UNIT I

Bacteria and it's Cultivation

Microbiology

Microbiology is the study of Microbes which are generally too small needed magnification to see. These microbes include viruses, bacteria, algae, fungi and protozoa. Microorganisms /Microbes are the living organisms that are less than 1 milli meter in diameter can exist as single cells or clusters.

Microbiology is the branch of Life Science and it can be applied or basic. Microbiology is linked to many other scientific disciplines including biochemistry, cell biology, evolution, ecology.

Branches 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 moulds.

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.

SCOPE OF MICROBIOLOGY

Microorganisms are recognized as the basicresearch tools as they help to understand the chemical and physical basis of life as they are the dominant group of living organisms in the biosphere and are actively involved in our day to day activities. Microbiology primarily paves way to analyse the biochemical and genetic background of living things. Moreover, as microbes

are the excellent models for understanding the cell functions and as they play important role in the field of medicine,

agriculture and industry that assures human welfare, microbiology is considered as one of thevital branch of science with utmost promising scopes.

By focussed **on application** microbiology can be divided as mentioned below; Eg;

***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.

Little History/Background of Microbiology

The theory of biogenesis proposes that new living organisms can be created only from other previously existing living organisms as a result of sexual or asexual reproduction. Spontaneous generation or abiogenesis is the exact opposite of biogenesis stating that life can come from non-living.

Antonie van Leeuwenhoek (1632–1723) was one of the first people to observe microorganisms, using a microscope. He reported on the ubiquity of microbes. Louis Pasteur performed several experiments to demonstrate that microbial life does not arise spontaneously. Robert Koch (1843–1910) established that microbes can cause disease. Robert Koch isolated the bacteria which cause anthrax and tuberculosis. Koch's four postulates are 1) The microorganism must be found in diseased but should not be found in healthy organisms. 2) The microorganism must be cultured from the diseased individual and should be cultured in pure culture. 3) The cultured microorganism should cause disease when introduced into a healthy organism. 4) The microorganism re-isolated from the inoculated, diseased individual and should be matched to the original microorganism.

The twentieth century saw numerous advances in bacteriology, indicating their diversity, ancient lineage, and general importance. Most notably, a number of scientists around the world made contributions to the field of microbial ecology, showing that bacteria were essential to food webs and for

the overall health of the Earth's ecosystems. The discovery that some bacteria produced compounds lethal to other bacteria led to the development of antibiotics, which revolutionized the field of medicine.

Prokaryotes & Eukaryotes

Based on the structure of nucleus, fundamentally two types of cells exist. They are

i. Prokaryotes and **ii.** Eukaryotes

Prokaryotes have a relatively simple morphology and lack a true membrane-delimited nucleus. All bacteria and archaea are prokaryotic. ARCHAEA are phylogenetically related prokaryotes that are distinguished from bacteria by many features, most notably-Bacteria contain peptidoglycan in the cell wall; archaea do not. The cell membrane in bacteria is a lipid bilayer; in archaea, it can be a lipid bilayer or a monolayer. Eg for Archaea: Methanobacterium which generate methane gas.

Prokaryote is a Greek word, pro - before and karyon – nucleus.

Eucaryotes are morphologically complex and have a true, membrane-enclosed nucleus eg: Algae, fungi, protozoa.

Eukaryote is a Greek word, eu - true.

DIFFERENCE BETWEEN PROKARYOTES AND EUKARYOTES

Sl. No	Characteristics	Prokaryotic	Eukaryotic
1	Cell Size	Generally, 1 to 10 µm in linear dimension	Generally, 5 to 100 µm inlinear dimension
2	Cell division	Binary fission	Mitosis
3	Cellular organism	Unicellular	Mostly multicellular withdifferentiation of manytypes.
4	Cell wall	Complex structure with peptidoglycan layer, proteinand lipids	Absent or composed of cellulose or chitin
5	Plasma membrane	Present, no sterols except inmycoplasma	Present, contain sterols
6	Metabolism	Anaerobic or	Aerobic

Table Contd…

		aerobic	
7	DNA	Circular DNA in cytoplasm.	Very long, linear DNA molecule bounded by nuclear envelope
8	Membrane bound Nucleus	Absent	Present.
9	Extra chromosomal DNA	Present	Absent
10	Histones	Absent	Present
11	Membrane bound organelles	Absent	Present (Nucleus, mitochondria, chloroplast, ER
12	Ribosomes	70S type	80S type
13	Lysosomes	Absent	Present
14	Locomotion	Rotating flagella and glidingmovement	Undulating flagella &ciliaand amoeboid movement
15	Flagella	Consists of two protein building blocks	Consists of multiple Microtubules
16	Pili	Present	Absent
17	Site for cellular respiration	Cell membrane	Mitochondria
18	Examples	Bacteria and Archaea	Fungi, Algae and Protozoa

Bacteria

Bacteria (singular: bacterium) are classified as prokaryotes, which are single-celled organisms with a simple internal structure that lacks a nucleus, and contains DNA that either floats freely in a twisted, thread-like mass called the nucleoid, or in separate, circular pieces called plasmids.

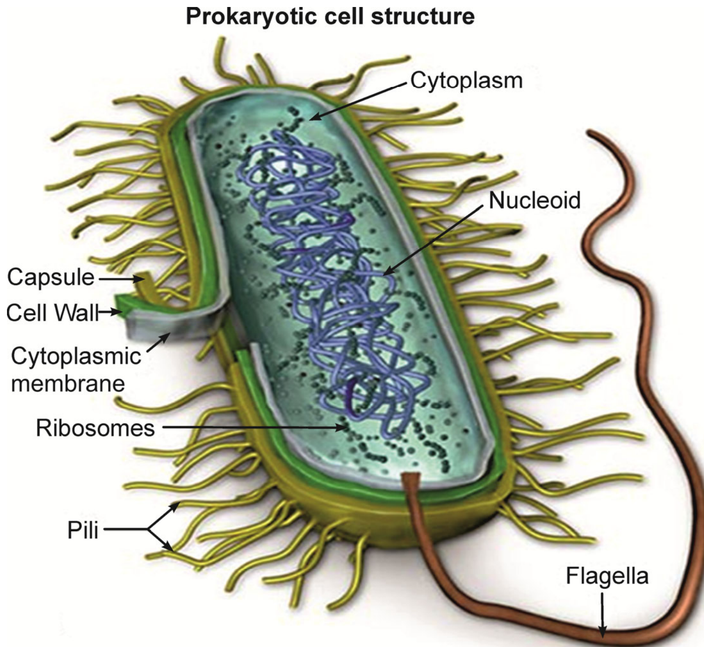


Fig 1.1. Prokaryotic Cell Structure.

For example, one species that lives symbiotically in the large intestine manufacture's vitamin K, an essential blood clotting factor. Other species are beneficial indirectly. Bacteria give yogurt its tangy flavour and sourdough bread its sour taste. They make it possible for ruminant animals (cows, sheep, goats) to digest plant cellulose and for some plants, (soybean, peas, alfalfa) to convert nitrogen to a more usable form.

Cell Envelope - The cell envelope is made up of two to three layers: the interior cytoplasmic membrane, the cell wall, and in some species of bacteria an outer capsule.

Capsule - It is an outer protective covering found in bacterial cells, in addition to cell wall. It helps in moisture retention and help in attachment of cells to absorb nutrients.

Cell Wall - Each bacterium is enclosed by a rigid cell wall composed of peptidoglycan, a protein-sugar (polysaccharide) molecule. The wall gives the cell its shape and surrounds the cytoplasmic membrane, protecting it from the environment. It also helps to anchor appendages like the pili and flagella, which originate in the cytoplasm membrane and protrude through the wall to the outside. The strength of the wall is responsible for keeping the cell from bursting when there are large differences in osmotic pressure between the cytoplasm and the environment.

Cytoplasm - The cytoplasm, or protoplasm, of bacterial cells is where the functions for cell growth, metabolism, and replication are carried out. It is a gel-like matrix composed of water, enzymes, nutrients, wastes, and gases and contains cell structures such as ribosomes, a chromosome, and plasmids. The cell envelope encases the cytoplasm and all its components. Unlike the eukaryotic (true) cells, bacteria do not have a membrane enclosed nucleus. The chromosome, a single, continuous strand of DNA, is localized, but not contained, in a region of the cell called the nucleoid. All the other cellular components are scattered throughout the cytoplasm.

