) or per gram (cfu/g) of test material. Because we generally have no idea of how many bacteria are in a sample, it is almost always necessary to prepare a dilution series to ensure that we obtain a dilution containing a reasonable number of bacteria to count.

Dilutions in the range 10-1 (1/10) to 10-8 (1/100,000,000) are generally used, although with particular types of samples the range of dilutions can be restricted. For example, for water that is not turbid, the maximal dilution needed is 10-6 because we know that if there were 107 or more bacteria per milliliter, the water would be turbid.

The Most Probable Number (MPN)

The most probable number procedure dates back to the earliest days of microbiology. However, it is still widely used in sanitary bacteriology to estimate numbers of coliforms in water, milk, and other foods. Coliforms are bacteria that reside in the intestine of warm-blooded mammals and are regularly excreted in the feces. They are Gram negative rods belonging to the Enterobacteriaceae family, ferment lactose and produce gas.

Not all members of Enterobacteriaceae are coliforms. The MPN procedure is a statistical method based upon the probability theory. Samples are serially diluted to the point of extinction, that is, to a point where there are no more viable microorganisms. To detect the end point, multiple serial dilutions are inoculated into a suitable growth medium, and the development of some recognizable characteristic, such as acid production or turbidity, is used to indicate growth (the presence of at least one viable microorganism in the diluted sample).

The pattern of positive tests (growth) in the replicates and statistical probability tables are used to determine the concentration (most probable number) of bacteria in the original sample. Statistical MPN tables are available for replicates of 3, 5, and 10 tubes of each dilution. The more replicate tubes used, the greater the precision of the estimate of the size of the bacterial population. In this exercise, we will use a three-tube MPN procedure to estimate the numbers of coliforms in a water sample. As the positive criterion for identifying coliforms, we will use the ability to ferment lactose with the production of acid and gas; acid production will be detected using bromcresol purple as a pH indicator (the change from purple to yellow = acid production) and gas production will be detected using inverted Durham tubes.

3 Direct Measurement of Microbial Biomass

Cell mass is determined directly by weighing whole cells; biomass can be correlated with cell numbers by reference to a standard curve. Wet weight or dry weight of bacteria may be used for estimation of cell numbers.

4Indirect Measurement of Microbial Biomass

Microbial biomass is estimated by measuring relatively constant biochemical components of microbial cells, such as protein, ATP, lipopolysaccharides, peptidoglycan, and chlorophyll; biomass can also be indirectly estimated by measured turbidity that can then be correlated with cell numbers by reference to a standard curve. Various procedures based on the detection of specific microbial macromolecules or metabolic products can be used to estimate numbers of microorganisms.

For example, peptidoglycan can be quantified, and because this biochemical occurs exclusively in the cell wall of bacteria, the concentration of peptidoglycan can be used to estimate bacterial numbers. Such biochemical approaches for determining bacterial numbers depend on the development of analytical chemical procedures for quantifying the particular biochemical and determining what proportion of bacterial cell is composed of the specific biochemical constituent.

III. LABORATORY SUPPLIES

Viable Plate Count

PROCEDURES

(Direct count is done by each student individually; however, the students at a table will form a group to perform the viable count and the MPN.)

First Session Direct Count Using a Counting Chamber

1. Clean a counting chamber with methanol and lens paper and then place it on the microscope stage.

2. Using the 4X objective find the ruled area on one side of the chamber and note the size and arrangements of larger squares and their small square subdivisions.

3. Shake the yeast suspension to distribute the cells evenly. Take out the counting chamber without changing the focus on the 4X objective. Place a coverslip over the calibrated surface of the counting chamber.

4. Using a transfer pipette, transfer some of the yeast suspension to the groove of the counting chamber to fill the chamber by capillary action.

5. Carefully place the counting chamber back onto the microscopic stage and observe the cells under 4X. You may need to reduce the amount of light by closing the diaphragm of the condenser to be able see the cells

6. Switch to the high-dry objective (40 X) and count the number of yeast cells in at least 50 of the small squares. If cells fall on a line, include in your count those on the top and left-hand lines and exclude those on the bottom and right-hand lines. (If the yeast cells are too dense to count, dilute your sample and start again.)

