

Introductory Microbiology



10. Introductory Microbiology (HBM 100) 2(1+ 1)

History and Scope of Microbiology: The discovery of micro-organism, spontaneous generation conflict, germ theory of diseases, microbial effect on organic and inorganic matter. Development of microbiology in India and composition of microbial world. **Microscopy and Specimen Preparation:** The bright field microscope, fixation, dyes and simple staining, differential staining. Difference between prokaryotic and eukaryotic cells. Prokaryotic cell structure and functions. Types of culture media and pre-culture techniques. Microbial growth in models of bacterial, yeast and mycelial growth curve. Measurement of bacterial growth. General properties of viruses and brief description of bacteriophages. General principle of bacterial genetics, DNA as genetic material. Antibiosis, symbiosis, in tramicrobial and extra microbial association.

Practical: Examination of natural infusion and living bacteria; examination of stained cells by simple staining and Gram staining. Methods for sterilization and nutrient agar preparation. Broth culture, agar slopes, streak plates and pour plates, turbidometric estimation of microbial Growth. .

INTRODUCTORY MICROBIOLOGY (1+1)

CHAPTER No 1

CONTENTS

- 1.1 Microbial evolution
- 1.2 History of Microbiology
- 1.3 Scope of Microbiology
- 1.4 Microbial effect on organic and inorganic matter
- 1.5 Composition of microbial world
- 1.6 Classification of living world

Microbiology is the study of living organisms of microscopic size, which include bacteria, fungi, algae and protozoa, and the infectious agents.

The organisms with a diameter of 1mm or less which cannot be seen by human eye, unaided are called **microorganisms**.

1. Groups of microorganisms

The major groups of protists are briefly described below. Although viruses are not protists or unicellular organisms, they are included for two reasons:

- a) The techniques used to study viruses are microbiological in nature and
- b) Viruses are causative agents of diseases, hence, diagnostic procedures for their identification are employed in the clinical microbiological laboratory as well as the plant pathology laboratory.

Algae:

Algae are relatively simple organisms. The most primitive types are unicellular. Others are aggregations of similar cells with little or no differentiation in structure or function. Still other algae, such as the large brown kelp, have a complex structure with cell types, specialised for particular functions. Regardless of size or complexity, algal cells contain chlorophyll and are capable of photosynthesis. Algae are found most commonly in aquatic environments or in damp soil.

Viruses:

Viruses are very small non-cellular parasites or pathogens of plants, animals and bacteria as well as other protists. They are so small that they can be visualized only by the electron microscope. Viruses can be cultivated only in living cells.

Bacteria:

Bacteria are unicellular prokaryotic organisms or simple associations of similar cells. Cell multiplication is usually by binary fission.

Protozoa:

Protozoa are unicellular eukaryotic organisms. They are differentiated on the basis of morphological, nutritional and physiological characteristics. Their role in nature is varied but the best known protozoa are the few that cause disease in human beings and animals.

Fungi:

Fungi are eukaryotic lower plants devoid of chlorophyll. They are usually multicellular but are not differentiated into roots, stems and leaves. They range in size and shape from single celled microscopic yeasts to giant multicellular mushrooms and puffballs. We are particularly interested in those organisms commonly called molds, the mildews, the yeasts and the plant pathogens known as rusts. True fungi are composed of filaments and masses of cells which make up the body of the organism known as mycelium. Fungi reproduce by fission by budding or by means of spores borne on fruiting structures that are quite distinctive for certain species.

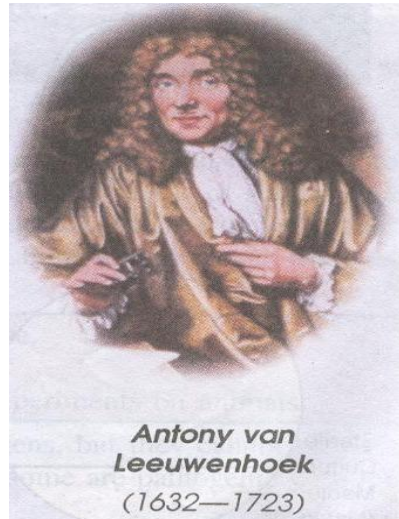
Microbiologists may specialize in the study of certain groups of microorganisms. Strictly speaking, bacteriology is the study of bacteria, but the term is often used as a synonym for microbiology, Protozoology is the study of protozoa; a special branch of Protozoology called parasitology deals exclusively with the parasitic or disease producing protozoa and other parasitic **micro** and macroorganisms. Mycology is the study of fungi such as yeasts and molds. Virology is the science that deals with viruses. Phycology is the study of algae. Further specialization in some aspect of the biology of a particular group of organisms is not uncommon.e.g., Bacterial genetics, algal physiology and bacterial cytology.

Importance of microorganisms:

Microorganisms affect the well being of people in a great many ways. As we have already stated, they occur in large numbers in most natural environments and bring about many changes, some desirable and others undesirable. The diversity of their activities ranges from causing diseases in humans, animals and plants to the production and deposition of minerals, the formation of coal and the enhancement of soil fertility.

There are many more species of microorganisms that perform important roles in nature than there are diseases producing species.

Although microorganisms have existed for a long time, their existence was unknown until the invention of the microscope in the 17th century. The year 1674, marks the birth of microbiology when Antony van Leeuwenhoek, a dutch merchant, looked at a drop of lake water through a glass lens which he had ground. He described a variety of microorganisms such as protozoa, algae, yeast and bacteria. He prepared 400 simple microscopes capable of enlarging objects from 50 to 300 times.



1.1 Microbial evolution:

The discovery of microbial world immediately raised questions regarding the origin of microorganisms. It was then recognized that living organisms such as plants and animals do not originate spontaneously. Yet, some believed that these microorganisms arose spontaneously and this theory came to be known as the theory of spontaneous origin or abiogenesis.

One of the first to provide evidence that microorganisms do not arise spontaneously in organic infusions was the Italian, **Lazzaro spallanzani**, who conducted number of experiments on this problem. He could show repeatedly that the growth of microorganisms in infusions can be prevented by heating. However, if such infusions were exposed to air after heating, these underwent spoilage. He concluded that, air contained microorganisms and these were carried into the infusions which were kept open after heating and subsequently caused spoilage. To further prove his point, he sealed the flasks after heating and when this was done, the infusions remained sterile. He concluded that microorganisms do not grow in the infusions, if the infusions are boiled and sealed to prevent the entry of air.

Francesco Redi (1665); Who put the theory of spontaneous generation. He demonstrated that the developing maggots coming from meat were the larvae of flies. The maggots could not develop when the meat was protected by placing it in vessel covered with fine gauze. He disproved the spontaneous formation of living being from non-living matter and is known as the doctrine of spontaneous generation or abiogenesis.

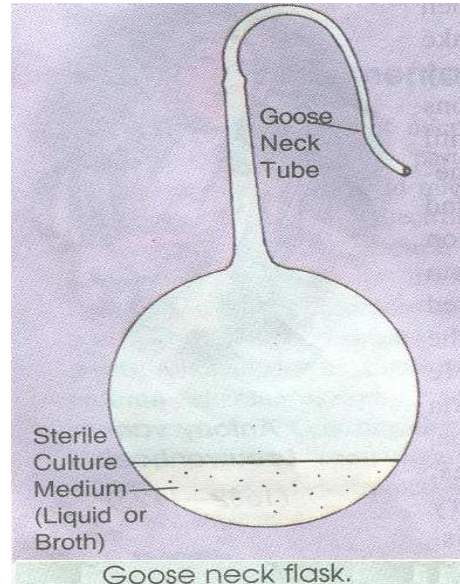
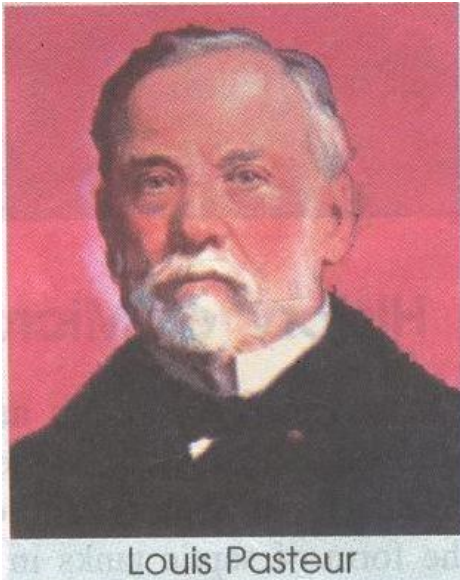
Louis Pasteur(1822-1895):Louis Pasteur began his brilliant career as professor of chemistry at the University Lille, French. A principal industry of France being the manufacture of wines and beer. Pasteur studied the methods and processes involved in order to help his neighbours produce a consistently good product. He found that fermentation of fruits and grains, resulting in alcohol was brought about by microbes by examining many batches of “Ferment”, he found microbes of different sorts. In good lots one type predominated, and in the poor products another kind was present. By proper selection of the microbe, the manufacturer might be assured of a consistently

good and uniform product. Pasteur suggested that the undesirable types of microbes might be removed by heating- not enough to hurt flavour of the fruit juice, but enough to destroy a very high percentage of the microbial populations. He found that holding the juices at a temperatures of 62.8⁰C (145⁰F) for half an hour did the job. Today pasteurization is widely used in fermentation industries, but most familiar with it in the dairy industry.

Pasteur's success in solving the problem of fermentation led the French government to request that he investigate pebrine, a silkworm disease that was ruining an important French industry. For several years Pasteur struggled with this problem, heartaches and disappointments following one after another. Eventually he isolated the parasite causing the disease. He also showed that silkworm farmers could eliminate the disease by using only healthy, disease free caterpillars for breeding stock.

Turning from silk to wool, Pasteur next tackled the problem of anthrax, a disease of cattle, sheep and sometimes human beings. In 1880, Pasteur isolated the organism causing chicken cholera. In one of his inoculation experiments, he found that the old cultures of the organism, when used as inocula, were non-infective, which incidentally led to the discovery of an avirulent bacterium, which could induce antibody formation in an experimental animal. He showed through further studies that the presence of antibodies in the animal would prevent subsequent infections by the virulent culture of the pathogen. His subsequent studies resulted in the development of 'attenuated cultures' or 'vaccines' for the prevention of anthrax. The success attained in these studies enabled him to concentrate on human diseases and so he turned his attention to rabies a disease transmitted to man by dog bite. He prepared vaccines against rabies from infected rabbits. In one of his experimental studies with the anthrax bacillus, he observed that a contaminating organisms in the bacterial culture completely suppressed the pathogenic quality of the culture and he stated that if the phenomenon could be explored, it would be of great use in curing diseases of man and animal. The phenomenon referred by Pasteur is 'antibiosis' and subsequent investigation on this have led to the discovery of antibiotics, the 'wonder drugs' now widely used to cure diseases of man and animal.

Louis Pasteur showed that growth did not occur in infusions which had been heated but exposed to air provided the incoming air was treated to remove the microorganisms. He first demonstrated that air contains microscopically observable organised structures. He aspirated a large quantity of air through a tube that contained a plug of gun cotton to serve as a filter. The gun cotton was then removed and dissolved in a mixture of alcohol-ether and the sediment was examined microscopically. He found that this sediment contained not only organic matter but also a large number of small round structures which were identical to microorganisms.



To prove that infusions which are once heated can be kept sterile, if kept away from air containing microorganisms, he sterilized infusions in flasks with open S-shaped tubes. These flasks containing sterile infusions had free access to air but the air has to pass through the S-shaped tube. Dust particles and microorganisms in the air settled at the bend and did not reach the infusion, thus keeping the infusion sterile.

Support to Pasteur's conclusion, John Tyndall—1828, who showed that sterile infusions placed in a dust free chamber could remain sterile indefinitely even if kept exposed to air.

Microorganisms exist in two forms—a heat labile form (vegetative) and heat resistant form (endospores). Intermittent heating and cooling to preserve food materials – Tyndallization.

1.2 History of Microbiology

In the poem *De rerum natura*, Lucretius (967-55 B.C) mentioned the existence of “seeds” of disease. India can also take pride in contributing to the development of ancient microbiology in the form of septic tanks in Mohenjodaro and Harappa regions (3000 B.C.). But the existence of microbes was not established until Antony van Leeuwenhoek (1677) could see them in simple (one-lens) microscope. Leeuwenhoek, a cloth merchant in Delft, Holland spent much of his time in grinding tiny lenses of high magnification (300 x or so), He took the scurf from the root of a decayed tooth and mixed it with clean rain water, and saw the mobility in animalcules. He discovered major classes of bacteria (spheres, rods, and spirals), protozoa, algae, yeasts, erythrocytes, spermatozoa, and the capillary circulation. Leeuwenhoek's discoveries were described in a flow of letters to the Royal Society of London.

Aristotle (384-322 B.C) emphasized that animals might evolve spontaneously from the soil, plants or other unlike animals. Virgil (70-19 B.C.) also gave opinion for the artificial propagation of bees. Discoveries about spontaneous generation persisted till 17th century.

1. Spontaneous Generation of Organisms

The theory of spontaneous generation states that the microbes arise automatically in decomposing organic matter. In the 17th century Francesco Redi worked out the appearance of maggots in decomposing meat depended on the decomposition of eggs by flies, but the idea of spontaneous generation persisted for the new world of microbes. T. Needham in 1748 experienced the appearance of organisms not present previously and concluded that these organisms appeared from the decomposition of the vegetables and meat. Later, Spallanzani (1729-1799) introduced the use of sterile culture media; he showed that infusion of meat, would remain clear indefinitely if boiled and properly sealed. This discovery was later on confirmed in the early 19th century, when a French confectioner, in the Appert, competing for a prize by Napoleon, developed the art of preserving food by canning. In 1837, Schwann obtained similar results even when air was allowed to fermentation and putrefaction were given by Schwann (1837) and Liebig (1839). To give more weightage, Schroder and von Dusch applied the use of cotton plug, to exclude air borne contaminants which is still in use.

2. Golden Era of Microbiology (1860-1910)

Golden era of microbiology started with the work of Louis Pasteur (France) and Robert Koch (Germany). John Tyndall (1820-1893) showed that the hay had contaminated his lab with an incredible kind of living organism. Ferdinand John (1877) demonstrated the resistant forms as small, refractile endospores, a special stage in the life cycle of hay bacillus (*Bacillus subtilis*). Since spores are readily sterilized in the presence of moisture at 120^o C, the autoclave, which uses steam under pressure, became hallmark of the bacteriology.