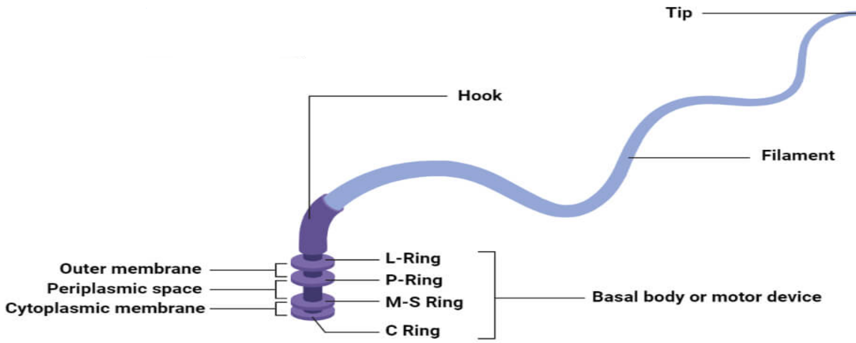
One of those components, **plasmids,** are small, extrachromosomal genetic structures carried by many strains of bacteria. Like the chromosome, plasmids are made of a circular piece of DNA. Unlike the chromosome, they are not involved in reproduction. Only the chromosome has the genetic instructions for initiating and carrying out cell division, or binary fission, the primary means of reproduction in bacteria. Plasmids replicate independently of the chromosome and, while not essential for survival, appear to give bacteria a selective advantage. While in the case of virus which encase their genetic material in a protective protein coat called a capsid.

Plasmids are passed on to other bacteria through two means. For most plasmid types, copies in the cytoplasm are passed on to daughter cells during binary fission. Other types of plasmids, however, form a tubelike structure at the surface called a pilus that passes copies of the plasmid to other bacteria during conjugation, a process by which bacteria exchange genetic information. Plasmids have been shown to be instrumental in the transmission of special properties, such as antibiotic drug resistance, resistance to heavy metals, and virulence factors necessary for infection of animal or plant hosts.

Cytoplasmic Membrane - A layer of phospholipids and proteins, called the cytoplasmic membrane, encloses the interior of the bacterium, regulating the flow of materials in and out of the cell.

Flagella - Flagella (singular, flagellum) are hair like structures or organelles provide locomotion to bacteria. Flagella have three parts namely basal body, hook and filament. The basal body consists of a rod and a series of rings which are attached to the cell wall and the cytoplasmic membrane. The rings which are basically proteins. There are three types of rings, namely the L-ring attached to the lipopolysaccharide, the P-ring attached to the peptidoglycan layer, and the M-S ring which is attached to the cytoplasmic membrane.

The hook is a flexible link between filament and the basal body proteins. The filament is a rigid, helical structure that extends from the cell surface.



Structure of Flagellum of Gram Negative Bacterium

A single flagellum at one end or the other is called monotrichous. They can be found at either or both ends of a bacterium (lophotrichous) or all over its surface (peritrichous). The flagella beat in a propeller-like motion to help the bacterium move toward nutrients; away from toxic chemicals; or, in the case of the photosynthetic cyanobacteria; toward the light.

Nucleoid - The nucleoid is a region of cytoplasm where the chromosomal DNA is located. It is not a membrane bound nucleus, but simply an area of the cytoplasm where the strands of DNA are found. Most bacteria have a single, circular chromosome that is responsible for replication, although a few species do have two or more. Smaller circular auxiliary DNA strands, called plasmids, are also found in the cytoplasm.

Pili - Many species of bacteria have pili (singular, pilus), small hairlike projections emerging from the outside cell surface. These outgrowths assist the bacteria in attaching to other cells and surfaces, such as teeth, intestines, and rocks. Without pili, many disease-causing bacteria lose their ability to infect because they're unable to attach to host tissue. Specialized pili are used for conjugation, during which two bacteria exchange fragments of plasmid DNA.

Ribosomes - Ribosomes are microscopic "factories" found in all cells, including bacteria. They translate the genetic code from the molecular language of nucleic acid to that of amino acids—the building blocks of proteins. Proteins are the molecules that perform all the functions of cells and living organisms.

Morphological classification

Bacteria can be classified into **six** major groups on morphological basis.

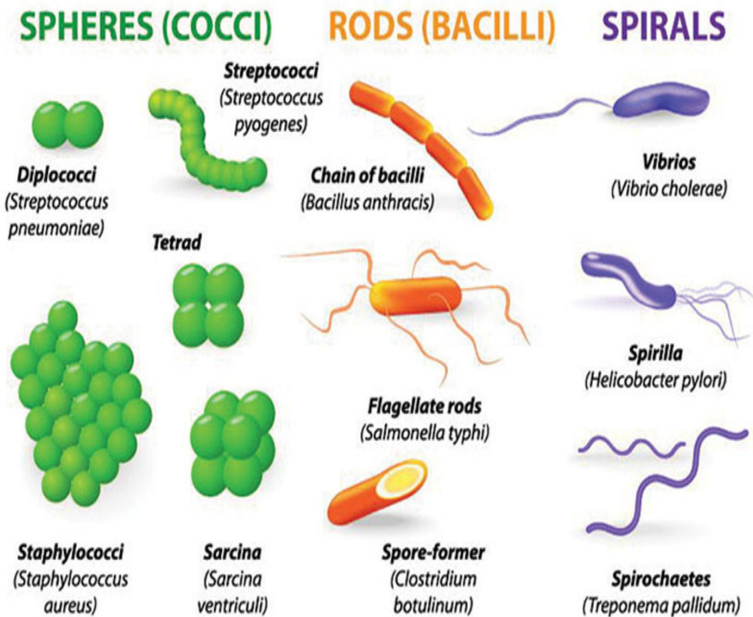


Fig 1.2. Classification of bacteria based on morphology.

1. **TRUE BACTERIA** – Cocci. These are spherical or oval cells. On the basis of arrangement of individual organisms, they can be described as – Monococci (Cocci in singles) – Monococcus species, Diplococci (Cocci in pairs) – Streptococcus pneumoniae, Staphylococci (Cocci in grape-like clusters) – Staphylococcus aureus, Streptococci (Cocci in chains) – Streptococcus pyogenes, Tetrad (Cocci in group of four) - Micrococcus species, Sarcina (Cocci in group of eight) Bacilli– These are rod-shaped bacteria.

2. **ACTINOMYCETES** (actin - ray, mykes-fungus) (actin - ray) These are rigid organisms like true bacteria but they resemble fungi in that they exhibit branching and tend to form filaments. They are termed such because of their resemblance to sun rays when seen in tissue sections.

3. **SPIROCHAETES** These are relatively longer, slender, non-branched microorganisms of spiral shape having several coils.

4. **MYCOPLASMAS** These bacteria lack in rigid cell wall (cell wall lacking) and are highly pleomorphic and of indefinite shape. They occur in round or oval bodies and in interlacing filaments.

5. **RICKETTSIAE AND CHLAMYDIAE** These are very small, obligate parasites, and at one time were considered closely related to the viruses.

Classification on the basis of Gram Stain and Bacterial Cell Wall

It allows a large proportion of clinically important bacteria to be classified as either Gram positive or negative based on their morphology and differential staining properties. Slides are sequentially stained with crystal violet, iodine, then distained with alcohol and counter-stained with safranin. Gram positive bacteria stain blue-purple and Gram-negative bacteria stain red.

Classification of Bacteria on the Basis of Mode of Nutrition

Phototrophs: These bacteria gain energy from light. Phototrophs are further divided into two groups on the basis of source of electron.

Photolithotrophs: These bacteria gain energy from light and ***uses reduced inorganic compounds*** such as H_2S as electron source. Photo organotrophs: These bacteria gain energy from light and ***uses organic compounds*** such as succinate as electron source.

Chemotrophs: Those bacteria gain energy from chemical compounds. Chemotrophs are further divided into two groups on the basis of source of electron.

Chemolithotrophs: These gain energy from oxidation of chemical compound and reduces inorganic compounds such as NH_3 as electron source. Eg. Nitrosomonas.

Chemoorganotrophs: These gain energy from chemical compounds and uses organic compound such as glucose and amino acids as source of electron. Eg. Pseudomonas pseudoflava.

Autotrophs: Those bacteria which uses carbondioxide as sole source of carbon to prepare its own food. Autotrophs are divided into two types on the basis of energy utilized to assimilate carbondioxide i.e., Photoautotrophs and chemoautotrophs.

Photoautotrophs: These utilized light to assimilate CO_2. Chemoautotrophs: These utilize chemical energy to assimilate CO_2.

Heterotrophs: Those bacteria which uses organic compound as carbon source. They lack the ability to fix CO_2. Most of the human pathogenic bacteria are heterotrophic in nature.