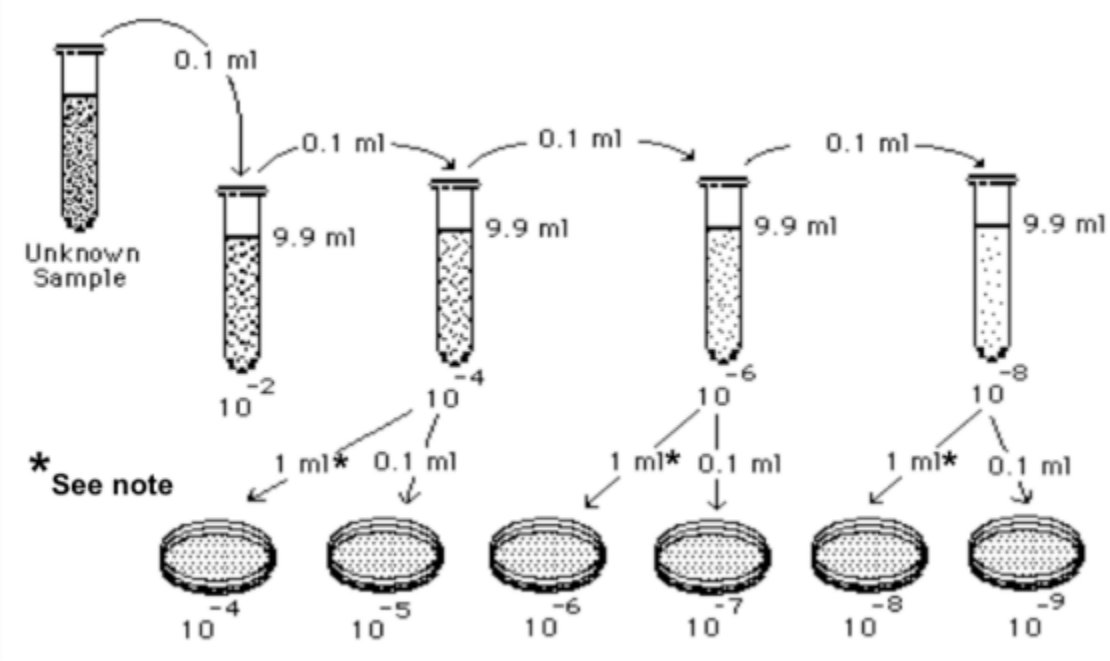
7. Calculate the average number of yeast cells per small square. Then calculate the number of yeasts per ml by dividing the average number of yeasts per small square by the volume of each small square which is 0.00025 µl. If you diluted the sample you must also multiply your results by the dilution factor to determine concentration of yeast cells in the original sample. Record your calculations and results.

Viable Plate Count

1. Label four 9.9 ml saline tubes 10-2, 10-4, 10-6, and 10-8, respectively. Label six BHI plates 10-4 to 10-9.

2. Vortex the unknown sample to ensure an even distribution of bacteria. Aseptically remove 0.1 ml of sample with a sterile pipette and transfer it to the 10-2 dilution tube (see diagram).

3. Vortex the 10-2 tube and transfer 0.1 ml to the 10-4 tube.



4. Again vortex the 10-4 dilution tube and transfer 0.1 ml to the 10-6 tube. Vortex this last tube well.

5. Vortex the 10-6 tube, transfer 0.1 ml to 10-8 tube and vortex again.

6. Using a new sterile pipette, aseptically transfer 1.0 ml from the 10-4 dilution tube to the plate labeled 10-4 and 0.1 ml to the plate labeled 10-5. Spread the inoculum on the surface of the agar in each plate using an alcohol-dipped, flamed, metal spreader. Dip the spreader into the alcohol jar and quickly take it through the flame and let the alcohol burn off after each spreading. Do not allow the spreader to get too hot. Never hold the spreader in the flame for more than a second.