Pasteur (1857) became interested in fermentation products and observed different kind of microbes associated with different kind of fermentation: spheres of variable size (now known as yeast cells) in the alcoholic fermentation and smaller rods (lactobacilli) in the lactic fermentation. During this Experiment, Pasteur established the study of microbial metabolism and in particular he showed that life is possible without air. Pasteur explained that in grape juice the high sugar concentration and the low protein content (i.e low buffering power) lead to a low pH, which allows the outgrowth of acid-resistant yeasts and thus yields an alcoholic fermentation. In milk in contrast, the much higher protein and lower sugar content favour the outgrowth of fast growing but more acid-sensitive bacteria, which cause a lactic fermentation. This finding led Pasteur to state that specific microbes might also be causes of specific disease in man.

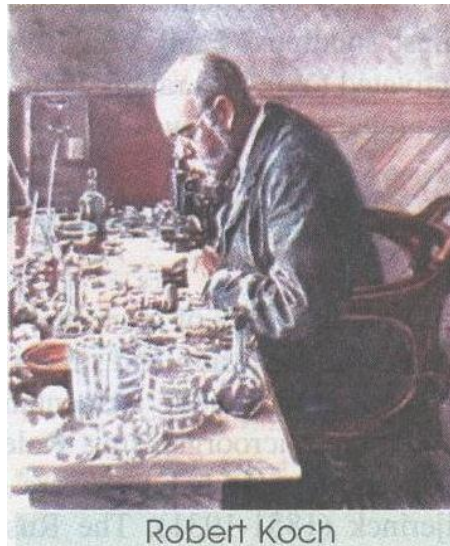
Pasteur developed the procedure of gentle heating (i.e. pasteurization) to prevent the spoilage of beer and wine by undesired microbes. This process was later used to prevent milk borne diseases of man. Of the great economic importance was the extension of industrial fermentations from the production of foods and beverages to that of valuable chemicals, such as glycerol, acetone, and later vitamins, antibiotics and alkaloids.

The unity of biology at a molecular level concept was developed when it was discovered that the carbohydrate metabolism pathways are similar in some microbes and in mammals. This discovery was made towards the end of the Pasteurian era notably in Russia and Beijerinck in Holland who discovered variety of metabolic patterns by different kinds of bacteria adapted to different ecological niches. The ecological niches is defined as the physical space occupied by an organism, but also its functional role in the community. These organisms were isolated by using

Pasteur's principle of selective cultivation: enrichment culture in which only a particular energy source is provided, and growth is restricted to those organisms that can use that source,

3. Germ Theory of Disease

The 'germ theory of disease' has presented a great stimulus in Microbiology and Medicine. Louis Pasteur and Robert Koch (1843-1910) were the national heroes. Preventive measures also supported the germ theory. Edward Jenner (1796) introduced vaccination (*L. vacce, cow*) against small pox, using material from lesions of a similar disease of cattle (cowpox). In 1860s Joseph Lister introduced antiseptic surgery, on the basis of Pasteur's evidence for the ubiquity of airborne microbes.



Recognition of agents of infection first to be recognized were fungi: Agostino Bassi (1836) demonstrated that a fungus was the cause of disease (of silk worm), the etiologic role of bacteria was established by Koch (1876) for anthrax. The pure culture preparation is the key to the identification. Koch perfected the technique of identification including the use of solid media and the use of stain. After identifying the tubercle bacillus Koch formalized the criteria, introduced by Henle in 1840 but known as Koch's postulates, for distinguishing a pathogenic form an adventitious microbe:

1. The organism is regularly found in the lesion of the disease.
2. It can be isolated in pure culture.
3. Inoculation of this culture produces a similar disease in experiments on animals.

These criteria have proved invaluable in identifying pathogens, but they cannot be met: some organisms such as viruses cannot grow on artificial media and some are pathogenic only for man.

Golden era of microbiology was established between 1860 and 1910 because of development of powerful methodology. Moreover, various members of the German school isolated (in addition to the tubercle bacillus), the cholera vibrio, Typhoid *Bacillus*, Diphtheria, *Bacillus*, Pneumococcus, Staphylococcus, Streptococcus, Meningococcus, Gonococcus and Tetanus bacillus.

4. Viral Diseases and Immunization

The discovery of viruses and their role in disease was made possible when Charles Chamberland (1851-1908), one of Pasteur's associates constructed a porcelain bacteria filter in 1884. The first virus to be recognized as filterable was tobacco mosaic virus, discovered by Russian, named Iwanowaskii (1882) and by Beijerinck (1899) in Holland. On the otherhand, filterable animal viruses were discovered for foot and mouth disease of cattle by Löffler and Frosch (1898), and for a human disease, yellow fever by the US army commission. Twort in England and d' Herelle in France in 1916-1917 discovered viruses that infect bacteria i.e. bacteriophages. The first crystallization of virus was made by Stanley (1935).

After this discovery, it was a matter of great surprise to the scientist that how animals resisted disease. Pasteur observed that old cultures of the bacterium attenuated i.e. lost their disease causing ability, they remain healthy but developed the ability to resist disease. He called the attenuated culture as **Vaccine** in respect of Edward Jenner because, several years back Jenner had used vaccination with material from cowpox lesion to protect people against smallpox. Emil Von Boehringer (1854-1917) and Shibasaburo Kitasato (1852-1931) used inactivated toxin into rabbit, inducing them for antitoxin production. This is how a tetanus antitoxin was prepared and now used in the treatment.

5. Microbiology in 20th Century

The discovery of microbial effects on organic and inorganic matter started with the discovery of Theodore Schwann and others (1857) who observed that yeast cells are able to convert sugar to alcohol i.e. alcoholic fermentation. It was Pasteur's observations that revealed about anaerobic and aerobic microorganisms. Role of microorganisms in the carbon, nitrogen and sulphur cycles in soil and aquatic habitats were discussed by Sergei N. Winogradsky (1856-1953) and Martinus Beijerinck (1851-1931), The Russian microbiologist Winogradsky also discovered that (i) soil bacteria oxidize Iron, Sulphur and Ammonia to obtain energy, (ii) isolated anaerobic N₂ fixers and (iii) studied the decomposition of cellulosic organic matter. On the other hand, Beijerinck, contributed a lot in the area of microbial ecology. Azotobacter, a free living nitrogen fixer was isolated. Later a root nodulating bacterium named as Rhizobium and sulphate reducers were also isolated. Both these microbiologists developed the enrichment culture techniques and the use of selective media in the microbiology.

In 20th century, microbiology developed from the angle of other disciplines of biological sciences in such a way so that problems of cell structure to the evolution are solved. Although, more emphasis were laid down on the agents of infectious disease, the immune response, chemotherapeutic agents and bacterial metabolism.

Beadle and Tatum (1941) used mutants of the bread mold, *Neurospora* while Salvatore Luria and Max Delbruck (1943) used bacterial mutants to show that gene mutations were truly spontaneous and not directed by the environment. Avery, Macleod, and McCarty (1944) evidenced that DNA was the genetic carried genetic information. Such discoveries made microbiology, genetics and biochemistry as modern molecularly oriented genetics. Microbiology contributed maximum in molecular biology which deals with the physical and chemical aspects of living matter and its function. The genetic code and the mechanism of DNA, RNA and protein

synthesis were also studied by using several microorganisms. Regulation of gene expression and the control of enzymes activity were also discussed in the light of microbiology in 1970's new discovery such as recombinant DNA technology and genetic engineering were also led to development of microbiology which gave the service of microbial biotechnology.

Scientists of West Chester University, Pennsylvania have revived a microbe that had been in suspended animation for 250 million years, a remarkable feat which boosts theories that the ancient seeds for life arrived on Earth from space. Russell Vreeland (2003) isolated a spore forming *Bacillus* sp. From 250 years old sample of salt crystal found below ground (1850 ft.) in New Mexico. The bacterium seems to be similar to *Bacillus marismortui*. Earlier, there were reports of oldest living creatures of 254-40 million years.

Table: 1.1 : Nobel prizes awarded in the subject related in Microbiology Research (1945 onwards)

Name of Scientist	Area of research	Year
A. Fleming, E.B. Chain & H.W. Florey	Discovery of Penicillin and its therapeutic value	1945
M. Theiler	Development of vaccine against yellow fever	1951
S.A. Waksman	Discovery of streptomycin	1952
J.F. Enders, T.H. Weller & F. Robbins	Cultivations of poliovirus in tissue culture	1954
D. Bovet	Discovery of the first antihistamine	1957
G.W. Beadle, E.I. Tatum & J. Lederberg	Microbial genetics	1958
S. Ochoa & A. Kornberg	Discovery of enzyme catalyzing nucleic acid synthesis	1959
F.H.C. Crick	Discoveries related to DNA	1962
F. Jacob, A. Lwoff & J. Monod	Discoveries about the regulation of genes	1965
F.P. Rous	Discovery of cancer virus	1966
R.W. Holley, H.G. Khorana & M.W. Nirenberg	Deciphering of the genetic code	1968
M. Delbruck, A.D. Hershey & S.E. Luria	Discoveries concerning viruses and viral infection of cells	1969
G. Edelman & R. Porter	Research on the structure of antibodies	1972
H. Temin, D. Baltimore & R. Dulbecco	Discovery of RNA dependent DNA synthesis by RNA tumor viruses; reproduction of DNA tumour virus	1975
B. Blumberg & D.C. Gajdusek	Mechanism and dissemination of hepatitis B virus; research on slow virus infection	1976
R. Yalow	Development of the Radioimmuno assay technique	1977
H.O. Smith, D. Nathans & W.	Discovery of restriction enzymes and their	1978

Arber	application to the problem of molecular genetics	1981
B. Benaclavaf, G. Snell & J. Dausset	Discovery of the histo-compatibilty antigens	1980
P. Berg, W. Gilberg & F. Sanger	Development of DNA technique (Berg); specially of DNA sequencing techniques	1981
A. Klug	Development of crystallographics electron microscopy	1982
C. Milstein, G.J.F. Kohler & N.K. Jerne	Development of the technique for formation of monoclonal antibodies; theoretical work in immunology.	1984
E. Ruska	Development of the transmission electron microscope.	1986
S. Tonegawa	The genetic principle for generation of antibody diversity	1987
J. Deisenhofer, R. Huber & H. Nichel	Crystallization and study of the photosynthetic feaction center from a bacterial membrane.	1988
J.M. Bishop & H.E. Varmus	Discovery of oncogenes	1989
S. Altman & T.R. Cech	Discovery of ribosome	1989
K.B. Mullis & M. Smith	For discovery of PCR technique and development of site directed mutagenesis	1993
E. Lewis, C. Nusslein & E. Wieschans	Physiology of Genetics of microbes	1995
S.B. Prussiner	Discovery of prions	1997

Noteworthy Events in the Development of Industrial Microbiology

Year	Events
1857	L. Pasteur showed that Lactic acid formation is due to microorganisms.
1881	L. Pasteur developed anthrax vaccine.
1885	Gave rise rabies vaccine.
1887	Buchner discovered that yeast extract ferment sugar.
1921	Fleming discovered Lysozyme.
1923	First edition of Bergey's manual.
1929	Fleming discovered penicillin.
1933	Ruska developed electron microscope.
1935	Domagk discovered sulfa drugs.
1937	Chatton divided living organisms into prokaryotes and eukaryotes.
1941	Beadle and Tatum gave one gene one enzyme theory.
1944	Waksman discovered streptomycin.
1982	Recombinant hepatitis B vaccine developed.
1986	First vaccine (hepatitis B vaccine) produced by genetic engineering approved for

human use.

Scope of Microbiology

Microbiology is the study of living organisms of microscopic size, which include bacteria, fungi, algae, protozoa and the infections agents at the border line of life that are called viruses. It is concerned with their form, structure reproduction, physiology, metabolism, and classification. It includes the study of their distribution in nature, their relationship to each other and to other living organisms, their effects on human beings and on other animals and plants, their abilities to make physical and chemical changes in our environment, and their reactions to physical and chemical agents.

Microorganisms are closely associated with the health and welfare of human beings; some microorganisms are beneficial and others are detrimental. For example, microorganisms are involved in the making of yogurt, cheese, and wine; in the production of penicillin and alcohol; and in the processing of domestic and industrial wastes. Microorganisms can cause disease, spoil food, and deteriorate materials like iron pipes, glass lenses, and wood pilings.

There are many more species of microorganisms that perform important roles in nature than there are disease producing species.

A summary of the major fields of applied microbiology are,

Field	Applied areas
1) Medical Microbiology-	Causative agents of disease; diagnostic procedures for identification of causal agents; preventive measures.
2) Aquatic Microbiology-	Water purification; microbiological examination; biological degradation of waste; ecology.
3) Aero-Microbiology-	Contamination and spoilage; dissemination of diseases.
4) Food Microbiology-	Food preservation and preparation; food borne diseases and prevention.
5) Agricultural Microbiology-	Soil fertility; plant and animal diseases
6) Industrial Microbiology-	Production of medicinal products such as antibiotics, vaccines, fermented beverages, industrial chemicals, production of proteins and hormones by genetically engineered microorganisms.
7) Exo-Microbiology-	Exploration of life in outer space.
8) Geochemical Microbiology-	Coal, mineral and gas formation; prospecting deposits of coal, oil and gas recovery of minerals from low grade ores.

Microbial effect on organic and inorganic matter:

There are two major groups of bacteria, the “eubacteria” and the recently discovered “archaeobacteria”. The eubacteria contain most of the common bacteria such as *Escherichia coli*,

and the cyanobacteria (blue green algae). The archaeobacteria are found mainly in the deep ocean near hydrothermal vents. What is striking from the stand point of the divergence of genetic material is that these two group of bacteria are more different than are animals and plants . In other words, these two groups of bacteria have evolutionary diverged further from one another than animals have diverged from plants.

Microbes must acquire certain elements to grow and reproduce these elements compose their protoplasm (52.4% protein, C,H,N,O and S, 19.9% nucleic acid 16.6% polysaccharide and 9.4% phospholipid). In addition, they must produce ATP in order to use the stored energy in this molecule to operate various cellular processes. Assimilative processes are used to bring needed elements into the cell and to incorporate them into the cell protoplasm. Dissimilative processes donot incorporate elements into the cell, but instead they use the energy gained in the process to form ATP.

Important impacts of microbes on ecosystems:

1.Generate oxygen in the atmosphere:

Almost all of the production of oxygen by bacteria on earth today occurs in the ocean by the cyanobacteria or “blue green algae”.

2. Recycle nutrients stored in organic matter to an inorganic form.

Decomposition releases the mineral nutrients (e.g. N,P,K) bound up in dead organic matter in an inorganic form that is available for primary producers to use. Without this recycling of inorganic nutrients, primary productivity on the globe would stop.