Classification of Bacteria on Basis of Temperature Requirement

major types on the basis of their temperatures response as indicated below:

a. **Psychrophiles**: Bacteria that can grow at 0°Cor below but the optimum temperature of growth is 15 °C or below and maximum temperature is 20°C are called psychrophiles.

Examples: Vibrio psychroerythrus, vibrio marinus, Psychroflexus.

b. Psychrotrops (facultative psychrophiles):

Those bacteria that can grow even at 0°C but optimum temperature for growth is (20-30)°C. Eg: Pseudomonas fluorescens, Micrococcus.

c. Mesophiles:

Those bacteria that can grow best between (25-40) °C but optimum temperature for growth is 37^0C, most of the human pathogens are mesophilic in nature.

Examples: E. coli, Salmonella, Klebsiella, Staphylococci.

d. Thermophiles: Those bacteria that can best grow above 45°C.

Examples: Streptococcus thermophiles, Bacillus stearothermophilus, Thermus aquaticus.

e. Hypethermophiles:

Those bacteria that have optimum temperature of growth above 80^0C.

Mostly Archeobacteria are hyperthermophiles.

Examples: Thermodesulfobacterium, Aquifex, Pyrolobus fumari,

Classification of Bacteria on the Basis of Oxygen Requirement

***Obligate Aerobes*:** Require oxygen to live.

Example: Pseudomonas, common nosocomial pathogen.

Facultative Anaerobes: Can use oxygen, but can grow in its absence.

They have complex set of enzymes. Most bacteria of medical importance arefacultative anaerobes.

Examples: E. coli, Staphylococcus, yeasts, & many intestinal bacteria.

Obligate Anaerobes:

Cannot use oxygen and are harmed by the presence of toxic forms of oxygen.

Examples: Clostridium bacteria that cause tetanus and botulism.

Aerotolerant Anaerobes: Cannot use oxygen, but tolerate its presence. Can break down toxic forms of oxygen.

Examples: Lactobacillus carries out fermentation regardless of oxygen presence.

Microaerophiles: Require oxygen, but at low concentrations. Sensitive to toxic forms of oxygen. Eg: Campylobacter.

Classification of Bacteria on the Basis of pH of Growth

***Acidophiles**:* These bacteria grow best at an acidic pH. The cytoplasm of these bacteria is acidic in nature. Some acidopiles are thermophilic in nature, such bacteria are called Thermoacidophiles.

Example: Thiobacillus thioxidans, Thiobacillus, ferroxidans, Sulfolobus

Alkaliphiles: These bacteria grow best at an alkaline pH.

Example: Vibrio cholerae optimum pH of growth is 8.2.

Neutrophiles: These bacteria grow best at neutral pH (6.5-7.5).

Example: E. coli

Classification of Bacteria on the Basis of Osmotic Pressure Requirement

Halophiles: Require moderate to large salt concentrations.

Cell membrane of halophilic bacteria is made up of glycoprotein with high content of negatively charged glutamic acid and aspartic acids. So high concentration of Na+ ion concentration is required to shield the –ve charge. Ocean water contains 3.5% salt. Most such bacteria are present in the oceans.

Archeobacteria, Halobacterium, Halococcus.

Extreme or Obligate Halophiles: Require a very high salt concentration (20 to 30%). Bacteria in Dead Sea.

Facultative Halophiles: Do not require high salt concentrations for growth, but tolerate up to 2% salt or more.

Classification of Bacteria on the Basis of Number of Flagella

Atrichos: These bacteria have no flagella. **Example:** Corynebacterium diptherae.

Monotrichous: One flagellum is attached to one end of the bacteria cell. **Example:** Vibro cholerae.

Lophotrichous: Bunch of flagella is attached to one end of the bacteria cell. **Example:** Pseudomonas.

Amphitrichous: Bunch of flagella arising from both end of the bacteria cell. **Example:** Rhodospirillum rubrum.

Peritrichous: The flagella are evenly distributed surrounding the entire bacterial cell. **Example:** Bacillus.

Classification of Bacteria on the basis of Spore Formation

1) Spore forming bacteria: Those bacteria that produce spore during unfavourable condition.

These are further divided into two groups:

i) ***Endospore forming bacteria*****:** Spore is produced within the bacterial cell. **Examples:** Bacillus, Clostridium, Sporosarcina etc

ii) ***Exospore forming bacteria:*** Spore is produced outside the cell.

Example: Methylosinus

2) Non-sporing bacteria:

Those bacteria which do not produce spores.

Example: E. coli, Salmonella.

Nutritional Requirements

The bacterial cell contains water (80% of total weight), proteins, polysaccharides, lipids, nucleic acids, mucopeptides and low molecular weight compounds.

For growth and nutrition of bacteria, the minimum nutritional requirements are water, a source of carbon, a source of nitrogen and some inorganic salts. Water is the vehicle of entry of all nutrients into the cell and for the elimination of waste products.

Their nutritional requirements vary widely. Some may require only a single organic substance like glucose. Others may need a large number of different compounds like amino acids, nucleotides, lipids, carbohydrates and coenzymes. Bacteria require a supply of inorganic salts. They require anions like phosphate and sulphate anions and cations like sodium, potassium, magnesium, iron and calcium. Some ions like cobalt may be required in trace amounts. Some bacteria require certain organic compounds in minute quantities. These are called growth factors or bacterial vitamins. Growth factors are called essential when growth does not occur in their absence. Accessory growth factors are those which enhance growth without being absolutely necessary for it. In many cases, bacterial vitamins are same as vitamins necessary for nutrition of mammals, for example, B group vitamins – thiamine, riboflavin, pyridoxine, nicotinic acid, folic acid and vitamin B12.

Gaseous Requirements: Depending on the influence of oxygen on growth and survival, bacteria are divided into **aerobes** and a**naerobes**. Aerobic bacteria require oxygen for growth. They may be ***obligate aerobes*** or *facultative anaerobe*s. Obligate aerobes grow only in the presence of oxygen, for eg. Cholera bacillus. Facultative anaerobes are ordinarily aerobic but can grow in the absence of oxygen, though less abundantly. Anaerobic bacteria, such as clostridia grow in the absence of oxygen. Obligate anaerobes may even die on exposure to oxygen. Microaerophilic bacteria are those that grow best in the presence of low oxygen tension.

In case of aerobes, atmospheric oxygen is the final electron acceptor in the process of respiration (aerobic respiration). In this case, the carbon and energy source may be completely oxidised to carbon dioxide and water. Energy is provided by the production of energy-rich phosphate bonds and the conversion of adenosine diphosphate (ADP) to adenosine triphosphate (ATP). This process is called ***oxidative phosphorylation***. Anaerobic bacteria use compounds like nitrates or sulphates instead of oxygen as final electron acceptors in the process of respiration (anaerobic respiration). A more common process used by these bacteria in anaerobic metabolism is ***fermentation.*** It is defined as the process by which complex organic compounds, such as glucose, are broken down by the action of enzymes into simpler compounds without the use of oxygen. This process leads to the formation of several organic end products such as organic acids and alcohols, as well as of gas (carbon dioxide and hydrogen). For example, Escherichia coli ferments glucose with the production of acid and gas. It also ferments lactose. During the process of fermentation, energy-rich phosphate bonds are produced by the introduction of organic phosphate into intermediate metabolites. This process is known as ***substrate-level phosphorylation***. The energy-rich phosphate groups so formed are used for conversion of ADP to ATP. All bacteria require some amounts of carbon dioxide for growth. This is obtained from the atmosphere or from the cellular metabolism of the bacterial cell. Some bacteria like Brucella abortus require much higher levels of carbon dioxide (5- 10%) for growth. They are called capnophilic.

Temperature Requirements: Bacteria vary in their requirement of temperature for growth. The temperature at which growth occurs best is known as the "***optimum temperature".*** In the case of most pathogenic bacteria, the optimum temperature is 37ºC. Bacteria which grow best at temperatures of 25-40ºC are called ***mesophilic***, for example Escherichia coli. Psychrophilic bacteria are those that grow best at temperatures below 20ºC. They are soil and water saprophytes and may cause spoilage of refrigerated food. ***Thermophilic bacteria*** are those which grow best at high temperatures, 55-80ºC. They may cause spoilage of under processed canned food. Some thermophiles, for example Geobacillus stearothermophilus, form spores that are highly thermoresistant.

The Effect of pH on Growth

The pH, or hydrogen ion concentration, [H^+], of natural environments varies from about 0.5 in the most acidic soils to about 10.5 in the most alkaline lakes. The range of pH over which an organism grows is defined by **three cardinal points**: the **minimum pH**, below which the organism cannot grow, the **maximum pH**, above which the organism cannot grow, and the **optimum pH**, at which the organism grows best.