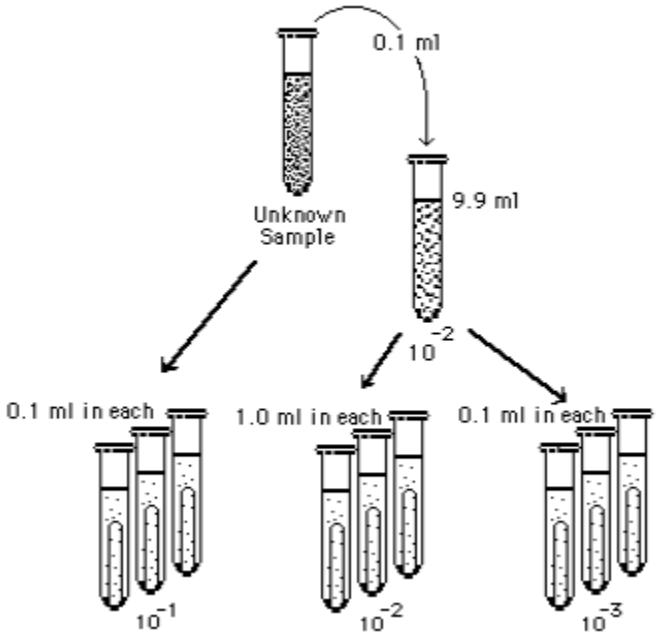
Note: (See stars on the figure above) Since it would take long for the agar in the petri dish to absorb the 1 ml sample, if the dish is moved during this time, it is possible to obtain wrong results as the dividing cells may move to different parts of the plate and produce their own colonies. To prevent this, place the 1.0 ml samples in Eppendorf tubes, microfuge the tubes for 1 minute, discard about 0.9 ml of supernatant, resuspend the pellet in the liquid left and spread the contents on their appropriate plates. Important Note: To sterilize the spreader, dip it in the 95% ethanol jar, shake the extra alcohol off by touching the inside of the jar above the alcohol level and then quickly take the spreader through a flame. Make sure that you do not hold the spreader in the flame for more than a second. It is the alcohol and not the heat that kills any bacteria present. Be extra careful as flaming alcohol drops may fall on objects and cause a fire hazard. Also while the alcohol is burning off, keep the spreader head down so the dripping alcohol will not spread to your fingers.

7. Repeat the above to transfer 1.0 and 0.1 ml from the 10-6 dilution tube to the 10-6 and 10-7 plates, respectively. In the same manner establish the 10-8 and 10-9 plates. Do not discard your dilution tubes. (See note above)

8. Allow the surface of the agar to dry before you move or invert the plates. Incubate the plates at 37°C for 2 days.

Most Probable Number (MPN)

1Label 9 lactose-bromcresol purple tubes for the following dilutions in triplicate: 10-1, 10-2 and 10-3.



2. Using a 5 ml pipette, take 3.3 ml of 10-2 dilution tube from the viable count procedure and dispense 0.1 ml into each of the 10-3 MPN tubes and 1.0 ml into each of the 10-2 MPN tubes. Use the same pipette to take 0.3 ml of the original sample and distribute 0.1 ml into each of the 10-1 MPN tubes. Mix contents of tubes very gently not to disturb the Durham tubes inside.

3. Incubate the tubes at 37°C until the next laboratory session.

Second Session Viable Plate Count

1. After incubation, count the colonies on each of the plates. Holding the plate to a light source, count the colonies by marking their position on the back of the petri plates with a marking pen. This aids in keeping track of those colonies previously counted and avoids recounts. If a plate has more than 300 colonies, record it as TNTC (too numerous to count).

2. From the plate count data, calculate the concentration of bacteria in the original sample. For statistical reasons use only data from plates which have between 30 and 300 colonies in this calculation.

Each colony forming unit (cfu) represents a single cell or a group of cells attached together and inseparable by shaking. Therefore, the number of cfu in the original sample is determined by multiplying the number of colonies on a dilution plate by the corresponding dilution factor. For example, if there are 200 colonies on the 10-4 plate, then there are 200 x10,000 = 2,000,000 colonies or 2 x 106 cfu/ml in the original sample. Generally replicates of each dilution are plated, and the mean count is recorded. Thus the mean of data from all groups in the lab would be an excellent estimate of the number of bacteria in the original sample.

Most Probable Number (MPN) Method

1 After incubation, examine tubes for the presence of BOTH acid and gas. Use the following MPN table to record the most probable number of coliforms in your sample

2 MPN TABLE MPN values & 95% Confidence limits for various combinations of positive results, when three replications of each sample are used. Number of tubes giving 95 % positive reaction out of 3 MPN Confidence

Some of the most important types of microscopes that used in biology are as follows:

1. Simple microscope

2. Compound microscope

3. Electron microscopes

4. Phase-Contrast microscope

5. Interference microscope.

The simple dissection microscope to advanced electron microscopes finds application in studies of living organisms.