On land most of the decomposition (also called ‘mineralization’) of dead organic matter occurs at soil surface, and the rate of decomposition is a function of moisture and temperature.

Fungi are important in terrestrial systems, but not in aquatic. They are present even before the leaves and twigs enter the soil and so decomposition starts in the living or senescent plant material. Fungi are the most important decomposers of structural plant compounds(cellulose and lignin-but not that lignin is not broken down when oxygen is absent). The fungi invade the organic matter in soils first and are then followed by bacteria.

In water, the decomposition of organic matter is mostly oxic in streams and in the ocean and anoxic in the bottoms of lakes or in swamps. Oxic decomposition proceeds faster than decomposition in environments where there is no oxygen. In the open ocean, the water is so deep (average 3900m) and contains so much oxygen, that most of the algal formed organic matter at the surface decomposes aerobically before it reaches the bottom. For example, only 2% of the primary productivity in the upper ocean sinks to a depth of 3500m. Most of the world is ocean, and most of the ocean is deep, so most of the aquatic decomposition must be aerobic. But, in shallow waters, coastal oceans lakes and estuaries 25-60% of the organic matter produced may settle out of the upper waters rapidly and be decomposed anaerobically.

Of course another important impact of decomposition besides generating inorganic nutrients is to produce CO₂ and CH₄ that is released to the atmosphere.

3) Fix nitrogen from the atmosphere into a useable form:

The only organisms capacity of removing N₂ gas from the atmosphere and “fixing” it into usable nitrogen form (NH₃) are bacteria. The specific bacteria that can perform N fixation are

scattered throughout the groups including the cyanobacteria. All organisms that fix nitrogen use same mechanisms and the same enzymes. This ability probably involved only once and early in the history of life. Symbiotic nitrogen fixation costs the plant photosynthate to support the fixation and the NH_3 assimilation; this cost could be from 15-30% of the total carbon assimilated by the plant. In fact, to fix one molecule of N_2 requires about 25 molecules of ATP, so it is expensive from the bacterial stand point and that means that the plant must support that energy requirement. In turn the plant receives nitrogen, which may otherwise be a limiting nutrient.

Another difficulty for the bacteria is that one of the enzymes necessary for N_2 fixation is destroyed by oxygen (which is necessary for efficient ATP formation). One solution to this problem is to form symbiotic relationships with other organisms that can provide carbohydrates; these include diatoms, the fungi of certain lichens, shipworms, termites and certain plants especially in nodules of the roots.

4). Allow herbivores to consume poor quality food:

In the ocean, most of the primary productivity is consumed by herbivores. In contrast in terrestrial systems most of the primary productivity is not consumed by the herbivores. The reason for this difference are :1) animals lack digestive enzymes capable of using cellulose and lignin and other structural plant compounds; 2) plants often have anti-grazing toxins, aromatic resins, or thorns; 3) most land plant tissue is poor in mineral nutrients compared to the tissue in the herbivore.

In a ruminant animal (cattle, deer, giraffe) the ingested food, possibly regurgitated and re-chewed, passes into the rumen together with saliva. The rumen is really a continuous fermenter where the complex carbohydrates of the plant material are fermented into methane, carbon dioxide and fatty acids. The biota of the rumen are found in about equal biomasses of bacteria (10^{11} /ml), protozoans (10^5 /ml to 10^6 /ml) and fungi (poorly known biomass). About 60-65% of the total energy removed from the plant food that is ingested by the animal comes from rumen fermentation. Plant tissues passing from the rumen undergo secondary fermentation in the caecum and large intestine where an additional 8-30% of the total energy is provided. In addition, many termites contain protozoans and bacteria in their guts that perform similar operations. The protozoans are capable of digesting cellulose and bacteria in the gut generate CH_4 from the organic compounds released from the cellulose degradation. Finally, some termites also have bacteria in their guts that are capable of fixing nitrogen from the atmosphere, providing a useable nitrogen source for the termite.

5) Give plant roots access to nutrients in the soil:

Plant roots create a zone of a nutrient depletion around themselves. To have access to new sources of nutrients, a plant can either grow more roots and small root hairs or form an association with a fungus whose hyphae provide an even more efficient absorptive structure. Most vascular plants can form such associations, which are called "mycorrhizae". Mycorrhizal fungi include those living on the surface of plants (ectotrophic or sheathing) and those which enter the host (endotrophic or vesicular-arbuscular or simply "V-A").

The added advantage to the plant is that the hyphae can secrete enzymes that break down organic molecules and make inorganic nutrients available. While the plants gain nutrients, the

fungi gain carbohydrate food from the plant. There is also a cost to the plant in this association; one study reported that mycorrhizal biomass was only 1% of a fir forest ecosystem but used 15% of the net primary production.

Importance of Microorganisms to the Biosphere

Microorganisms are the foundation of the biosphere. Without them other life forms would not have evolved and could not exist. Microorganisms established the geochemical conditions on earth that enabled evolution of plants and animals. Plants and animals are descended from microorganisms and their cells are now known to be composites of microorganisms. For example the mitochondria of all plants and animals are derived from bacteria. Similarly, the photosynthetic organelle the chloroplast, found in all plants and algae are descended from a group of photosynthetic bacteria, the cyanobacteria. Cyanobacteria are believed to be the first organisms on earth to produce the free oxygen gas and concomitantly, the protecting ozone layer around earth, thereby providing condition for evolution of land plants and animals.

Humans and other animals, as well as plants are completely dependent on microorganisms for life. Like all animals, humans harbor billions of microorganisms in their digestive tracts, microorganisms necessary to digest food and provide nutrients for growth and a source of energy. Plants also require microorganisms to provide nutrients for growth an activity that takes place largely in root systems. There the organic materials such as nitrogen and phosphorus , the natural fertilizers made available by microorganisms and required by plants for growth and development.

Microorganisms exist everywhere physical conditions permit. Although lake water may appear transparent to the eye, a liter of the water can harbor a billion bacteria. A gram of soil can also contain over a billion bacteria. Many microorganisms have special dispersal cells that can be carried by wind across and between continents. In addition, birds and insects transport microorganisms as they fly. Thus, we live in a world teeming with microbial life that carries out a myriad of activities essential for sustaining the biosphere of earth.

Microorganisms are highly diverse genetically and metabolically, for more so than plants and animals. This should not seem surprising because microorganisms have existed on earth for over 3.5 billion years. From analysis of molecular sequences of genes such as 16s and 18s ribosomal RNA, approximately 20 separate main phylogenetic groups of microbial life have been identified, comparable in depth and breadth to the animal and plant kingdoms.

One of the most surprising characteristics of microorganisms in range of physiological conditions under which they flourish they grow across broad ranges of temperatures, pH, salt concentration and oxygen concentration. Some thrives at boiling temperature in hot spring and at temperatures higher than 100⁰ C in submarine vents. Other are found in sea ice off Antarctica and at the North pole. Some produce sulphuric and nitric acids, and many microbial species live without oxygen. Other live in saturated salt brines and some are resistant to high levels of radioactivity.

The variety of metabolic types of microorganisms is enormous. Some are photosynthetic and like plants produce oxygen in this process. In fact this “biotechnology” first occurred in the cyanobacteria, which subsequently evolved endosymbiotically to form chloroplasts that enable algae and plants to conduct photosynthesis. Other bacterial groups carry out photosynthesis by different pathways and produce products such as sulfur. Microorganisms are the primary, if not

sole agents responsible for degradation of a great variety of organic compounds including cellulose, hemicellulose, lignin, and chitin (the most abundant organic matter on earth). If it were not for microbial activities involved in natural decay, excessive amounts of organic matter would accumulate in forests and aquatic sediments. In addition microorganisms are responsible for degradation of toxic chemicals derived from anthropogenic sources, such as PCBs (polychlorinated biphenyls), Dioxins and other pesticides. Because microorganisms are so versatile, they are relied upon to digest water in sewage treatment plants, landfills, and toxic waste sites. It is in this regard that the field of bioremediation, encompassing all of the processes is still in its infancy. Much needs to be learned before microbial breakdown processes can be controlled and enhanced *in situ*.

Microorganisms play important roles in geochemical processes. For example the global nitrogen cycle in nature is dependent on microorganisms unique processes carried out by microorganisms include nitrogen fixation (the natural conversion of atmospheric denitrogen gas to utilizable organic cell nitrogen), oxidation of ammonia and nitrite to nitrate, and nitrate reduction with formation of dinitrogen and nitrous oxide gases. Similar important and unique roles are played in other cycles, such as sulfur and carbon cycles as well as in oxidation and reduction of metals. If it were not for microorganisms, substances such as cellulose and lignin would not be recycled; they would accumulate in the environment. Induced almost all organic substance are recycled via activities of bacteria, fungi and protozoa.

The importance of microorganisms in agriculture is enormous and extends beyond geochemical cycles. Indeed, most of the fertility of soil derived from microbial mineralization and in production of nitrogen for plant growth. These processes extended to lichen and cyanobacteria dominated soils which occupy a larger surface area on earth than in tropical rain forests. Mycorrhizal fungi form important rhizosphere associations with almost all plants. Such associations are essential for optimum growth and in fact permit some plants to grow in areas they could not otherwise colonize. Recent advances in agriculture stem from breakthroughs in the genetic engineering of plants; one of the most dramatic examples is that of the bacterium.

Agrobacterium tumefaciens normally the curative of crown gall disease in plants, this bacterium has been used to transfer favorable properties into an agriculturally important plant species there by providing a mechanisms for introducing genes that provide resistance to plant diseases, insect or pesticides into plants. Microorganisms are important in recycling waste materials. Sewage (waste water) treatment and the breakdown of garbage in landfills occur because of microorganisms. These microorganisms do this “for free” because in most cases they derive energy from the process.

A recent discovery indicates that microorganisms may influence weather. Some marine algae produce dimethyl sulphide (DMS). This compound is volatile and escapes into the atmosphere where it is photooxidized to form sulfate. The sulfate acts as a water nucleating agent and when enough sulfate is formed, clouds are produced; these clouds have three major impacts. First they shade the ocean and, thereby, slow further algal growth and DMS production, eventually decreasing cloud formation. Second the clouds lead to increased rainfall. And third, because clouds are reflective of incoming sunlight, the clouds reduce the amount of heat that reaches earth moderating global warming.

Microorganisms are at the core of biotechnology. Many antibiotics and antitumor agents are derived from microorganisms including penicillin, streptomycin and chloramphenicol. The emergence of multiple antibiotic resistant pathogenic bacteria has necessitated the search for new antibiotics. Because there are so many types of microorganisms they produce many unique products currently useful in biotechnology and offer great promise for exploitation in the future.

Composition of microbial world

Although most microorganisms are too small to be seen, their importance cannot be ignored. Microorganisms are the foundation of the biosphere both from an evolutionary and an ecological perspective. Microorganisms were the first organisms on earth; they have lived on this planet for a period of at least 3.7 billion years of the 4.6 billion year existence of the earth. Microorganisms were living inhabitants for more than 3.0 billion years before the appearance of plants and animals. Not only did plants and animals evolve rather recently in earth's history but they evolved from microbial life on mars also is consistent with the concept that microorganisms preceded plants and animals on earth.

The earth's biosphere is largely shaped by geochemical activities of microorganisms that have provided conditions both the evolution of plants and animals and for the continuation of all life on earth. Many microorganisms carry out unique geochemical processes critical to the operation of the biosphere. Therefore, it is not surprising that the diversity of microorganisms from genetic metabolic and physiological aspects is for greater than that found in plants and animals.

In contrast to plants and animals, the diversity of microbial world is largely unknown and of that, which is known, the diversity is spectacular. Some microorganisms live at boiling temperatures, or higher in hot spring and deep sea thermal vents; other live at temperature below freezing in sea ice. Some produce sulphuric and nitric acids. Many grow without oxygen and anaerobic activities of their microorganisms are necessary for carrying out the many essential processes in the environment that cannot be accomplished by plants and animals including methane production and nitrogen fixation. Such familiar activities are leavening bread and production of yogurt, pickles, wine, beer and cheeses rely on microorganisms carrying out the key processes.

Microorganisms also play other essential and beneficial functions for society. For example, we rely on them for production of antibiotics, antitumor agents, and a variety of biotechnology products. We use microorganisms to produce human insulin via genetic engineering and to provide enzymes for manufacturing. They are important in agriculture; their metabolic activities enhance soil fertility especially in their often unique roles in the nitrogen, phosphorus, sulphur & carbon cycles.

A new awareness of microbial diversity has developed in recent years. Advances in molecular biology have allowed biologist to compare all living organisms to one another on the basis of highly conserved genes. Initial studies focused on those genes that code for ribonucleic acid (RNA) of ribosome, the cellular structure responsible for protein synthesis in all organisms. In particular, the sequence on the bases of the small subunit (16s or 18s) of ribosomal RNA (r RNA) has been used to map the relationship of all living organisms.

The place of microorganisms in the living world:

In biology, as in any other field, classification means the orderly arrangement of units under study into groups of larger units. Present day classification in biology was established by the work of Carolus Linnaeus (1707-1778), a Swedish botanist. His looks on the classification of plants and animals are considered to be the beginning of modern botanical and zoological nomenclature, a system of naming plants and animals. Nomenclature in microbiology, which came much later, was based on the principles established for the plant and animal kingdoms.

Until the eighteenth century, the classifications of living organisms placed all organisms into one of the two kingdoms, plant and animal. As previously stated in microbiology we study some organisms that are predominantly plantlike, others the animallike and some that share characteristics common to both plants and animals. Since there are organisms that donot fall naturally into either the plant or the animals kingdom, it was proposed that new kingdoms be established to include those organisms which typically are neither plants nor animals.

Haeckel's kingdom Protista:

One of the earliest of these proposals was made in 1866 by a German zoologist, E. H. Haeckel. He suggested that a third kingdom, protista, be formed to include those unicellular microorganisms that are typically neither plants nor animals. These organisms **the** protists, include bacteria, algae, fungi and protozoa. (viruses are not cellular organisms and therefore are not classified as protists). Bacteria are referred to as lower protists; the others-algae, fungi and protozoa are called higher protists.

Bacteria are procaryotic microorganisms. The eucaryotic microorganisms include the protozoa, fungi and algae (plant and animals cells are also eukaryotic). Viruses are left out of this scheme of classification.