Microorganisms which grow at an optimum pH well below neutrality (7.0) are called **acidophiles**. Those which grow best at neutral pH are called **neutrophiles** and those that grow best under alkaline conditions are called **alkaliphiles**.

In the construction and use of culture media, one must always consider the optimum pH for growth of a desired organism and incorporate buffers in order to maintain the pH of the medium in the changing milieu of bacterial waste products that accumulate during growth. Many pathogenic bacteria exhibit a relatively narrow range of pH over which they will grow. Most diagnostic media for the growth and identification of human pathogens have a pH near 7.

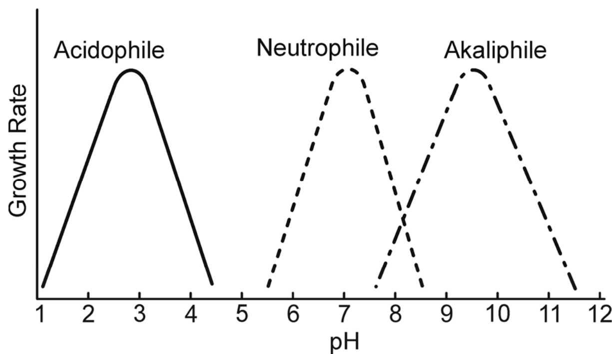


Fig 1.3. Growth rate vs pH for three environmental classes of procaryotes. Most free-living bacteria grow over a pH range of about three units. Note the symmetry of the curves below and above the optimum pH.

Trace Elements

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. The usual cations that qualify as trace elements in bacterial nutrition are Mn, Co, Zn, Cu, and Mo.

Culture Media for the Growth of Bacteria

For any bacterium to be propagated for any purpose it is necessary to provide the appropriate biochemical and biophysical environment. The biochemical (nutritional) environment is made available as a **culture medium**, and depending upon the special needs of particular bacteria (as well as particular investigators) a large variety and types of culture media have been developed with different purposes and uses. Culture Medium is defined as Nutrient material prepared for microbial growth in the laboratory. Requirements: Must be sterile & contain appropriate nutrients. Must be incubated at appropriate temperature.

Culture media are employed in the isolation and maintenance of pure cultures of bacteria and are also used for identification of bacteria according to their biochemical and physiological properties. Culture is defined as microbes that grow and multiply in or on a culture medium.

Media Can be used to-Enrich the numbers of bacteria, select for certain bacteria and suppress others, Differentiate among different kinds of bacteria.

The manner in which bacteria are cultivated, and the purpose of culture media, varies widely. **Liquid media** are used for growth of pure batch cultures, while solidified media are used widely for the isolation of pure cultures, for estimating viable bacterial populations, and a variety of other purposes. The usual gelling agent for solid or **semisolid medium** is **agar**, a hydrocolloid derived from red algae. Agar is used because of its unique physical properties (it melts at 100°C and remains liquid until cooled to 40°C, the temperature at which it gels) and because it cannot be metabolized by most bacteria. Hence as a medium component it is relatively inert; it simply holds (gels) nutrients that are in aqueous solution.

BASIC REQUIREMENTS OF CULTURE MEDIA

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.

1 **Classification of bacterial culture media on the basis of consistency**

  1 Solid medium
  2 Semisolid medium
  3 Liquid (Broth) medium

2 **Classification of Bacterial Culture media on the basis of purpose/ functional use/ application**

  1 General-purpose media/ Basic media
  2 Enriched medium (Added growth factors):
  3 Selective and enrichment media
    a. Selective medium
    b. Enrichment culture medium

4 Differential/ indicator medium: differential appearance:
5 Transport media
6 Anaerobic media:
7 Assay media

Solid medium

Solid medium contains agar at a concentration of 1.5-2.0% or some other, mostly inert solidifying agent. Solid medium has physical structure and allows bacteria to grow in physically informative or useful ways (e.g. as colonies or in streaks). Solid medium is useful *for* ***isolating bacteria*** or for determining the colony characteristics of the isolate.

Semisolid medium

Semisolid medium is prepared with agar at concentrations of 0.5% or less. Semisolid medium has a soft custard-like consistency and is useful for the cultivation of microaerophilic bacteria or for the determination of bacterial motility.

Liquid (Broth) medium

These media contain specific amounts of nutrients but don't have a trace of gelling agents such as gelatin or agar. Broth medium serves various purposes such as propagation of a large number of organisms, fermentation studies, and various other tests. e.g. sugar fermentation tests, MR-VR broth

General-purpose media/ Basic media

Basal media are basically simple media that supports most non-fastidious bacteria. Peptone-water,nutrient broth,and nutrient agar(NA)are considered as basal medium. These media are generally used for the primary isolation of microorganisms.

Enriched medium (Added growth factors)

Blood agar addition of extra nutrients in the form of blood, serum, egg yolk, etc, to basal medium makes enriched media. *Enriched media are used to grow nutritionally exacting (fastidious) bacteria.* Blood agar, chocolate agar, Loeffler's serum slope, etc are few of the enriched media. Blood agar is prepared by adding 5-10% (by volume) blood to a blood agar base. **Chocolate agar** is also known as heated blood agar or lysed **blood agar.**

Selective and enrichment media

These media are designed to inhibit unwanted commensal or contaminating bacteria and help to recover pathogens from a mixture of bacteria. While selective media are agar-based, enrichment media are liquid in consistency. Both these media serve the same purpose. Any agar media can be made selective by the addition of certain inhibitory agents that don't affect the pathogen of interest. Various approaches to making a medium selective include addition of antibiotics, dyes, chemicals, alteration of pH, or a combination of these.

a. Selective medium

Principle: Differential growth suppression Selective medium is designed to suppress growth of some microorganisms while allowing the growth of others. Selective medium is agar-based (solid) medium so that individual colonies may be isolated.

Examples of selective media include

Thayer Martin Agar used to recover *Neisseria gonorrhoeae* contains antibiotics; vancomycin, colistin and nystatin.

Mannitol Salt Agar and Salt Milk Agar used to recover S.aureus contains 10% NaCl.

Potassium tellurite medium used to recover *C.diphtheriae* contains 0.04% potassium **MacConkey's Agar** used for **Enterobacteriaceae** members contains bile salt that inhibits most gram positive bacteria.

Pseudosol Agar (Cetrimide Agar) used to recover *P. aeruginosa* contains cetrimide (antiseptic agent).

Crystal Violet Blood Agar used to recover *S. pyogenes* contains 0.0002% crystal violet.

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

Wilson and Blair's Agar for recovering *S. typhi* is rendered selective by the addition of dye brilliant green.

Selective media such as **TCBS Agar** used for isolating *V. cholerae* from faecal specimens have elevated pH (8.5-8.6), which inhibits most other bacteria.

b. Enrichment culture medium

Enrichment medium is used to increase the relative concentration of certain microorganisms in the culture prior to plating on solid selective medium. Unlike selective media, enrichment culture is typically used as a broth medium. Enrichment media are liquid media that also serves to inhibit

commensals in the clinical specimen. **Selenite F broth**, tetrathionate broth, and **alkaline peptone water (APW)** are used to recover pathogens from faecal specimens.

Differential/ indicator medium: differential appearance:

Certain media are designed in such a way that different bacteria can be recognized on the basis of their colony colour. Various approaches include incorporation of dyes, metabolic substrates, etc, so that those bacteria that utilize them appear as differently coloured colonies. Such media are called differential media or indicator media. Differential media allow the growth of more than one microorganism of interest but with morphologically distinguishable colonies.

Examples of differential media include

Mannitol salts agar (mannitol fermentation = yellow)

Blood agar (various kinds of hemolysis i.e. α, β and γ hemolysis)

Mac Conkey agar (lactose fermenters, pink colonies whereas non- lactose fermenter produces pale or colourless colonies.

TCBS (*Vibrio cholera* produces yellow colonies due to fermentation of sucrose)

Transport media

Clinical specimens must be transported to the laboratory immediately after collection to prevent overgrowth of contaminating organisms or commensals. This can be achieved by using transport media. Such media prevent drying (desiccation) of a specimen, maintain the pathogen to commensal ratio, and inhibit the overgrowth of unwanted bacteria. Some of these media (Stuart's & Amie's) are semi-solid in consistency. Addition of charcoal serves to neutralize inhibitory factors.

Cary Blair transport medium and Venkatraman Ramakrishnan (VR) medium are used to transport faeces from suspected cholera patients.