Microscope as the name suggests are instruments that help to enlarge minute (micro = very small) organisms or their parts. A microscope not only presents a magnified view of the object but also ‘resolves’ it better.

Resolution is the feature which makes it possible to differentiate between two points present close together in the objects being viewed. The first microscope was constructed by Anton Van Leeuwenhoek (1632-1723). This, microscope consisted of a single biconvex lens fitted in a small window of a “board” and the object was viewed through it. This was a simple microscope.

After this compound microscope, were developed using combinations of two lenses. Improvements continued, newer and newer’ microscopes were designed and are still being improved.

Different types of microscopes being used in biological studies are the following:

Resolving Power:

It is the ability of a microscope to show two closely lying points as two distinct points.

Magnification:

It is the ratio of the size of the image to that of the object:

1. Simple microscope

2. Compound microscope

3. Electron microscopes

4. Phase-Contrast microscope

5. Interference microscope

1. Simple Microscopes:

Simple/ Dissecting Microscope:

As shown in the figure 6.1, dissecting microscope consists of a biconvex lens which is moved up and down by an adjustment screw to bring the object in sharp focus. The object is placed on the platform and light is focused with the help of a concave mirror fitted below.

In simple microscope, convex lens of short focal length is used to see magnified image of a small object. The object is placed between the optical centre and the focus of a convex lens, its image is virtual, erect and magnified and on the same side as the object. The position of the object is so adjusted that the image is formed at the least distance of distinct vision (D).

Magnifying power (M) of a simple microscope is the ratio of the angle subtended by the image at the eye to the angle subtended by the object seen directly, when both lie at the least distance of distinct vision or the near point.

$M = 1 + D/ f$

Where D is the least distance of distinct vision and f is the focal length of the lens.

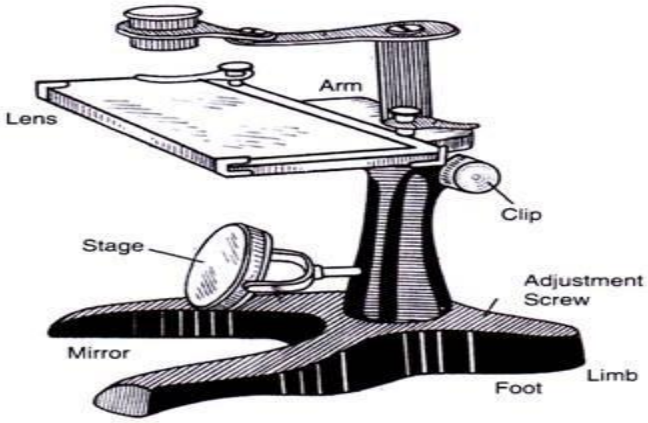


Fig. 6.1 Simple/dissecting microscope

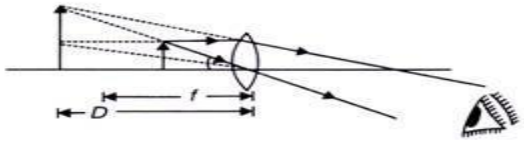


Fig. 6.1

2. A Compound Microscope:

A compound microscope consists of two set of convex lenses. A lens of short aperture and short focal length facing the object is called objective. Another set of lens of relatively moderate focal length and large aperture facing the eye is called the eye piece. The objective and the eye piece are placed coaxially at the two end of a tube (Fig. 6.2).

The object is placed between the centre of curvature and focus of the objective – it forms real, inverted and magnified image on the other side of the objective. This image acts as an object for the eye piece which then acts as a simple microscope to produce virtual, erect and magnified image.

Magnifying power (M) of a compound microscope will be

$M = L/ f_0 (1+D/f_e)$

Where $f_0$and f_e are focal length of objective and eye piece respectively, L is the length a the microscope tube and D is the least distance of distinct vision.