Whittakar's five kingdom concept:

A more recent and comprehensive system of classification, the five kingdom system, was proposed by R.H. Whittaker (1969). This system of classification, shown in figure, is based on three levels of cellular organization which evolved to accommodate three principal modes of nutrition; photosynthesis, absorption and ingestion. The prokaryotes are included in the kingdom **Monera**; they lack the indigestive mode of nutrition. Unicellular eukaryotic microorganisms are placed in the kingdom **protista**; all three nutritional types are represented here. Infact the nutritional modes are continuous; the mode of nutrition of the microalgae is photosynthetic; the mode of the nutrition of the protozoa is indigestive; and the mode of nutrition in some other protists is absorptive, with some overlap to the photosynthetic and indigestive modes. The multicellular and multinucleate eukaryotic organisms are found in the kingdoms Plantae (multicellular green plants and higher algae), Animalia (multicellular animals), and fungi (multinucleate higher fungi). Their diversified nutritional modes lead to a more diversified cellular organization. Microorganisms are found in three of the five kingdoms. Monera (bacteria and cyanobacteria), protista (microalgae and protozoa), and fungi (yeasts and molds).

FIGURE ?

Kingdom procarayote after Bergey's manual of systematic bacteriology:

Bergey's manual of systematic bacteriology places all bacteria in the kingdom prokaryotae which in turn is divided into four divisions as follows:

- 9) Division 1: Gracilicutes: Prokaryotes with a complex-cell wall structure characteristic of gram negative bacteria.
- 10) Division 2: Firmicutes: Prokaryotes with a cell-wall structure characteristic of gram positive bacteria.
- 11) Division 3: Tenericutes: Prokaryotes that lack a cell wall.
- 12) Division 4: Mendosicutes: Prokaryotes that show evidence of an earlier phylogenetic origin than those bacteria included in Division 1 and 2.

CHAPTER No 5

Microbiological stains

CONTENTS

5.1 Fixed, stained smears

5.2 Simple staining

5.3 Differential staining

5.4 Gram staining

5.5 Other differential stains

MICROBIOLOGICAL STAINS

Microbiological stains:

A large number of colored organic compounds (dyes) are available for staining microorganisms. These compounds are generally rather complex in terms of molecular structure. On the basis they may be classified into groups such as triphenylmethane dyes, oxygen dyes, and thiazine dyes.

A more practical classification for the cytologist is one based on the chemical behavior of the dye, namely, acid, basic or neutral. An acid (or anionic) dye is one in which the charge on the dye ion is negative; a basic (or cationic) dye is one in which the charge carried by the dye ion is positive. A neutral dye is a complex salt of a dye acid with a dye base, e.g. eosinate of methylene blue. Acid dyes generally stain basic cell components, and basic dyes generally stain acidic cell components.

The process of staining may involve ion-exchange reactions between the stain and active sites at the surface of or within the cell. For example, the colored ions of the dye may replace other ions on cellular components. Certain chemical groupings of cell proteins or nucleic acids may be involved in salt formation with positively charged ions such as Na^+ or K^+ . Thus, we might view these peripheral areas of the cell as carrying a negative charge in combination with positively charged ions; for example, (Bacterial cell-) (Na^+).

In a basic dye like methylene blue, the colored ion is positively charged (a cation) and if we represent this ion by the symbol methylene blue, the dye, which is actually methylene blue chloride, may be represented as **$\text{MB}^+ \text{Cl}^-$** .

The ionic exchange which takes place during staining can be represented by the following equation, in which the MB^+ cation replaces the Na^+ cation in the cell: (Bacterial cell-) (Na^+) + (MB^+) (Cl^-) – (Bacterial cell-) (MB^+) + (Na^+Cl^-).

5.1 Fixed, stained smears:

Fixed stained preparations are most frequently used for the observation of the morphological characteristics of bacteria. The advantages of this procedure are that 1) The cells are made more clearly visible after they are colored, and 2) Differences between the cells of different species and within the same species can be demonstrated by use of appropriate staining solutions.

The essential steps in the preparation of a fixed, stained smear 1) preparation of the film or smear 2) fixation and 3) application of one or more staining solutions.

5.2 Simple staining:

The coloration of bacteria by applying a single solution of stain to a fixed smear is termed simple staining. The fixed smear is flooded with a dye solution for a specified period of time, after which this solution is washed off with water and the slide is blotted dried. The cells usually stain uniformly. However, with some organisms, particularly when methylene blue is used, some granules in the interior of the cell may appear more deeply stained than the rest of the cell, indicating a different type of chemical substance.

5.3 Differential staining:

Staining procedures that make visible the differences between bacterial cells or parts of a bacterial cell are termed differential staining techniques. They are slightly more elaborate than the simple staining technique in that the cells may be exposed to more than one dye solution or staining reagent.

5.4 Gram staining:

One of the most important and widely used differential staining technique in microbiology is gram staining. This technique was introduced by Christian gram in 1884. In this process the fixed bacterial smear is subjected to the following staining reagents in the order listed; crystal violet, iodine solution, alcohol (decolorizing agent) and safranin or some other suitable counter stain. Bacteria stained by the gram method fall into two groups; gram positive bacteria, which retain the crystal violet and hence appear deep violet in color and gram negative bacteria which lose the crystal violet are counter stained by the safranin, and hence appear red in color.

The most possible explanations for this phenomenon are associated with the structure and composition of cell wall. Differences in the thickness of cell walls between these two groups may be important; the cell wall of gram negative bacteria are generally thinner than those of gram positive bacteria. Gram negative bacteria contain a higher percentage of lipid than gram positive bacteria. Experimental evidence suggests that during staining of gram negative bacteria the alcohol treatment extracts the lipid, which results in increased porosity or permeability of cell wall. Thus the crystal violet iodine (CV-I) complex can be extracted and the gram negative organism is decolorized. These cells subsequently take on the color of the safranin counterstain. The cell walls of gram-positive bacteria because of their different composition (lower lipid content) become dehydrated during treatment with alcohol. The pore size decreases, permeability is reduced and the CV-I complex cannot be extracted. Therefore these cells remain purple violet.

The gram stain has its greatest use in characterizing bacteria. This staining technique is not generally applicable for other groups of microorganisms such as protozoa and fungi, however, yeasts consistently stain gram-positive.

5.5 Other differential stains:

There are numerous other staining techniques designed to identify some particular feature of cell structure or composition. These techniques are summarized below.

Sl. No	Name of the staining technique	Application
1	Acid fast stain	Distinguishes acid fast bacteria such as <i>Mycobacterium Spp.</i> from non-acid fast bacteria.
2	Endospore stain	Demonstrates spore structure in bacteria as well as free spores.
3	Capsule stain	Demonstrates presence of capsules surrounding cells.
4	Flagella stain	Demonstrates presence and arrangement of flagella.
5	Cytoplasmic inclusion stains.	Identifies intracellular deposits of starch, glycogen, poly phosphates, hydroxyl butyrate and other substances.
6	Giemsa stain	Particularly applicable for staining rickettsia and some protozoa.

Study questions:

Q. No.I. Choose the correct answers from the following choices:

- The characterization of bacteria is done by
 - Simple stain
 - Gram stain
 - Both
 - Differential stain
- Yeasts consistently stain
 - Gram positive
 - Gram negative
 - Both
 - None of these
- Protozoa are stained by
 - Gram stain
 - Geimsa stain
 - Capsule stain
 - None of these
- Endospores are stained by

- a. Crystal violet b. Methylene blue c. Both d. None of these
5. The cell wall of Gram negative bacteria is
- a. Thinner b. Thicker c. Both d. None of these
6. The cell wall of Gram positive bacteria is
- a. Thinner b. Thicker c. Multilayer d. None of these
7. Decolorizing agent is
- a. Methanol b. Acetone c. Ethyl alcohol d. None of these
8. Lipid content is more in
- a. Gram-ve bacteria b. Gram +ve bacteria c. Both d. None of these
9. Capsule stain demonstrates
- a. Endospore b. Exospore c. Capsule d. Slime
10. Non-acid bacteria is distinguished by
- a. Flagella stain b. Endospore stain c. Acid fast stain d. All of these

Q.No.II. Match the following:

- | | |
|--------------------------|----------------------------|
| 1. Geimsa stain | a. Christian Gram |
| 2. Acid fast Stain | b. Rickettsia |
| 3. Counter stain | c. Mycobacterium |
| 4. Differential staining | d. Safranin |
| 5. Neutral dye | e. Positively charged |
| 6. Acid dye | f. Pink or red |
| 7. Basic dye | g. Purple violet |
| 8. Gram-ve bacteria | h. Crystal violet |
| 9. Gram+ve bacteria | i. Negatively charged |
| 10. Primary stain | j. Eosinate methylene blue |

Chapter 6-7. STRUCTURE AND ORGANIZATION OF MICROORGANISMS.

CONTENTS

6.1 Structure and Organization of Microorganisms

6.1.2 The size, shape and arrangements of bacterial cells

6.1.3 Shape and arrangement

6.1.4 Size

6.2 Bacterial structures

6.5.1 Structures external to the cell wall

6.6 Flagella and Motility

6.6.1 Movement

6.7 Archaeobacteria

6.8 Capsules and slime

6.9 Pili (*Fimbriae*)

6.10 Sheaths

7.1 Prosthecae and stalks

7.2 Cell envelope

7.3 Techoic acids

7.4 Cell membrane

7.5 Cytoplasm

7.6 Ribosomes

7.7 Cellular reserve materials

7. 7.1 Spores and cysts

7. 7.2 Endospores

7.7.3 Cysts

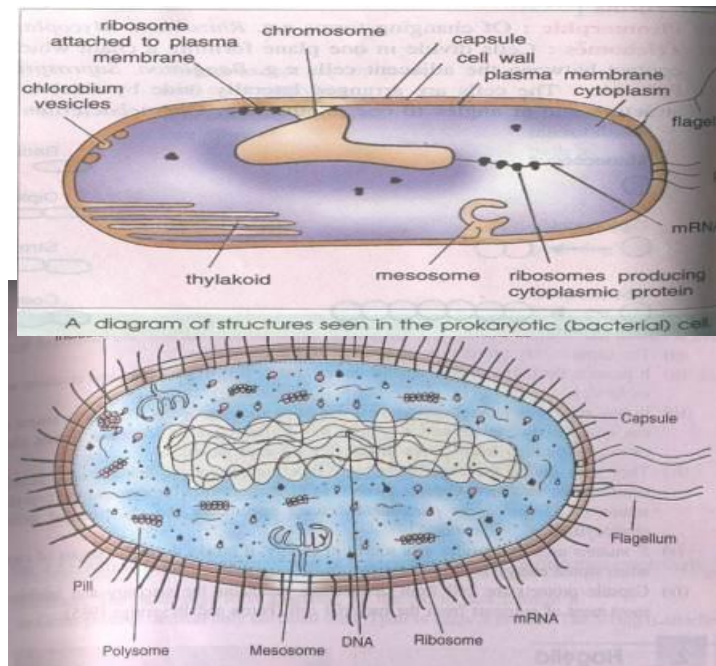
6.1 Structure and organization of bacteria

Prokaryotes: Pro=Primitive **Karyote**=Nucleus

Are those microorganisms which lack distinct nucleus and plastids. Among the major characteristics of bacterial cells are their size, shape, structure and arrangement. These characteristics constitute the morphology of cells. Depending on the species, individual cells are spherical, rod like or helical, although many variations of these three basic shapes occur.

The bacterial cell possess a detailed internal structure. The discovery of this internal structure was made possible by the development of electron microscope techniques and of instruments for slicing a bacterial cell into extremely thin sections. The terms microbial cytology and bacterial anatomy have become common place in microbiological literature.

The various structure of a bacterial cell differ from one another not only in their physical features but also in their chemical characteristics and in their functions. Thus biologists today seek to integrate the structure, chemical and functional properties of the bacterial cell.



A Typical bacterial cell

prokaryotic

The size, shape and arrangements of bacterial cells

Size:

Bacteria are very small, most being approximately 0.5 to 1.0 micrometer in diameter. A relatively large surface through which nutrients can enter compared to small volume of cell. Hence, bacteria have high rate of growth and metabolism.

Shape and arrangement:

The shape of the bacterium is governed by its rigid cell wall, however, exactly what attribute of this rigid material determines that a cell will have a particular shape is not yet understood. Typical bacterial cells are spherical (cocci), straight rods (Bacilli), or rods that are helically curved (spirilla), although most bacterial species have cells that are of fairly constant and characteristic shape, some have cells that are pleomorphic i.e that can exhibit a variety of shape.

Bacterial cells are usually arranged in a manner characteristics of their particular species. Although, it is rare that all the cells of a species are arranged in the same manner, it is the predominant arrangement that is the important feature.

Cocci appear in several characteristic arrangements depending on the plane of cellular division and whether the daughter cells stay together following division.

- A. **Diplococci** - Cells divide in one plane and remain attached predominantly in pairs.

- B. **Streptococci**- Cells divide in two planes and remain attached to form chains.e. g., *Streptococcus*
- C. **Tetrads (Tetracocci)**-Cells divide in two planar and characteristically forms groups of four cells. e. g., *Aerococcus*
- D. **Staphylococci**- Cells divide in three planes, in an irregular pattern, producing in bunches of cocci. E. g., *Staphylococcus*
- E. **Sarcinae**- Cells divide in three planes in a regular pattern producing a cuboidal arrangement of cells.

Bacilli are not arranged in patterns as complex as those of cocci, and most occur singly or in pairs (Diplobacilli). But some species such as *B. subtilis*, form chains (Streptobacilli); others such as *Beggiatoa* and *Saprospira* spp, form trichomes, which are similar to chains but have a much larger area of contact between the adjacent cells.

In other *Bacillus* spp, such as *Corynebacterium diphtheriae* the cells are lined side by side like match sticks (palisade arrangement) and at angles to one another.

Still others, such as *Streptomyces* spp, form long, branched, multinucleate filaments called hyphae, which collectively form mycelium.

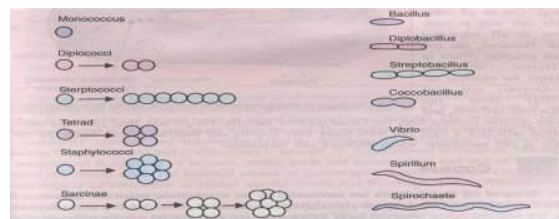
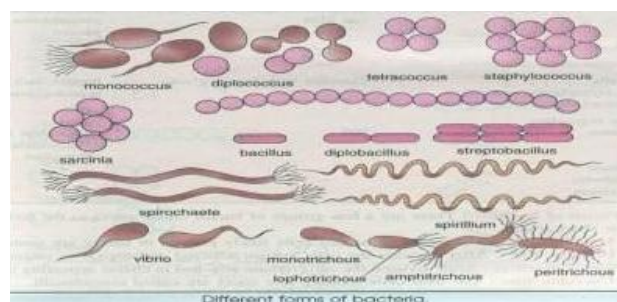


Diagram of cocci, bacilli and spiral bacteria.