Sach's buffered glycerol saline is used to transport faeces from patients suspected to be suffering from bacillary dysentery.

Pike's medium is used to transport streptococci from throat specimens.

Anaerobic media

Anaerobic bacteria need special media for growth because they need low oxygen content, reduced oxidation-reduction potential and extra nutrients.

Media for anaerobes may have to be supplemented with nutrients like hemin and vitamin K. Such media may also have to be reduced by physical or chemical means. Boiling the medium serves to expel any dissolved

oxygen. Addition of 1% glucose, 0.1% thioglycollate, 0.1% ascorbic acid, 0.05% cysteine, or red-hot iron filings can render a medium reduced. Before using the medium must be boiled in a water bath to expel any dissolved oxygen and then sealed with sterile liquid paraffin.

Robertson Cooked Meat (RCM) medium that 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.

Methylene blue or resazurin is an oxidation-reduction potential indicator that is incorporated in the medium. Under reduced condition, methylene blue is colourless.

Assay media

These media are used for the assay of vitamins, amino acids, and antibiotics. E.g. antibiotic assay media are used for determining antibiotic potency by the microbiological assay technique.

Other types of medium include;

Media for enumeration of bacteria,

Media for characterization of bacteria,

Maintenance media etc.

Mueller-Hinton agar is a microbiological growth medium that is commonly used for antibiotic susceptibility testing. Originally formulated for isolation of *Neisseria* species.

It is also used to isolate and maintain *Neisseria* and *Moraxella* species.

Growth and Multiplication of Bacteria

Bacteria divide by binary fission. When a bacterial cell reaches a certain size, it divides to form two daughter cells. Nuclear division is followed by cell division. The interval of time between two cell divisions, or the time required for a bacterium to give rise to two daughter cells under optimum conditions, is called the generation time or the population doubling time. In Escherichia coli and many other medically important bacteria, the generation time is about 20 minutes. Some bacteria are slow-growing. The generation time in tubercle bacilli is about 20 hours. In leprae bacilli, it is as long as about 20 days. When bacteria are grown in a vessel of liquid medium, multiplication is arrested after a few cell divisions due to depletion of nutrients or accumulation of toxic products. This is a batch culture. By the use of special devices for replenishing nutrients and removing bacterial cells (chemostat or turbidostat), it is possible to maintain a continuous culture of bacteria for industrial or research purposes. When bacteria multiply in host tissues, the situation may be intermediate between a batch culture and a

continuous culture. The source of nutrients may be inexhaustible but the bacteria have to fight the defence mechanisms of the host.

Bacteria growing on solid media (for example blood agar, MacConkey agar) form colonies. Each colony represents a cluster of cells derived from a parent cell. In liquid media, growth is diffuse. Bacterial growth may be considered at two levels: increase in the size of the bacterial cell and increase in the number of cells.

Growth in numbers can be studied by bacterial counts. Two types of bacterial counts can be made: total count and viable count. The total count gives the total number of cells in the sample, irrespective of whether they are living or not. It can be done by various methods, for example direct counting under the microscope using counting chambers. The viable count measures the number of living cells, that is, cells capable of multiplication. Viable counts are obtained by dilution or plating methods. In the dilution method, the suspension, whose cell count is to be determined, is serially diluted. The dilutions are made to the point beyond which unit quantities do not yield growth when inoculated into suitable liquid media. Each dilution is inoculated into the respective tubes containing liquid media. The viable count is statistically evaluated from the number of tubes showing growth. This method is not accurate but is used for the estimation of "presumptive coliform count" in drinking water. The presumptive coliform count is a method of estimating the level of pollution of drinking water. In the plating method, appropriate dilutions are inoculated on solid media, either on the surface of plates or as pour plates. The number of colonies that develop after incubation gives an estimate of the viable count. The method commonly employed is that described by Miles and Misra (1938) in which serial dilutions are dropped on the surface of dried plates and colony counts obtained.

Bacterial growth curve

When a bacterium is seeded into a suitable liquid medium and incubated, its growth follows a definite course. If bacterial counts are made at intervals after inoculation and plotted in relation to time, a growth curve is obtained.

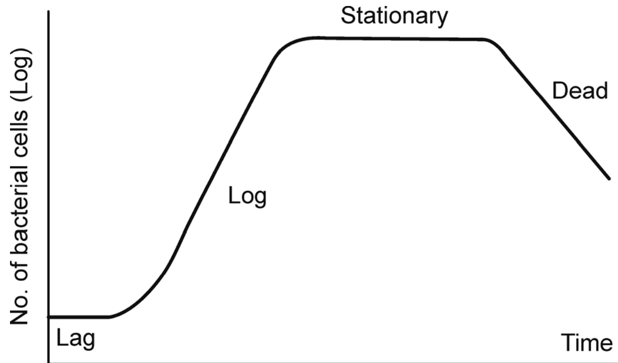


Fig 1.4. Bacterial growth curve.

The curve shows the following phases:

Lag phase: Immediately following the seeding of a culture medium, there is no appreciable increase in number, though there may be an increase in the size of the cells. This initial period is the time required for adaptation to the new environment. The necessary enzymes and metabolic intermediates are built up in adequate quantities for multiplication to proceed. The maximum cell size is obtained towards the end of lag phase. The duration of the lag phase varies with the species, size of the inoculum, nature of the culture medium and environmental factors such as temperature.

Log (logarithmic) or exponential phase: Following the lag phase, the cells start dividing and their numbers increase exponentially or by geometric progression with time. If the logarithm of the viable count is plotted against time, a straight line will be obtained. In this phase, cells are smaller and stain uniformly.

Stationary phase: After a varying period of exponential growth, cell division stops due to depletion of nutrients and accumulation of toxic products. The number of new cells formed is just enough to replace the number of cells that die. Equilibrium exists between the dying cells and the newly formed cells. So, the viable count remains stationary. In this phase, cells are frequently gram variable and show irregular staining. Sporulation occurs at this stage.

Phase of decline: This is the phase when the population decreases due to cell death. Besides nutritional exhaustion and toxic accumulation, cell death may also be caused by autolytic enzymes.

Isolation and preservation methods for pure cultures

Culture: Act of cultivating microorganisms or the microorganisms that are cultivated. Mixed culture has more than one microorganism.

Pure culture: containing a single species of organism.

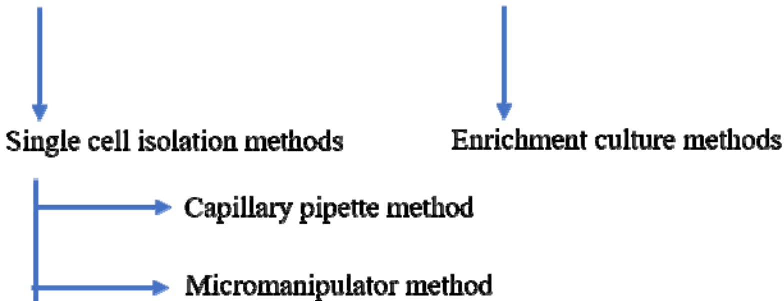
A pure culture is usually derived from a mixed culture (one containing many species) by transferring a small sample into new, sterile growth medium in such a manner as to disperse the individual cells across the medium surface or by thinning the sample many times before inoculating the new medium.

Pure cultures are important in microbiology for the following reasons-

- Once purified, the isolated species can then be cultivated with the knowledge that only the desired microorganism is being grown.
- A pure culture can be correctly identified for accurate studying and testing, and diagnosis in a clinical environment.
- Testing/experimenting with a pure culture ensures that the same results can be achieved regardless of how many times the test is repeated.
- Pure culture spontaneous mutation rate is low
- Pure culture clone is 99.999% identical.

Common isolation techniques

Streak plate method, pour plate method, Spread plate method & Roll tube method.



Streak plate method

Streaking is the process of spreading the needle on the surface of the media. Sterilize the inoculating needle by flame to make red hot and allow it to cool for 30seconds.