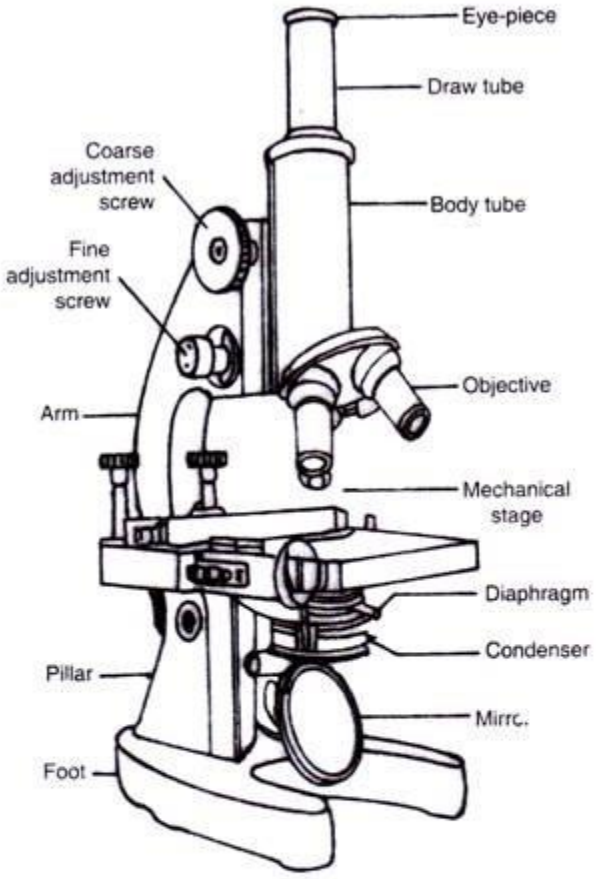


Fig. 6.2 A compound microscope

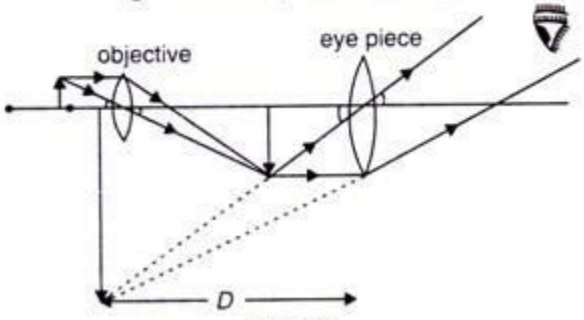


Fig. 6.2

3. The Electron Microscope:

The organelles of the cell became known after the electron microscope was invented. The electron microscope was developed in 1932 by M. Knoll and Ruska in Germany. It consist of a source of supplying, a beam of electron of uniform velocity, a condenser lens for concentrating the electron on the specimen, a specimen stage for displacing the specimen which transmits the electron beam, an objective lens, a projector lens and a fluorescent screen on which final image is observed (fig. 6.3).

For permanent record of the image, the fluorescent screen is replaced by photographic film. This microscope utilizes a stream of high speed electrons which are deflected by an electromagnetic field in the same way as a beam of light is reflected when it crosses a glass lens. There are two types of electron microscope.

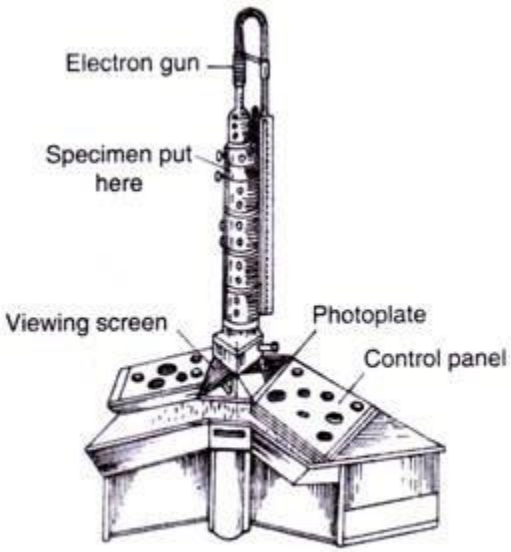


Fig. 6.3 An electron microscope

(a) Transmission electron microscope (TEM):

This is used to observe fine structure of cells. Ultra thin sections of the object are prepared and they are stained with a heavy metal (gold or palladium) to make certain part dense, and inserted in the vacuum chamber of the microscope. A 100, 00 volt electron beam is focused on the section and manipulated prepared from the image may be enlarged with enough resolution to achieve a total magnification of over 20 million times.