Curved bacteria are usually curved with a twist. Bacteria with less than one complete twist or turn have a *Vibrioid* shape. Whereas those with one or more complete turns have a helical shape, *Spirilla* are rigid helical bacteria, whereas spirochetes are highly flexible.

Bacterial structures

Examination of bacterial cells reveals various component structures. Some of these are external to the cell wall. Others are internal to the cell wall. Some structures are present in only certain species; some are more characteristic of certain species than of others.



Structures

external to the cell wall

Flagellar arrangement

Flagella are arranged differently on different bacteria. In polar flagellation, the flagella are attached at one (e.g., *Pseudomonas aeruginosa*) or both the ends of the cell. Occasionally a tuft (group) of flagella may arise at one end of the cell (e.g., *Pseudomonas fluorescens*), an arrangement called lophotrichous. Tufts of flagella of this type can be seen in living cells by dark field microscopy, where the flagella appear light and attached to light colored cells against a dark background. In extremely large prokaryotes, tuft of flagella can also be observed by phase contrast microscopy (e.g., *Aquaspirillum serpens*). In peritrichous flagellation, the flagella are inserted by many locations around the cell surface (e.g., *Salmonella typhi*). The type of flagellation, polar or peritrichous, is used as one characteristic in the classification of bacteria as shown in the figure.

Flagella and Motility

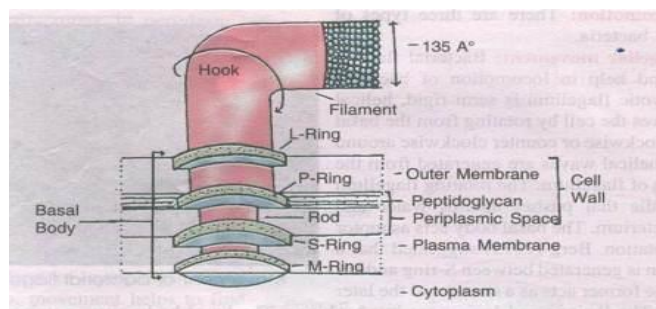
Bacterial flagella are hair like helical appendages that protrude through the cell wall and are responsible for swimming motility. They are much thinner than eukaryotes flagella and or cilia. Their location on the cell varies depending on the bacterial sp. and may be polar (at one end or both the ends of the bacterium) or lateral (along the sides of the bacterium).

A flagellum is composed of 3 parts, a basal body associated with the cytoplasmic membrane and the cell wall, a short hook and a helical filament which is usually several times as long as the cell. Some gram^{-ve} bacteria have a sheath surrounding the flagellum; this sheath continuous with the outer membrane of the gram^{-ve} bacterial cell wall. The chemical composition of the basal body is unknown but the hook and filament are composed of protein subunits arranged in helical fashion. The protein of the filament is known as flagellin.

Unlike a hair, a flagellum grows at its tip rather than at the base. Flagellin monomers synthesized within the cell are believed to pass along the hollow center of the flagellum and are added to the distal end of the filament.

Different parts flagellum in bacterium

Movement:



of attachment of a Gram-negative

The rings found in the basal body are probably involved. It is known that the flagellor motor is driven by the proton motive force (pmf) i.e the force derived from the electrical potential in the H^+ ion gradient across the cytoplasmic membrane. Recent studies suggests that the concentration of cGMP (guanosine 3', 5' cyclic phosphoric acid) within the cell governs the direction in which the rotation occurs.

Prokaryotic cell is characterized by the absence of the endoplasmic reticulum(ER) and the cytoplasm membrane is the only unit membrane of the cell. However, the membrane may be some times infolded deep into the cytoplasm. Electron microscopy of most prokaryotes reveals

only two internal regions, the cytoplasm and the nucleoplasm. The cytoplasm is granular in appearance and contains ribosomes that are smaller in size as compared to eukaryotic ribosomes. The nucleoplasm is fibrillar and contains DNA. Except, in mycoplasmas, other prokaryotes contain a defined rigid cell wall. No membranous structures similar to the mitochondria or chloroplasts are seen in the prokaryotes. Cytoplasm membrane is the site of respiratory electron transport in prokaryotes. In photosynthetic bacteria, the photosynthetic apparatus is located in a series of membranous, flattened structures similar to the thylakoids and these structures are not organised into chloroplasts but are dispersed in the cytoplasm. The cytoplasmic membrane contains specific sites for DNA attachment and also has a major role in cell division. The cell membrane, unlike in the eukaryotic cell does not generally contain sterols and poly unsaturated fatty-acids. The genetic material located in the nucleolus which lacks a defined nuclear membrane. It consists of double helical DNA without any associated basic proteins and also contains extra chromosomal DNA, which can replicate autonomously, called as plasmids.

Most prokaryotes possess a cell wall which is different in composition from that of eukaryotes and invariably contains a rigid polymer known as the peptidoglycan. Some prokaryotes possess this rigid structure show active movement with the aid of flagella. Some prokaryotes also show a gliding motility as in the blue green bacteria.

Table: Major differences in Eukaryotic and prokaryotic cellular organization.

Sl No	Cellular organisation	Eukaryotes	Prokarotes
1	Nuclear membrane	+	-
2	Chromosome	>1	1
3	Nucleus	+	-
4	Histones	+	-
5	Golgi	+	-
6	ER	+	-
7	Mitochondria	+	-
8	Chloroplastids	+	-
9	Lysosomes	+/-	-
10	Cytoplasmic ribosomes	80s	70s
11	Organellular ribosomes	70 s	-
12	Presence of peptidoglycan in cell wall	-	+ or -

Bacteria can be broadly divided into two major groups based on their gram staining characteristics such as Gram-positive and Gram-negative. The gram staining technique, the most widely used differential staining technique employed in bacteriology was first developed by

Christian gram in the 19th century and is used primarily to divide bacteria into two broad groups.

Table: The general features of the gm +ve and gm-ve bacteria are as follows

Gram + ve	Gram –ve
1) Capsules	1) Capsules
2) Peptidoglycan	2) Fimbriae
3) Cytoplasm	3) Cytoplasm
4) Flagellum	4) Flagellum
5) Mesosome and cytoplasmic membrane	5) Peptidoglycan
6) Genetic material	6) Genetic material
7) Plasmids	7) Outer membrane
8) Ribosomes	8) Mesosomes & cytoplasmic membrane
	9) Plasmids
	10) Ribosomes

Most bacteria have at least two structures around the cytoplasm namely cell wall (envelope) and the cytoplasmic membrane. Some may have additional components such as the outer membrane as in the gm –ve bacteria, a capsule and slime.

Some bacteria which are motile have surface appendages with the aid of which they can move. In addition some others such as the gm -ve bacteria have short hair like structures called pilli (fimbriae) these are not essential for the growth and survival of the bacterial cells.

2. Capsules and slime:

Many prokaryotic organisms synthesise loose amorphous organic exopolymers which are deposited outside the cell wall called the capsules or the slime. The term capsule refers to the layer tightly attached to the cell wall while the slime layer is the loose structure that often diffuses into the growth medium. These structures are not essential for growth and development of bacterial cells but their presence confers certain advantages to the bacterial cells that possess these structures. In fact, many bacteria do not produce neither a capsule nor a slime and those which can produce can lose the ability to synthesize these compounds without any ill effects. Primary interest in these exopolymers was for determining their role in pathogenicity since most pathogenic organisms are found to produce a capsule or slime.

Composition of exopolymers varies with bacteria which contains amino acids (D- Glutamic acid L- Glutamic acid) or sugars (glucose, rhamnose, galactose, fructose and fructose and sugar derivatives).

Functions of capsule:

1. Capsule may prevent the attachment of bacteriophages.
2. It protects the bacterial cell against desiccation as it is hygroscopic and contains water molecules.
3. It may survive in natural environment due to its sticky property. After attachment they can grow on diverse surfaces. E.g., root surfaces, of human teeth and tissues, rocks in fast growing stream etc.

4. They may inhibit the engulfment by white blood cells (WBC's) and therefore contribute to virulence.
5. Protects the cell from desiccation, maintains the viscosity and inhibit the movement of nutrient from the bacterial cell.
6. Acts as sources of energy for some bacterial species.

3. Fimbriae and pili:

Fimbriae and pili are short filamentous structures composed of protein that extend from the surface of a cell. Fimbriae enable organisms to stick to surfaces, including animal tissues in the case of some pathogenic bacteria or to form pellicles or biofilms on surfaces. Notorious among these pathogens include *Salmonella typhimurium* (salmonellosis), *Neisseria gonorrhoeae* (gonorrhoea), and *Bordetella pertussis* (whooping cough).

Pili are structurally similar to fimbriae but are typically longer and only one or a few pili are present on the surface. Because they serve as receptors for certain types of viruses, pili can be seen under the electron microscope when they become coated with virus particles. Although possibly involved in attachment as for fimbriae, pili are clearly involved in the process of conjugation (a form of genetic exchange) in prokaryotes.

Many classes of fimbriae/pili are known, distinguished by their structure and function. One class called type IV fimbriae/pili is involved in an unusual form of motility in certain bacteria called twitching motility. Twitching motility is a type of movement on solid surfaces, where it is thought that rapid and reversible extension and retraction of the fimbriae allow the cell to crawl along the surfaces. Unlike other fimbriae, type IV fimbriae are found only at the poles of the cells and besides motility have been implicated as key host colonization factors in a variety of pathogens including *Vibrio cholerae* (cholera), and *Neisseria meningitidis*. Type IV fimbriae are also thought to mediate genetic transfer by the process of transformation in a wide variety of bacteria.

Pili are hollow, non-helical filamentous appendages that are thinner, shorter, and more numerous than flagella. They do not function in motility since they are found on non-motile as well as motile species. There are, however, several functions associated with different types of pili. One type known as the *F. pilus* (or sex pilus), serves as the port of entry of genetic material during bacterial mating. Some pili plays a major role in human infection by allowing pathogenic bacteria to attach to epithelial cells lining the respiratory, intestinal, or genitourinary tracts. This attachment prevents the bacteria from being washed away by the flow of mucous or body fluids and permits the infection to be established.

4. Sheaths:

Some species of bacteria, particularly those from fresh water and marine environments, form chains or trichomes that are enclosed by a hollow tube called a sheath. This structure is most readily visualized when some of the cells have migrated from it. Sheaths may some times becomes impregnated with ferric or manganese hydroxides, which strengthen them.

5. Prosthecae and stalks:

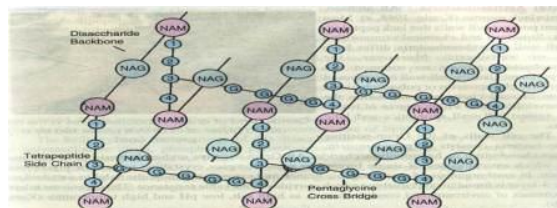
Prosthecae are semi-rigid extensions of the cell wall and cytoplasmic membrane and have a diameter that is always less than that of the cell. They are characteristic of a number of aerobic bacteria from fresh water and marine environments. Some bacterial genera such as *Caulobacter* have a single prosthecae; others such as *Stella* and *Ancalamicrobium* have several prosthecae increase the surface area of the cells for nutrient absorption, which is advantageous in dilute environments. Some prosthecae bacteria may form a new cell at the end of a prosthecae; others have an adhesive substance at the end of a prosthecae that aids its attachment to surfaces.

Although the term stalk is sometimes used interchangeably with the terms prosthecae or hypha, it is perhaps better to restrict its use to certain non-living ribbon like or tubular appendages that are excreted by the cell, such as those found in the genera *Gallionella* or *Planctomyces*. These stalks aid in attachment of the cells to surfaces.

II. Cell wall:

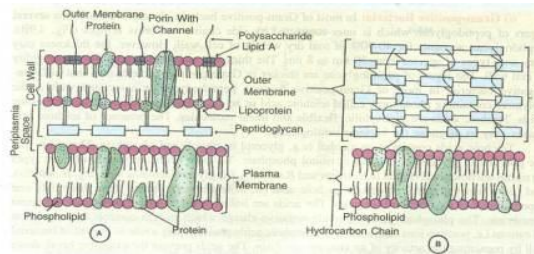
In the gram +ve bacteria, the cell envelope contains mainly the peptidoglycan and the teichoic acids. The peptidoglycan is a substituted sugar polymer found only in prokaryotic organisms. It consists of two acetylated amino-sugars, n-acetyl glucosamine and n-acetyl muramic acid and a small number of amino acids mainly D-glutamic and D and L-alanine. The two amino-sugars forms $\beta,1-4$ linkage and each strand contains 10 to 65 disaccharide residues. Short peptides containing 4 amino acids are attached to the muramic acid residues and the most common sequence is L-alanine, D-glutamic acid, L-diaminopimelic acid D-alanine.

The third amino acid varies with different bacteria and may either be lysine, Di-aminopimelic acid or threonine. Cross linked by varying number of amino acids.

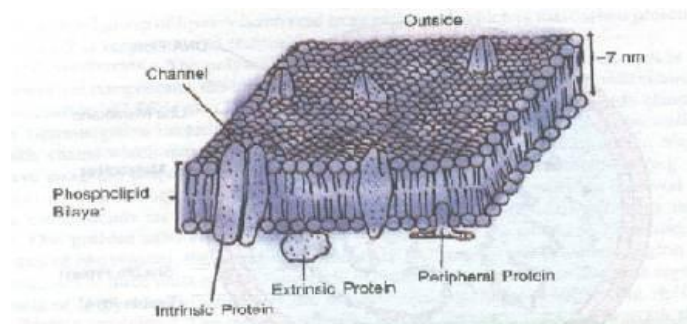


Peptidoglycan layer of *Staphylococcus aureus*

In the cell envelopes of gram -ve bacteria, two distinct layers are recognized. One, a uniform inner layer about 2-3 nm wide and a thicker outer layer about 8-10 nm wide. The outer layer also called the outer membrane and the peptidoglycan is confined to the inner layer. The outer membrane contains proteins, lipoproteins and lipopolysaccharides.