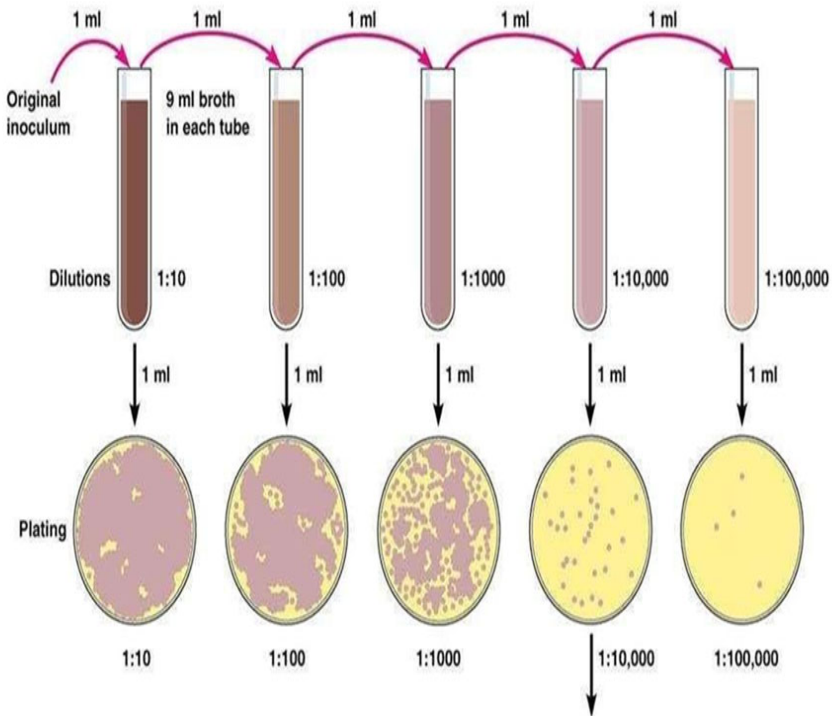
The sample is streaked in such a way to provide series of dilution. The purpose is to thin out inoculum to get separate colonies. Culturing can be done by streaking well isolated colonies from streak plate to new plate.

Pour plate method

The bacterial culture and liquid agar medium are mixed together. After mixing the medium, the medium containing the culture poured into sterilized Petri dishes (Petri plates), allowed solidifying and then incubated. After incubation colonies appear on the surface.

Demerits

1. Microorganism trapped beneath the surface of medium hence surface as well as subsurface colonies are developed which makes the difficulties in counting the bacterial colony.
2. Tedious and time-consuming method, microbes are subjected to heat shock because liquid medium maintained at 45°C.
3. Unsuitable-Psychrophile.



Spread plate method

This is the best method to isolate the pure colonies.

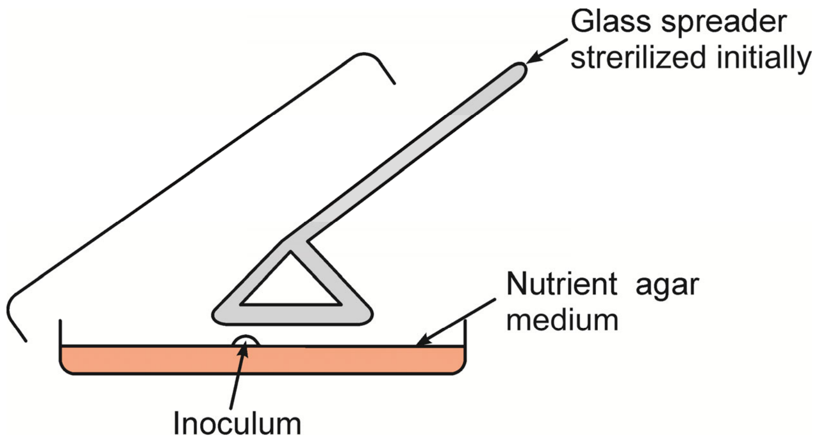
In this technique, the culture is not mixed with the normal saline and serially diluted agar medium. Instead, it is mixed with 0.1ml of sample taken

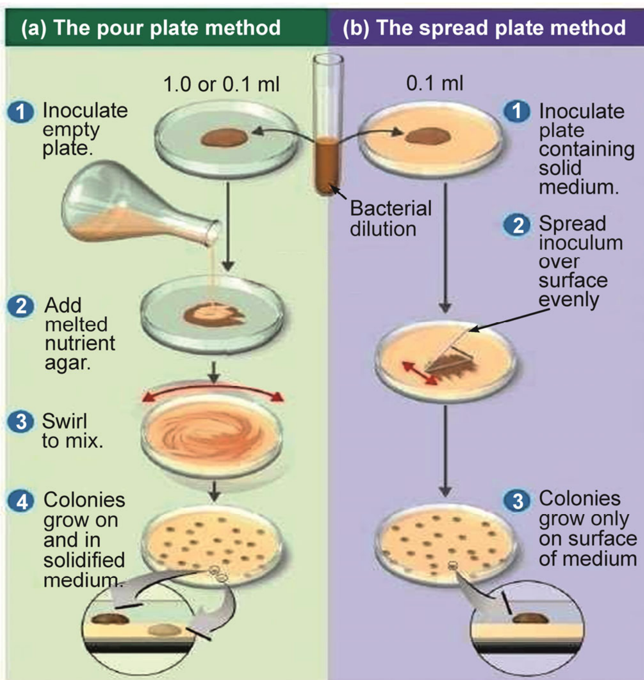
from diluted mixture, which is placed on the surface of the agar plate and spread evenly over the surface by using L shaped glass rod called spreader.

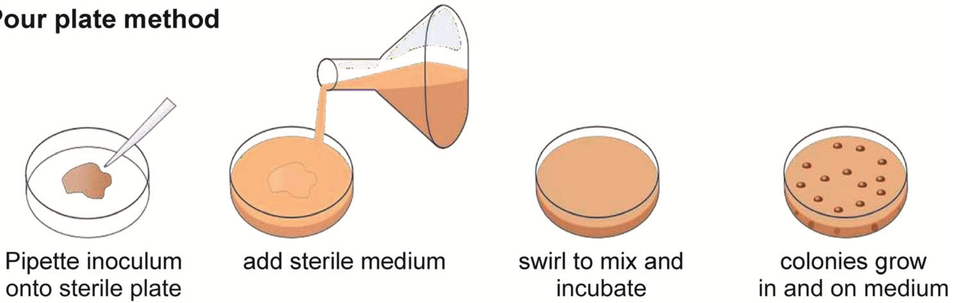
Incubate the plates, after incubation, colonies are observed on the agar surface.

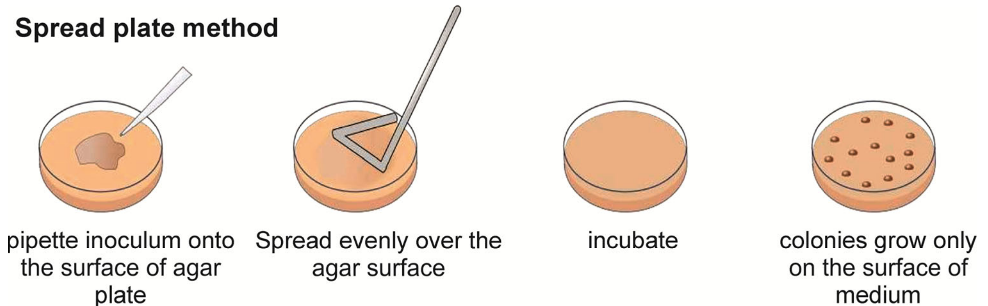
Merits/Advantages

It is a simple method. In this method only, surface colonies are formed. Micro-organisms are not exposed to higher temperature.









Roll tube method

A roll-tube method was developed in which agar medium was distributed as a thin layer over the internal surface of test tubes charged with an anaerobic atmosphere for the isolation of obligately anaerobic bacteria of the rumen. In the roll-tube method, exposure of bacteria and culture medium to air is avoided by displacing the air in the culture vessel with an oxygen-free gas, such as carbon dioxide, hydrogen, nitrogen, or mixtures of these gases. Carbon dioxide is the gas of choice because it is heavier than air, relatively cheap, and valuable in buffering. Vessels are stoppered under conditions preventing access of air. The cultures require no special incubators and can be removed and examined with no anaerobic precautions if kept stoppered. If opened, anaerobiosis can be continuously maintained during necessary manipulations, and the culture again closed without exposure to oxygen.

Micromanipulator method

Micromanipulators have been built, which 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 single cell of microbe sucked into micropipette and transferred to large amount of sterile medium.

Advantages of micromanipulator method

The advantages of this method are that one can be reasonably sure

from a single cell and one can obtain strains within the species.

Disadvantages

The disadvantages are that the equipment is expensive, its

manipulation is very tedious, and it requires a skilled person.

Preservation of Pure Culture

To maintain pure culture for extended periods in viable condition without any genetic change is referred as Preservation. During preservation most important factor is to stop microbial growth or at least lower the growth rate.

Aim of Preservation

To maintain isolated pure culture for extended periods in a viable condition. To avoid contamination and to restrict Genetic Mutation.