(b) Scanning electron microscope (SEM):

It is used to study the surfaces of the cell and organisms. In this microscope, the image is formed by electrons reflected back from the object. The image formed by this microscope has a remarkable three dimensional appearance. Typically magnification of scanning electron microscope is around 20,000 times.

4. Phase-Contrast microscope:

This is used to study the behavior of living cells, observe the nuclear and cytoplasmic changes taking place during mitosis and the effect of different chemicals inside the living cells. By using the phase-contrast microscope, an image of strong contrast of the object is obtained (fig. 6.4).

It is a contrast-enhancing optical technique that can be utilized to produce high-contrast images of transparent specimens, such as living cells (usually in culture), microorganisms, thin tissue slices, fibers, glass fragments, and sub-cellular particles (including nuclei and other organelles). In effect, the phase contrast technique employs an optical mechanism to translate minute variations in phase into corresponding changes in amplitude, which can be visualized as differences in image contrast.

One of the major advantages of phase contrast microscopy is that living cells can be examined in their natural state without previously being killed, fixed, and stained. As a result, the dynamics of ongoing biological processes can be observed and recorded in high contrast with sharp clarity of minute specimen detail.

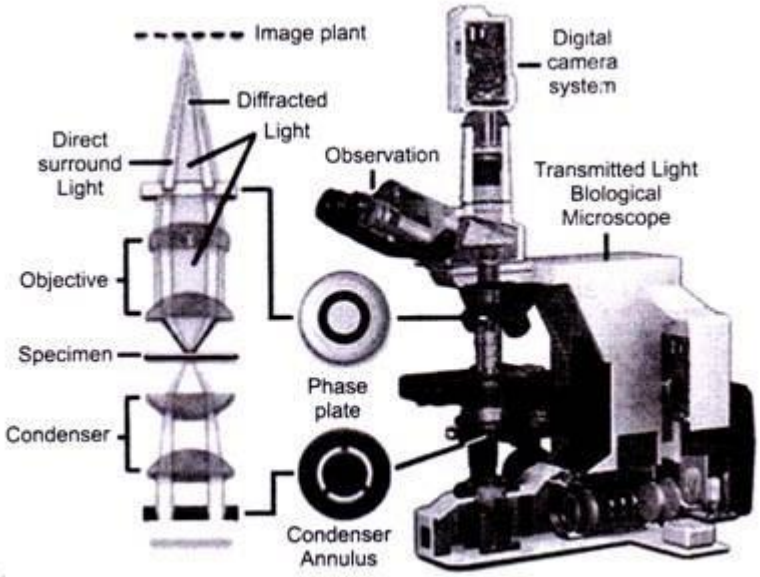


Fig. 6.4 Phase-contrast microscope

5.

Interference microscope:

Interference microscope is used for quantitative studies of macromolecules of the cell components, for example it is used for determination of lipid, nucleic acids and protein contents of the cell. Interferometry is a traditional technique in which a pattern of bright and dark lines (fringes) result from an optical path difference between a reference and a sample beam.

The incoming light is split inside an interferometer, one beam going to an internal reference surface and the other to the sample. After reflection, the beams recombine inside the

interferometer, undergoing constructive and destructive interference and producing the light and dark fringe pattern.

A precision translation stage and a CCD camera together generate a 3D interferogram of the object that is stored in the computer memory. This 3D interferogram of the object is then transformed by frequency domain analysis into a quantitative 3D image providing surface structure analysis (fig. 6.5).

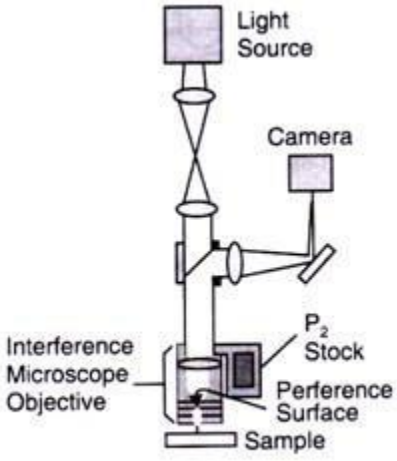


Fig. 6.5 Interference microscope