Structure and chemical composition of cell walls of Gram-negative (A) and Gram-positive (B) bacteria



Fluid mosaic model of bacterial membrane

of bacterial

III.Outer membrane:

The peptidoglycan of the gram -ve bacteria displays a rather low degree of cross linkages within the glycan strands. The fine structure of the outer membrane, like the cell membrane, consists of a lipids bilayer in which both phospholipids and lipopolysaccharides are present. The peptidoglycan of the wall has specific types of lipoproteins on its outer surface, which are linked by peptide bonds to some of the Dipicolonic acid residues in the peptidoglycan. The lipoproteins thus serves as the bridge from the peptidoglycan to the outer wall layer. The lipopolysaccharides form the major component of the outer membrane and is are extremely complex molecule varying in chemical composition. The lipopolysaccharides are the major antigenic determinants and also the receptors for adsorption of many bacteriophages. The outer membrane of gram -ve bacteria acts as a barrier to the passage of substances such as antibiotics, dyes and bile salts into the cell.

Table: Differences between cell walls of gram positive and gram negative bacteria.

Sl.No.	Characters	Gram positive	Gram negative
1.	Grams staining	Retain crystal violet and appear dark violet.	Pass crystal violet and counter stained by safranin and appear red
2.	Outer membrane	Absent	Present
3.	Peptidoglycan	Several layers (thick)	Single layer (thin)
4.	Lipid and lipo-proteins	Low	High
5.	Lipopolysaccharides	Absent	High

6	Teichoic acid	Mostly present	Absent
7	Periplasmic space	Absent	Present
8	Flagella	2 rings at basal body	4 rings
9	Toxin production	Exotoxin	Endotoxin
10	Strength	High	Low
11	Resistance to drying	High	Low
12.	Susceptibility to streptomycin, tetracycline and chloramphenicol	Low	High

The lipid found are mostly phosphatidyl ethanolamine and to a small extent phosphatidyl-serine and phosphatidyl-choline.

IV. Teichoic acids;

The teichoic acids (ribitol and glycerol phosphates) are a family of large molecular weight polymers which are found mostly in gram +ve bacteria and are located in the membrane and the wall. They are grouped mainly as the wall teichoic acids and the membrane teichoic acids although originally the word 'teicho' was given to indicate their presence only in the wall.

V. Cell membrane:

Cell membrane is the bounding layer of the cytoplasmic contents and is the principal osmotic and permeability barrier. It is a lipoprotein devoid of any polysaccharide and when examined under an electron microscope appears as a 3 layer unit with a unit membrane structure.

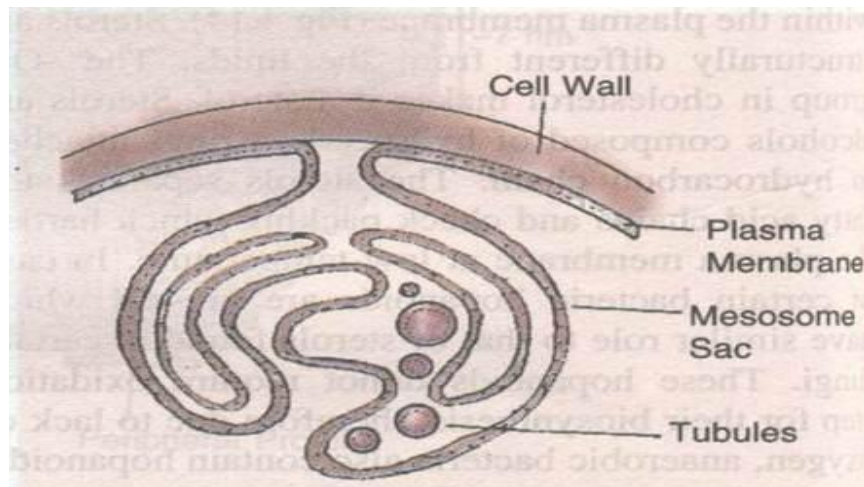
The polar head region of the phospho-lipids are located at the two outer surfaces while hydrophobic fatty acid chains extend to the centre of the membrane. The middle protein layer is intercalated into the phospholipids bilayer.

Functions:

- 1) It behaves as an osmotic barrier and contains permeases responsible for the transport of chemicals and nutrients in and outside the cell.
- 2) It contains the enzymes involved in the bio synthesis of membrane lipids and other various macromolecules of bacterial cell wall
- 3) It contains the components of the energy generation system. In addition to these, there is also evidence to show that the cell membrane has specific attachment sites for the replication and segregation of the bacterial DNA to the plasmids.

VI. Mesosomes:

In some bacteria, particularly in the gram +ve bacteria depending upon growth conditions the membrane appears to be infolded at more than one point. Such infoldings are called mesosomes. The presence of such structures in large numbers have also been found in organisms that have a higher respiratory activity as in the nitrogen fixing bacteria namely *Azotobacter* during logarithmic growth phases. In photosynthetic bacteria the extent of membrane infolding has been related to pigment content and photosynthetic activity. In sporulating bacteria, the appearance of such infolding (mesosome formation) is a prerequisite for and sporulation.



Bacterial Mesosome

VII. Cytoplasm:

The major cytoplasmic contents of bacterial cell include the nucleus, ribosomes, proteins and other water soluble components and reserve material. In most bacteria, extrachromosomal DNA or plasmid DNA is also present. The plasmid DNA is circular and double stranded.

VIII. Ribosomes:

The most notable structures in the bacterial cytoplasm are the ribosomes which are involved in protein synthesis. Their number varies with rate of protein synthesis. The greater the rate of protein synthesis, the greater is the number of ribosomes. These are ribo-nucleo protein particles, have a diameter of 200 \AA and are characterized by their sedimentation properties. The bacterial ribosomes are referred to as 70s ribosomes (S=svedbery unit, the unit of sedimentation). These ribosomes, during active protein synthesis are associated with the mRNA and such associations are called polysomes.

IX. Cellular reserve materials:

A variety of reserve materials are found in the prokaryotic cells and are termed as granular cytoplasmic inclusions. Of these, starch, glycogen and polyhydroxy butyric acid are important. Poly β -hydroxy butyric acid is found only in prokaryotes. For example, glycogen or starch in clostridia, while poly β -hydroxy butyric acid in many pseudomonads. Generally, prokaryotes do not store nitrogenous organic materials except blue green algae which accumulate nitrogenous reserve material as cyanophycin. Many prokaryotes accumulate volutin (metachromatic) granules due to presence of inorganic polyphosphate which accumulate under phosphate starvation conditions. Sulphur bacteria, such as photosynthetic purple sulphur bacteria and filamentous non-photosynthetic bacteria accumulate S during hydrogen sulphide production. In BGA, contain Thylakoids involved in photosynthesis. In certain BGA, and purple bacteria contain polyhedral structures called carboxysomes involved in carbon dioxide fixation by carboxy-dismutase enzyme, site of CO_2 fixation.

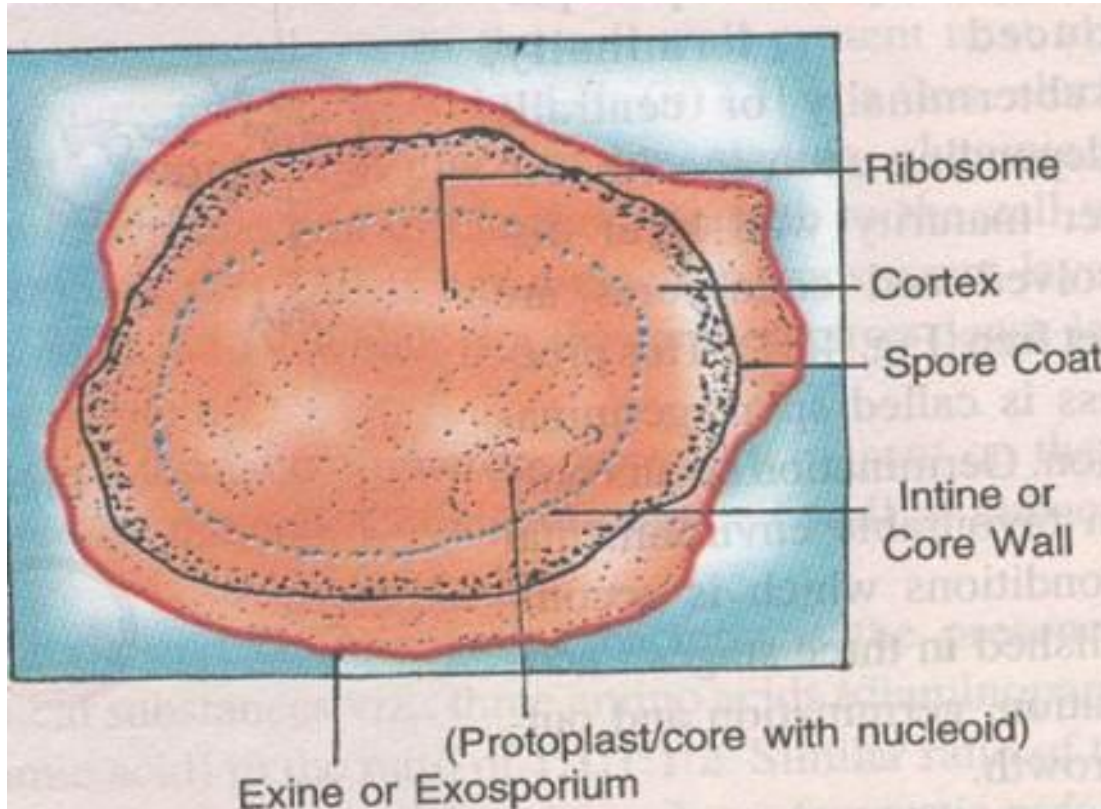
X. Endospores

Certain species of bacteria produce spores, either within the cell (endospores) or external to the cell (exospores). The spore is a metabolically dormant form, which under appropriate conditions, can undergo germination and out growth to form a vegetative cell. Endospores help the bacterial cell to tide over unfavourable environmental conditions.

Endospores are thick walled, lightly refractive bodies that are produced by *Bacillus*, *Clostridium* and *Sporosarcina*, *Thermoactinomyces*, and a few other genera. Endospores are usually produced by cells growing in rich media but which are approaching the end of active growth. They are extremely resistant to desiccation, staining, disinfecting chemicals, radiation and heat.

All endospores contain large amounts of dipicolinic acid (DPA) accounts for 10-15% of the spores dry weight and occur in combinations with calcium and probably located in the core i.e., central part of the spore. The Ca-DPA complex may possibly play a role in the heat resistance of endospores. Synthesis of DPA and uptake of calcium occur during advanced stages of sporulation.

During germination, lose their resistance to heat and staining. Subsequently, out growth occurs characterized by synthesis of new cell material and development of the organism into growing cell.



Structure of an Endospores of *Bacillus anthracis*

XI.Cysts:

Cysts are dormant, thick walled, desiccation resistant forms that develop by differentiation of a vegetative cell and which can later germinate under suitable conditions. In some ways cysts resemble endospores; however, their structure and chemical composition are different and they do not have the high heat resistance of endospores. The classic example of a cyst is the structurally complex type produced by the genus *Azotobacter*.

XII. Archaeobacteria

The archaeobacteria are unusual organisms and the group is known to include 3 different kinds of bacteria, the methanogenes, the extreme halophiles and the thermoacidophiles.

Table: The major differences between archaeobacteria and eubacteria

	Cellular organization	Archaeobacteria	Eubacteria
1	Cell wall	Variety of types not containing muramic acid	Variety with one type, contain muramic acid
2	Membrane lipids	Ether linked, branched aliphatic chains	Ester linked, straight aliphatic chains
3	Thymine in common arm of t-RNA	Absent	present in t-RNA's of most sps.
4	Sensitivity to chloramphenicol	Insensitive	sensitive
5	Structure of RNA polymerase core	Ten subunits	four subunits

CHAPTER No 8

TYPES OF CULTURE MEDIA AND PRECULTURE TECHNIQUES:

CONTENTS

8.1 Bacteriological Media:

8.2 Types of media:

8.2.1 Selective media

8.2.2 Differential media

8.2.3 Assay media

8.3 Media for enumeration of bacteria

8.4 Media for characterization of bacteria

8.5 Maintenance media

8.6 Solid and semi-solid media

8.7 Preparation of media:

8.1 Bacteriological Media:

1. GROWTH MEDIA AND CLASSIFICATION:

Growth media:

Growth media are the nutrient solutions used to grow microorganisms in the laboratory. Because laboratory culture requires for the detailed study of a microorganism, careful attention must be paid to both selection and preparation of media for successful culture to take place.

Classes of culture media:

Two broad classes of culture media are used in microbiology; chemically defined and undefined (complex). Chemically defined media are prepared by adding precise amounts of highly purified inorganic or organic chemicals to distilled water. Therefore, the exact chemical composition of a defined medium is known. Of paramount importance in any culture medium is the carbon source, since all cells need large amounts of carbon to make new cell material. In a simple defined medium, a single carbon source is present. The nature of carbon source and its concentration depends on the organism to be cultured.

Chemically defined media are needed for the cultivation of autotrophs and also useful for defining the nutritional requirements of heterotrophs. However, for the routine cultivation of heterotrophs, chemically defined media are not generally used. Instead, certain complex raw materials such as peptones, meat extract, and yeast extract are used. And the resulting media support the growth of a wide variety of heterotrophic bacteria. Agar is included as a non-nutritive solidifying agent when a solid medium is desired. A description of these raw materials is given

in **table**. Examples of relatively simple liquid and solid media that support the growth of many common heterotrophs are nutrient broth and nutrients agar (**Table**). The addition of yeast extract to each of these formulas improves the nutrient quality. Since, yeast extract contains several of the B vitamins and other growth promoting substances. Other complex supplements such as bovine rumen fluid, animal blood, blood serum or extract of plant and animal tissues may be required for the cultivation of fastidious heterotrophs.

Table: Characteristics of several complex materials used as ingredients of media

Sl.No	Raw material	Characteristic	Nutritive value
1.	Beef extract	An aqueous extract of lean beef tissue concentrated a paste	Carbohydrates, organic nitrogen compounds, water soluble, vitamins and salts.
2	Peptone	The product resting from the digestion of proteinaceous materials eg: meat, casein and gelatin	Contains organic nitrogen vitamins, carbon hydrates.
3.	Agar	Accomplish carbohydrates contain from marine algae	Used as a solidifying agent, agar dissolved in aqueous solutions, gels when temperature is reduced below 45 ⁰ C
4	Yeast extract	An aqueous extract of yeast cells, available as a powder	A very rich source of the B vitamins; also contains organic nitrogen and carbon components.

Table: Composition of nutrient broth and nutrient agar.