Why to Preserve Bacteria

- ❖ In nature there are only 1% bacteria which is pathogenic and harmful to Animalia and Plantae.
- ❖ 99% of bacterial populations are of economic importance for human beings and plants.
- ❖ In soil for nutrient uptake in food industry, in sewage treatment, in medical industry.

So, the preservation of bacteria is one of the most profitable practices economically as well as environmentally. For Academic purpose, Research Purpose, Biotechnology field and in Fermentation Industry.

Preservation methods of Bacteria

1. Periodic transfer to fresh medium
2. Storage at low temperature
3. Storage in sterile soil
4. Preservation by overlaying culture with mineral oil
5. Lyophilization or freeze drying

Periodic transfer to fresh medium

Strains can be maintained by periodically preparina fresh culture from the previous stock culture. The culture medium, the storage temperature, and the time transfers are made vary with the species.

The temperature and the type of medium chosen should support a slow rather than a rapid rate of growth so that the time interval between transfers can be as long as possible. Many of the more common heterotrophs remain viable for several weeks or months on a medium like Nutrient Agar. The transfer method has the disadvantage of failing to prevent changes in the characteristics of a strain due to the development of variants and mutants.

Storage at Low Temperature

By Refrigeration & Cryopreservation Pure cultures can be successfully stored at 0-4°C either in refrigerators or in cold-rooms.

This method is applied for short duration (2-3 weeks for bacteria and 3-4 months for fungi) because the metabolic activities of the microorganisms are greatly slowed down but not stopped.

Thus, their growth continue slowly, nutrients are utilized and waste products released in medium.

This results in finally the death of the micro-organism.

Cryopreservation

Cryopreservation (i.e., freezing in liquid nitrogen at -196°C or in the gas phase above the liquid nitrogen at -150°C) helps survival of pure cultures for long storage times.

In this method, the microorganisms of culture are rapidly frozen in liquid nitrogen at - 196°C in the presence of stabilizing agents such as glycerol or Dimethyl Sulfoxide (DMSO) that prevent the cell damage due to formation of ice crystals and promote cell survival.

This liquid nitrogen method has been successful with many species that cannot be preserved by lyophilization and most species can remain viable under these conditions for 10 to 30 years without undergoing change in their characteristics, however this method is expensive.

Storage in sterile soil

Storing organisms in soil fall into two groups

Sterile soil infested with small amount of inoculum, immediately dried and stored in refrigerator. Soil infested with the organism, then incubated allowing

The organism to grow thus, the mycelium and propagative unit of second generation are preserved. The soil preservation method is useful for fungi, and by this method actinomycetes are maintained in soil for 4 to 5 years, and there are several bacterial spp which are also maintained in soil for several years.

Merits

We can remove some of the growth under the oil with a transfer needle, inoculate afresh medium, and still preserve the original culture. The simplicity of the method makes it attractive, but changes in the characteristics of a strain can still occur.

Paraffin Method/Preservation by Overlaying Cultures with Mineral Oil

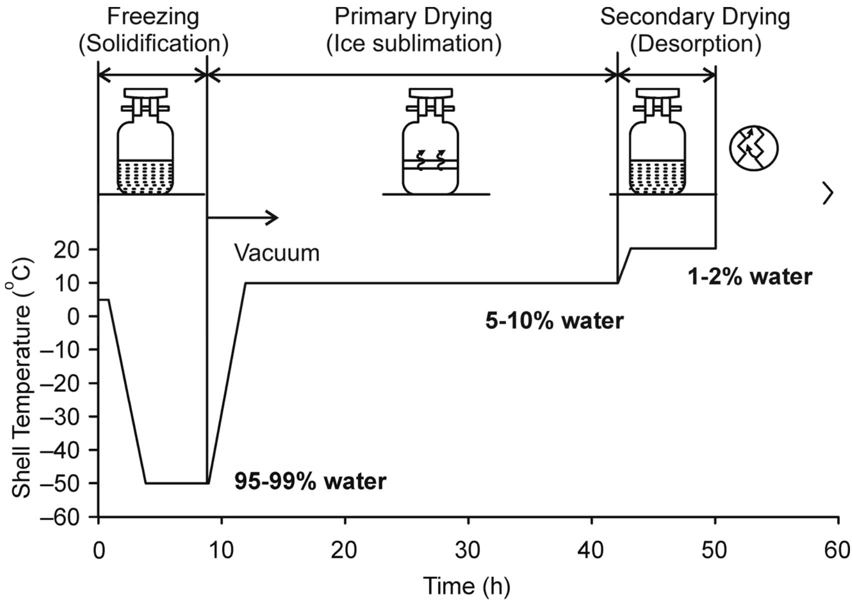
- Simple, most economical method. Agar slants are inoculated & incubated.
- Then, covered with sterile mineral oil to a depth of 1cm above the tip of slant surface.
- Transfers are made by removing a loop full of growth touching the tip to the glass surface to drain off excess il-inoculating a fresh medium-preserving the initial stock culture.
- Functions-provinding an aerobic condition, prevents the dehydration of the medium and decreases the metabolic rate of the organisms.

Lyophilization or freeze drying

Freeze drying is a stabilizing process in which a substance is first frozen and then the quantity of the solvent is reduced, first by sublimation (primary drying stage) and then desorption (secondary drying stage).

Better preservation occurs with freeze-drying than with other methods because freeze-drying reduces the risk of intracellular ice crystallization that compromises viability, removal of water from specimen effectively prevents this damage. Lyophilisation is greatest with gram-positive bacteria (spore formers) and decrease with gram -negative bacteria but viability can be maintained as long as 30 years.

Large numbers of vials of dried microorganisms can be stored with limited space, and organisms can be easily transported long distances at room temperature. The process combines freezing and dehydration- Organisms are initially frozen and then dried by lowering the atmospheric pressure with a vacuum apparatus. Specimens can be connected individually to the condenser (manifold method) or can be placed (in a chamber) where they are dehydrated in one larger airspace.



Storage Vials

Glass vials are used for all freeze-dried specimens. When freeze-drying is performed in a chamber, double glass vials are used.

Chamber method: an outer soft glass for protection and preservation of the dehydrated specimen. Silica gel granulesare placed in the bottom vial before the inner vial is inserted and cushioned with cotton.

Manifold method - a single glass vial is used. storage vial must be sealed to maintain the vacuum and the dry atmospheric condition.

Cryoprotective Agents Most commonly used agent is Skim milk for chamber lyophilisation.

Advantages: Removal of water at low temperature, Thermolabile materials can be dried, Sterility can be maintained, Reconstitution is easy.

Disadvantages

Many biological molecules are damaged by the stress associated with freezing, freeze- drying, or both. E.g. the process of drying causes extensive damage to moulds, protozoa, and most viruses, Hence, these microorganisms cannot be stored by this method. The product is prone to oxidation, due to high porosity and large surface area. Therefore, the product should be packed in vacuum or using inert gas.

Anaerobic bacteria culture

An anaerobic bacteria culture is a method used to grow anaerobes from a clinical specimen. Obligate anaerobes are bacteria that can live only in the

absence of oxygen. Obligate anaerobes are destroyed when exposed to the atmosphere for as briefly as 10 minutes. Some anaerobes are tolerant to small amounts of oxygen. Facultative anaerobes are those organisms that will grow with or without oxygen. The methods of obtaining specimens for anaerobic culture and the culturing procedure are performed to ensure that the organisms are protected from oxygen.

Cultivation of Anaerobic Bacteria

Main Principle is to 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 procedures 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. redox indicator dye called resazurin is added to the medium because the dye changes colour 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:

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 N2 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 CO2 by means of a cannula, restoppered, and incubated.

Fig 1.5. Anaerobic Chambers.

1. 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 hydrogen, 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).

2. Anaerobic Jar

GasPak systemAnaerobic 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.

Counting of microorganisms:

Counting of microorganisms can be done by different methods. In most cases the sample to be counted is first diluted to avoid overwhelming the whole counting procedure. However, in some cases, the sample may be too dilute to give the required minimum count to be able to estimate the microbial population of that sample. In such cases, concentration of the sample is carried out.

Counting can be done by use of a slide and a cover slip. A drop of the diluted sample is put on the slide with a suitable agent for proper visualization of the sample. It is then covered with a cover slip and put under a microscope and observed at a suitable magnification. The centre area can be dimensioned with etched grids. The number of microbes in the grids is multiplied by the dilution factor to get the number in the original sample.