Nutrient broth		Nutrient agar	
Beef extract	-3.0g	Beef extent	-3.0g
Peptone	-5.0g	Peptone	-5.0g
Water	-1000ml	Agar	-15.0g
		Water	-1000ml

8.2 Types of media:

Many special media are needed to facilitate recognition, enumeration and isolation of certain types of bacteria. To meet these needs, the microbiologist has available numerous media which, on the basis of their application or function, consistency and nature may be classified as follows.

8.2.1 Selective media

These media provide nutrients that enhance the growth and predominance of a particular type of bacterium and do not enhance other types of organisms that may be present. For instance, a medium in which cellulose is the only carbon source will specifically select for or enrich the growth of cellulose utilizing organisms when it is inoculated with a soil sample containing many kinds of bacteria. e.g., Eosine methylene blue agar

8.2.2 Differential media

Certain reagents or supplements, when incorporated into culture media, may allow differentiation of various kinds of bacteria. For example, if a mixture of bacteria is inoculated into a blood containing agar medium (blood agar), some of the bacteria may hemolyze (destroy) the red blood cells, others do not thus one can distinguish between hemolytic and non-hemolytic bacteria on the same medium. e.g., Congored yeast extract mannitol agar

8.2.3 Assay media

Media of prescribed composition are used for the assay of vitamins, amino acids and antibiotics. Media of special composition are also available for testing disinfectants.

8.3 Media for enumeration of bacteria

Specific kinds of media are used for determining the bacterial content of such materials as milk and water. Their composition must adhere to prescribed specifications. e.g., Nutrient agar

8.4 Media for characterization of bacteria

A wide variety of media are conventionally used to determine the types of growth produced by bacteria, as well as to determine their ability to produce certain chemical changes.

8.5 Maintenance media

Satisfactory maintenance of the viability and physiological characteristics of a culture over time may require a medium different from that which is optimum for growth. Prolific, rapid growth may also be associated with rapid death of the cells at the end of the growth phase. For example, glucose in a medium frequently enhances growth, but acid harmful to the cells is likely to be produced. Therefore, omission of the glucose is preferable in a maintenance medium.

Based on consistency, media are classified into two types.

Solid media

In addition to liquid media, solid and semi-solid media are useful for isolating bacteria or for determining the characteristics of colonies. The solidifying agent is usually agar, which at concentrations of 1.5 to 2.0 percent forms firm, transparent gels that are not degraded by most bacteria. Silica gel is sometimes used as an inorganic solidifying agent for autotrophic bacteria. e.g., Nutrient agar

Semi-solid media

Semi-solid media, prepared with agar at concentrations of 0.50 percent or less have a soft, custard like consistency and are useful for the cultivation of microaerophilic bacteria or for determination of bacterial motility. e.g., Nitrogen free malic acid BTB medium

Based on nature and chemical composition, the media are classified into two types.

- i) **Synthetic or chemically defined medium:** Those media are prepared by mixing all the pure chemicals of known composition, for eg. Czapek dox medium.
- ii) **Semi-synthetic or un-defined medium:** Such are those media, where exact chemical composition is unknown, e.g. potato dextrose agar.

8.7 Preparation of media:

Some naturally occurring substances are used for the cultivation of bacteria. Notable among these is milk, usually skimmed rather than whole. Such natural materials are merely dispensed into tubes or flasks and sterilized before use. Media of the nutrient both or nutrient agar types are

prepared by compounding the required individual or ingredients or more conveniently by adding water to a dehydrated product which contains all the ingredients. Practically, all media are available commercially in powdered form. The preparation of bacteriological media usually involves the following steps:

- 1) Each ingredient or the complete dehydrated medium is dissolved in the appropriate volume of distilled water in earlenmeyer flasks (250 ml or 500 ml capacity).
- 2) The pH of the liquid medium or broth is adjusted by adding 1N HCl or 1N NaOH.
- 3) If a solid medium is desired, agar is added and the medium is boiled to dissolve the agar. Flasks are then plugged with non-adsorbent cotton plugs. The plugged flasks should be wrapped by brown paper and placed for sterilization by autoclaving at a pressure of 15lbs/inch² (at temperate 121⁰c) for 15min. Some media that are heat labile are sterilized by filtration. It is first to be noted that all the glassware in use should be sterilized in an oven at 170⁰c for 3hr. before using them. Such sterilized glassware (Petri dishes) are needed for pouring the medium used for culturing the microorganisms.

Each and every biological process requires energy for their vital activities. The basic cell building requirements are supplied by the nutrition, which is manipulated according to its requirements. Nutrition not only provides energy but also acts as precursors for growth of microorganisms. The nutritional requirement of an organism depends upon the biochemical capacity. If an organism is capable of synthesizing its own food using various inorganic components, requires a simple nutritional diet whereas organisms unable to meet such synthesis requires complex organic substances.

CHAPTER NO 9

MICROBIAL NUTRITION

CONTENTS

9.1 Nutrition and growth of microorganisms

9.2 Carbon requirement

9.3 Nitrogen and sulphur requirement

9.4 Classification based on O₂ requirement

9.5 Nutritional classification

9.6 Other mineral requirement:

9.7 Growth factors

9.8 Vitamins and their functions

9.1 Nutrition and growth of microorganisms

Nearly 70% of the weight of cell is water and the rest is solid matter. In addition to oxygen and hydrogen the microbial cell contains four other major elements such as C, N, P and S. These six together accounts 95% of cellular dry weight. Other elements are found in lesser amounts are K, Mg, Ca, Na, Fe, Mn, Cl, Cu, Mo and Zn. Microorganisms, therefore, need a large number of elements for survival and growth. Even microorganisms differ with respect to the chemical form in which these elements are utilized as nutrients.

Carbon is a constituent of all organic cell material and therefore represents nearly 50% of the cell dry weight. Nitrogen is found mostly in proteins, nucleic acids, co-enzymes etc. Phosphorous is a major constituent of nucleic acids while S is a constituent of mainly proteins and co-enzymes.

9.2 Carbon requirement

The most oxidized form of 'C' is carbon dioxide. Organisms which are photosynthetic reduce CO_2 to organic cell constituents. Energy for this process is derived from light or the oxidation of reduced inorganic compounds such as H_2S . On the other hand, all non-photosynthetic organisms obtain their carbon requirement mainly from organic nutrients which contain reduced carbon compounds. They not only provide the carbon for synthesis but also meet the energy requirement by entering into energy yielding metabolic pathways and are eventually oxidized to CO_2 .

9.3 Nitrogen and sulphur requirement

Nitrogen and sulphur taken up as NO_3^- and SO_4^{2-} by most organisms and are subsequently reduced within the cell and utilized in other biosynthetic processes. Some microorganisms use N as NH_4^+ salts and some use N and S as organic nutrients in reduced and organic combinations such as amino acids. A few organisms can reduce element N to NH_3 this process of N assimilation is biological nitrogen fixation (BNF).

9.4 Classification based on O_2 requirement

Although O_2 is found as a cellular component, most organisms need O_2 for respiration. In these organisms O_2 serves as the terminal electron acceptor and such organisms are referred to as obligate aerobes e.g. *Nitrobacter*. As opposed to this, there are organisms which do not use molecular O_2 as terminal electron acceptor although O_2 is a component of their cellular material. In fact molecular O_2 is toxic to these organisms and these are called as obligate anaerobes e.g. *Clostridium*. In these organisms NO_3^- , SO_4^{2-} or organic compounds serve as electron acceptors.

Some microorganisms can also grow either in the presence or absence of molecular O_2 are termed as facultative anaerobes e.g. *E. coli*. In addition to these major classes, there are organisms which grow best at reduced O_2 pressure but are obligate aerobes and these are called microaerophilic e.g. most *Lactobacilli*.

9.5 Nutritional classification

Microorganisms are grouped in relation to nutritional requirement into two groups;

- I. **Autotrophs:** which can utilize inorganic nutrients or light as a source of energy. Ex: Nitrifying bacteria, algae etc.
- II. **Heterotrophs:** Which requires organic compounds as a source of energy. Ex: Many bacteria, fungi and protozoa.

Currently microorganisms are grouped into four major groups based on the above parameters.

1. **Photoautotrophs:** which use light as energy source and CO_2 as carbon source. Ex: Algae and many photosynthetic bacteria.

2. **Photoheterotrophs:** Which use light as energy source and reduced organic compounds as C source
EX: purple non-sulphur bacteria.
3. **Chemoautotrophs:** Which use inorganic chemicals as energy source and CO₂ as carbon source. Obtain their energy by the oxidation of reduced inorganic compounds such as NH₃, NO₂, H₂, H₂S, Fe²⁺. These organisms are also called as **chemolithotrophs**. Since these can grow in an inorganic medium in the absence of light.
4. **Chemoheterotrophs:** Which use organic compounds as an energy source as well as principal carbon source. The clear distinction between the energy source and the carbon source which is the characteristic of the above 3 groups is lost since this group can derive both carbon and energy from a single organic compound.

Microorganisms	Energy	Carbon	Examples
Photolithotrophs	Light	Carbon dioxide	Algae, Purple sulphur bacteria, Green sulphur bacteria
Photo organotrophs	Light	Organic	Purple non-sulphur bacteria
Chemolithotrophs	Oxidation of inorganic compounds	Carbon dioxide	Nitrifying bacteria, Iron bacteria, Hydrogen bacteria
Chemo organotrophs	Oxidation of organic compounds	Organic compounds	Most bacteria fungi and protozoa

9.6 Other mineral requirement:

In addition to C,N,P, and S, microorganisms also need a large number of metals in trace quantities. They are essential for the functioning of various enzymes and co-enzymes in the cells.

Elements	Functions
1) Potassium	-Co-factor of some enzymes
2) Mg	-Co-factor in enzymatic reactions, binds enzymes to substrates.
3) Fe	-Constituent of cytochromes and other heme and non heme proteins, co-factor of many enzymes
4) Ca	-Co-factor for enzymes such as protease
5) Mn	-Co-factor for some enzymes, can replace Mg in some cases
6) Cu, Zn, Mo.	-Inorganic constituent of some enzymes
7) Cobalt	-Constituent of VB ₁₂ and its co-enzymes.

9.7 Growth factors :

Organic substances that cannot be synthesized from simple carbon or nitrogen sources and which must be supplied to the organisms to allow growth are called as growth factors. These include vitamins and are required in small quantities. Vitamins function as co-enzymes in various enzymatic reactions.

9.8 Vitamins and their functions:

Sl. No.	Vitamins	Co-enzyme form	Reactions involving co-enzymes
1	Thiamine (B ₁)	Thiamin pyrosulphate	decarboxylation and some group transfer reactions .
2	Riboflavin (B ₂)	FAD, FMN	some dehydration reactions electron transport.
3	Pyridoxine (B ₆)	pyridoxal phosphate	Amino acids metabolism transamination, Demination
4	Nicotinamide	NAD, NADP	Dehydrogenation reactions

5	Pantathonic acid	Co-enzyme A	Keto and oxidation for and
6	Biotin	Biotin	CO ₂ fixation, carboxyl transfer
7	Folic Acid	Tetrahydrofolic acid	Transfer of one carboxyl units
8	Lipoic acid	Lipoic acid	Hydrogen transport and acyl transfer
9	Cobamide (B ₁₂)	Cobamide co-enzymes	Isomerisation of methyl melonyl coenzyme-A

CHAPTER No 11

Lecture 11. MICROBIAL GROWTH

CONTENTS

11.1 Microbial growth

11.2 Batch culture

11.3 Methods of determining growth

11.4 Mathematics of bacterial growth

11.5 Continuous cultures

11.6 Synchronous culture

11.7 Di-auxic growth

11.8 Factors affecting growth and growth rates

11.9 Measurement of yeast growth

12.0 Measurement of fungal growth

11.1 Microbial growth:

Growth is defined as an orderly increase in cellular components. However, an increase in mass alone need not reflect growth since microorganisms sometimes can accumulate mass without a concurrent increase in cells number. To distinguish orderly, growth form from disorderly growth, the term **balanced growth** has been used. **Campbell** defined balanced growth, is the doubling of every biochemical unit of the cells within the time period of a single division without a change in the rate of growth.

11.2 Batch culture:

Growth of microorganisms in a limited volume of liquid medium is generally termed as a batch culture. The growth of bacteria in a culture medium in which conditions for balanced growth are favourable, changes in cell mass can be related to three or four phases, i.e., lag phase, log phase, stationary phase and death or autolytic phase.

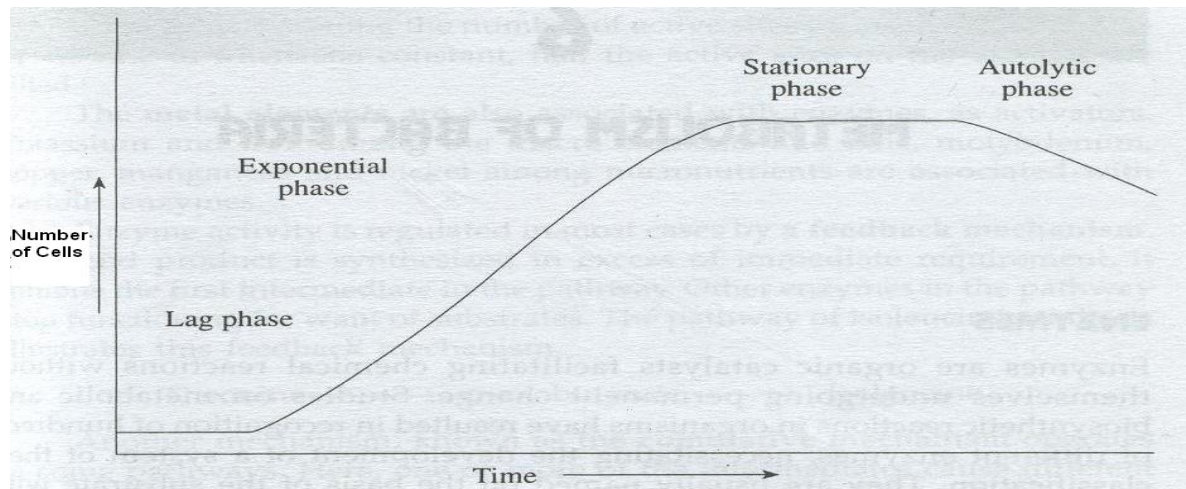
- **In lag phase**, there is no increase in the number of viable cells. However, cell growth occurs as indicated by the increase in cell mass. During this period cell increase in size as a result of extensive macromolecular synthesis. This period represents a period of **active growth** without cell division and the cells prepare for cell division.

- **In the log phase**, the cell population increases logarithmically and the cells divide at the maximum rate permitted by the composition of the medium and environmental conditions. The growth rate can be expressed in terms of the number of cell divisions that occur per hour or unit time.