The Petri dish count is where the sample is diluted to a point where the colonies will be statistically significant to be counted but not so many to overgrow each other. This method takes time for the individual cells to grow into colonies. The colonies counted are multiplied by the dilution factor to get the number in the original sample. The results here are expressed in colony forming units per milli liter i.e. CFU/ML. The time taken for the cells to grow into individual colonies is called the incubation period.

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 employment of a microscope.

For instance, when determining total microbial count in water by pour plate, a known volume of water is mixed with molten yeast-malt extract agar and given time to solidify. This is done on several plates. One set of plates are incubated at 37°C for about 24 hours and the other set of plates are incubated at 20-22°C for 3 days. Most bacteria capable of growth in water do so well at 22°C than at higher temperatures. While the microbes that grow well at 37°C will not grow very well in water. This means that the two types of microorganisms need to be counted differently since they differ in their growth pattern. In this case, carrying out of total count on water is beneficial in several ways.

It helps to evaluate the efficiency of certain water treatment processes like coagulation, flocculation and disinfection. It also gives an indication of the level of cleanliness of the water distribution system. It can also be used it determine the suitability of water supply to firms where food and drinks are prepared on large scale.

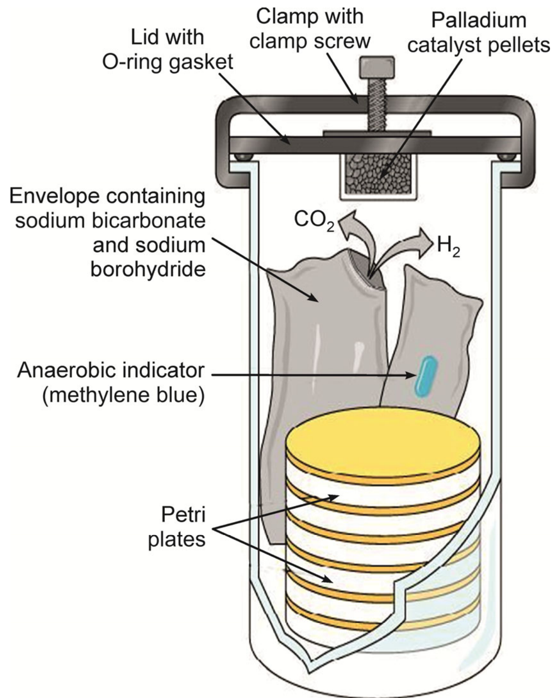
Total count is achieved either by use of direct or indirect counts. One method of direct count is the use of a haemocytometer. A haemocytometer is a specialized microscope slide important in cell counting. The central part of this slide has etched grids with precisely spaced lines to enable accurate 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. If this requirement is not met, necessary adjustments by either dilution or concentration are done as necessary.

In indirect counts, one method is by use of a colorimeter. As the microorganisms grow with time, they make the agar more and more turbid. This turbidity can be measured by use of a colorimeter where optical density is measured. The greater the optical density the greater the number of microbes.

There is also a measure of dry weight. This method involves centrifugation followed by weighing to get the dry weight. The limitation of this method is that cells are destroyed.

The other indirect count method involves the use of a coulter counter. A coulter counter is a probe which measures variation in conductivity of a solution as a bacteria passes through a narrow gap.

The advantage of direct and indirect counts is that the process can be automated but the disadvantage is that they cannot differentiate dead cells from living ones.



Viable count:

Viable count involves counting of colonies produced by only viable cells under favourable growth conditions. 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.

In pour plating, 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.

Phase-Contrast Microscopy

Phase-contrast microscopy visualizes differences in the refractive indexes of different parts of a specimen relative to unaltered light.

Phase-contrast microscopy is a method of manipulating light paths through the use of strategically placed rings in order to illuminate transparent objects. Dutch physicist Fritz Zernike developed the technique in the 1930s; for his efforts he was awarded the Nobel Prize in 1953. In phase-contrast microscopy, parallel beams of light are passed through objects of different densities. The microscope contains special condensers that throw light "out of phase" causing it to pass through the object at different speeds. Internal details and organelles of live, unstained organisms (e.g. mitochondria, lysosomes, and the Golgi body) can be seen clearly with this microscope.

A phase ring in condenser allows a cylinder of light to pass through it while still in phase. Unaltered light hits the phase ring in the lens and is excluded. Light that is slightly altered by passing through a different refractive index is allowed to pass through. Light passing through cellular structures, such as chromosomes or mitochondria is retarded because they have a higher refractive index than the surrounding medium. Elements of lower refractive index advance the wave. Much of the background light is removed and light that constructively or destructively interfered is let through with enhanced contrast.

Phase-contrast microscopy allows the visualization of living cells in their natural state with high contrast and high resolution. This tool works best with a thin specimen and is not ideal for a thick specimen. Phase-contrast images have a characteristic grey background with light and dark features found across the sample. One disadvantage of phase-contrast microscopy is halo formation called halo-light ring.

Electron Microscopy

Electron microscopy uses magnetic coils to direct a beam of electrons from a tungsten filament through a specimen and onto a monitor.

Fig 1.6. Electron Microscope a Modern Electron Microscope.

Electron microscopy uses a beam of electrons as an energy source. An electron beam has an exceptionally short wavelength and can hit most objects in its path, increasing the resolution of the final image captured. The electron beam is designed to travel in a vacuum to limit interference by air molecules. Magnets are used to focus the electrons on the object viewed.

There are two types of electron microscopes. The more traditional form is the transmission electron microscope (TEM). To use this instrument, ultra-thin slices of microorganisms or viruses are placed on a wire grid and then stained with gold or palladium before viewing, to create contrast. The densely coated parts of the specimen deflect the electron beam and both dark and light areas show up on the image. TEM can project images in a much higher resolution—up to the atomic level of thinner objects.

The second and most contemporary form is the scanning electron microscope (SEM). It allows the visualization of microorganisms in three dimensions as the electrons are reflected when passed over the specimen. The same gold or palladium staining is employed.

Electron microscopy has multiple applications. It is ideal to:

- explore the in vivo molecular mechanisms of disease;
- visualize the three-dimensional architecture of tissues and cells;
- determine the conformation of flexible protein structures and complexes;
- observe individual viruses and macromolecular complexes in their natural context.

Sample preparation can be critical to generate a successful image because the choice of reagents and methods used to process a sample largely depends on the nature of the sample and the analysis required.

Dark-Field Microscopy

Dark-field microscopes show a light silhouette of an organism against a dark background. In dark-field microscopy, the light reaches the specimen from an angle with the help of an opaque disk. The specimen appears lit up against a dark back ground. Dark-field microscopy is most useful for extremely small living organisms that are invisible in the light microscope Dark-field microscopes show a light silhouette of an organism against a dark background. Radiance Against a Dark Background

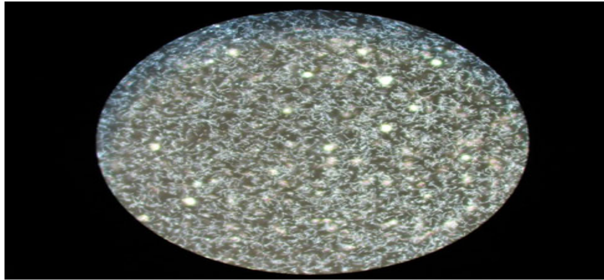


Fig 1.7. Visualization of live bacteria: Spirochetes bacteria observed under dark field microscopy.

Dark-field microscopy is ideally used to illuminate unstained samples causing them to appear brightly lit against a dark background. This type of microscope contains a special condenser that scatters light and causes it to reflect off the specimen at an angle. Rather than illuminating the sample with a filled cone of light, the condenser is designed to form a hollow cone of light. The light at the apex of the cone is focused at the plane of the specimen; as this light moves past the specimen plane it spreads again into a hollow cone. The objective lens sits in the dark hollow of this cone; although the light travels around and past the objective lens, no rays enter it.

The entire field appears dark when there is no sample on the microscope stage; thus the name dark-field microscopy. When a sample is on the stage, the light at the apex of the cone strikes it. The rays scattered by the sample and captured in the objective lens thus make the image.

Samples observed under dark-field microscopy should be carefully prepared since dust and other particles also scatter the light and are easily detected. Glass slides need to be thoroughly cleaned of extraneous dust and dirt. It may be necessary to filter sample media (agar, water, saline) to exclude confusing contaminants. Sample materials need to be spread thinly; too much material on the slide creates many overlapping layers and edges, making it difficult to interpret structures.

Dark-field microscopy has many applications in microbiology. It allows the visualization of live bacteria, and distinguishes some structure (rods, curved rods, spirals, or cocci) and movement.