- **In the stationary phase**, the total number of viable cells remain constant. In fact the stationary phase is reached when the viable cell number does not increase.

- **In death phase or phase of decline**, is characterized by an exponential decrease in the number of viable cells. The phase of decline generally seen in bacteria.

Some bacteria such as the sporulating bacteria may form endospores as they reach the stationary phase of growth and these would be resistant to lysis or death. In such cases the number of viable cells will remain constant after attaining the stationary phase and phase of decline may not be seen.



Typical Growth Curve of Bacteria in Culture

11.3 Methods of determining growth:

A variety of direct as well as indirect methods are available to determine growth and growth rates of microorganism. The direct methods involve determining the increase in cell number, dry weight or the increase in any other cellular component as a function of time while indirect methods include measurement of optical density, turbidity etc.

The total number of cells in a population can be determined either microscopically or electronically. In the microscopic method, the number of cells are determined by haemocytometer, by the dilution plating technique.

Other direct methods of estimating growth include the estimation of cell nitrogen or increase in cell dry weight or any other cellular components. For determining the dry weight, the culture is centrifuged in pre weighed tubes and the pellet is dried to constant weight at 80°-85⁰C.

The most popular indirect method of determining growth in bacteria or yeast is by the use of colorimeters or turbidometers in which, the density of the cell suspension can be determined.

11.4 Mathematics of bacterial growth:

Bacteria divide by binary fission, that is one cell gives rise to 2; 2 to 4, 4 to 8 and so on. The increase in the cell number therefore is a function of the exponent, $2^1, 2^2, 2^3, 2^4 \dots 2^n$.

Where 'n' is the exponent is the number of cell divisions. If M_1 is the number of cells at time T_1 and M_2 is the number of cells at time T_2 ,

$$\text{then } M_2 = M_1 2^n$$

Taking logarithm at both the ends $\text{Log } M_2 = \text{Log } M_1 + n \log 2$

Simplifying $\text{Log } M_2 / M_1 = n \log 2$

$$n = \frac{\text{Log } M_2 - \text{Log } M_1}{\text{Log } 2} \quad \text{(Growth equation)}$$

If 't' is mean generation time

Then, $n \times t = T_2 - T_1$ or $n = (T_2 - T_1) / t$

Where 'n' is the number of divisions or generation that the population has undergone during an interval of time $(T_2 - T_1)$.

Therefore, $\text{Log } M_2/M_1 = (T_2 - T_1) / t \times \log 2$ -----(1)

$$M_2/M_1 = k (T_2 - T_1)$$
-----(2)

Where k is growth rate constant and M is the number of cells.

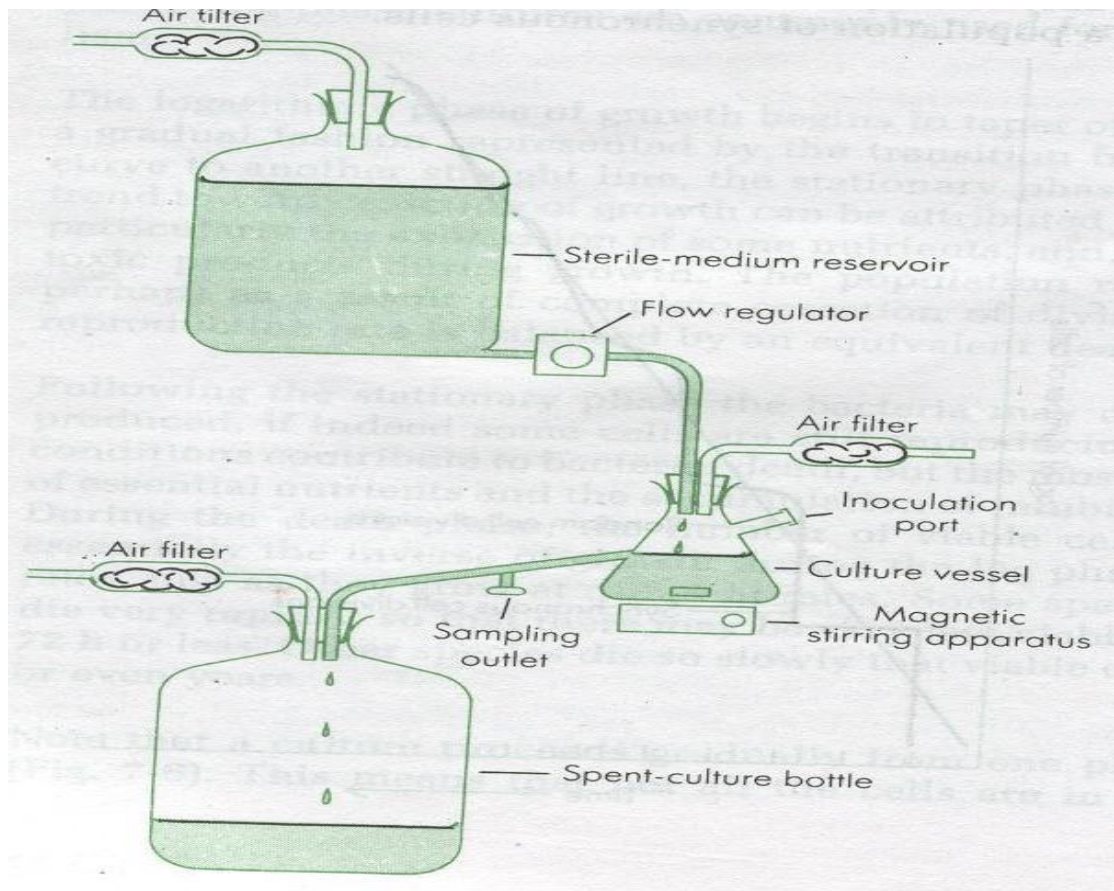
Combining the equations (1) and (2)

$$K (T_2 - T_1) = (T_2 - T_1) / t \times n \log 2$$

$$\text{Or } k = 2.303 \log 2 / t = 0.693 / t$$

11.5 Continuous cultures:

In a liquid batch culture, cells grow in a continuously changing environment with the result that the composition of the cells and the growth rate will be constantly changing. Novick and Szillard in 1959, devised a method called chemostat, which permits growth at a constant rate. In this apparatus fresh medium flows into a growth chamber at a carefully controlled rate. The volume of the culture is maintained by controlling the rates of inflow and outflow. The rate of growth is then controlled by regulating the inflow rates. In a chemostat therefore, indefinite growth at any constant rate can be maintained.



Design of a chemostat (Continuous cultures)

The development of chemostat has allowed measurement of growth rates of microorganisms under a variety of conditions.

The rate of loss of cells through the over flow can be expressed as $dM / dt = F/V = DM$

Where F is flow rate, V is culture volume / hr, D is dilution rate.

In a continuous culture, $KM = DM$

$K = D$ where, K is growth rate, D is dilution rate.

Continuous culture methods are extremely useful both for genetic and biochemical studies.

The Turbidostat is one in which growth is monitored by controlling and monitoring the turbidity of culture. By this, a culture of constant turbidity can be maintained. In a Photostat, which is used to get steady state cultures of photosynthetic organisms, growth rates can be controlled by controlling the light supply.

11.6 Synchronous culture:

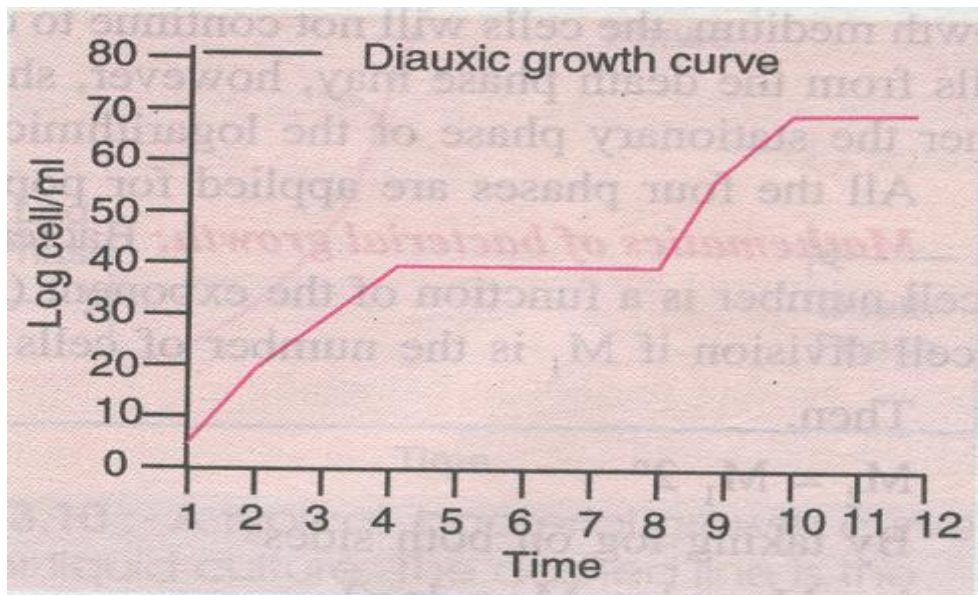
A system that closely resembles and amplifies the behaviour of single cells is synchronous culture which contain cells that are physiologically identical and are in the same stage of division cycle. A synchronous population can be generated either by physically separating cells in the same stage of division or by forcing a cell population to attain an identical physiological condition by a change in the environment. Several methods are available to get synchronous cultures are by centrifugation, filtration or by periodic changes in nutritional and environmental conditions. In a synchronous culture since the cells are physiologically identical, cell division occurs periodically at constant intervals.

Fig:

11.7 Di-auxic growth:

In a medium containing two carbon sources bacteria such as *E. coli* display a growth curve which is called diauxic. J. Monod, about 35 years ago observed this phenomenon when he grew *E. coli* in a medium containing glucose and lactose. Under these conditions, glucose is first utilized and after the exhaustion of glucose, lactose is utilized. In between a short lag period is seen. This led him to conclude that bacteria preferentially utilize certain carbon substrates.

Di-auxic growth can be obtained by using any pair of carbon compounds as long as other nutrients are not limiting.



Diauxic growth curve

11.8 Factors affecting growth and growth rates:

- 1) Temperature
- 2) pH
- 3) Oxygen requirement
- 4) Osmotic pressure

1) Temperature:

The growth rate of microorganisms falls abruptly at high temperatures is caused by the thermal denaturation of enzymes. Every organisms, has a minimum, maximum and optimum temperature for growth.

On the basis of temperature, microorganisms are divided into:

- 1) The psychrophiles – 0-5⁰C
- 2) The mesophiles – 20-45⁰C
- 3) The thermophiles – > 55⁰C

2) pH:

Many yeasts and molds and certain bacteria grow well in acidic media (5.00 or below) while most bacteria require a pH of near neutrality (6.5-8.0) for optimal growth. The pH of the medium profoundly affects the growth of microorganisms, since enzymes are sensitive to alterations in pH.

3) O₂ requirement:

Growth rate of microorganisms depends on optimum aeration conditions. Aerobes depend on O₂ whereas anaerobes sensitive to O₂ and grow in the absence of O₂. In between there are facultative anaerobes which can grow either in the presence or in the absence of O₂.

4) Osmotic pressure:

All organisms do not need a high sugar or salt concentration for optimal growth. Some yeasts and molds grow in medium containing a high concentration of sugar are called as 'osmophiles'. Certain bacteria of marine origin require high concentration of NaCl (>20%) for their optimal growth and these are termed as 'halophiles'.

11.9 Measurement of yeast growth:

An important component of fermentation processes is to continually monitor yeast growth and viability. The most common method for doing this is using the hemocytometer count method. In this method, samples are taken from the fermentation vessel, stained with methylene blue and then counted manually under a microscope using a hemocytometer.

While this method is well known and documented, it is at best an estimate based upon a very small sample count. The hemocytometer, when viewed under a microscope, presents a grid of measurement areas.

Because of the time involved for an operator to do manual counting, only a small number of actual grid cells are counted, with the results then being interpolated as an average number. Not only is the sample size very small, which yields low statistical significance, but it is known that up to 25% error can be introduced merely by “operator interpretation”.

It was desired to develop a method for making the yeast counts more precise, increase the statistical significant by looking at a larger sample and it eliminate the time and potential operator error for the procedure.

Method:

The flow camera(CAM) is ideally suited to automate this process. It can image count and measures thousands of individual yeast cells in the time it takes for an operator to count only tens of cells using the hemocytometer method. The visual spreadsheet software automatically produces a count of live, dead and budding yeast cells without any operator being involved. This normalizes out human error, and provides extremely precise and repeatable results. Further, the numbers have a much higher statistical significance due to the larger data populations obtained by the flow CAM.

The yeast sample are taken from the fermentation vessel and prepared just as they are for the hemocytometer method. The sample is then run through the flow CAM is auto image mode at reverse frame per second as it flows through the flow cell. Every yeast cell is imaged, stored and measured during acquisition.

The flow CAM automatically captures each yeast cell as a single stored image from the fluid flow. During image capture, up to 26 different spatial and gray-scale measurements are recorded and indexed to the individual cell images. When the yeast cells are stained with the methylene blue, dead cells will uptake the stain, causing them to appear blue to the camera.

For the Flow CAM, differentiating between the live and dead cells is quite straight forward, and is based primarily on the “average blue” value recorded for the cell image (along with several shape measurements). The budding cells present a bit more difficult challenge, however, due to the fact that the resolution needed to accurately differentiate a single “live” cell from a “budding” cell is much higher than can be obtained from the flow CAM.

12.0 Measurement of fungal growth

Kinetic analyses show that fungal filamentous growth can be interpreted on the basis of a regular cell cycle, and therefore encourage the view that mycelial growth and morphology can be described mathematically. Here, mathematical models that attempt to describe fungal growth and branching in the vegetative (mycelial) phase are presented.

Measurement methodologies:

In order to describe and quantify hyphal growth and branching, measurement of the parameters such as hyphal diameters (hd) and hyphal length (hl) is essential. These allow hyphal volume (hv), to be calculated, which when multiplied by the average density of the composite hyphal material (p), gives an estimate of biomass (X). If these measurements are taken over a series of time intervals it is possible to calculate hyphal extension rate (E), and hence rate of increase of biomass. Currently, automated image analysis systems permit real time analysis of these microscopic parameters. Some of these analyses suggest that hyphal tips grow in pulses, although this has been contested, particularly because the observations use video techniques and the pixelated image generated by analogue and digital cameras will cause pulsation artefacts.

The most important macroscopic parameter is total biomass. Total hyphal length is proportional to total biomass, if hd and p are assumed to be constant, but measurement can be difficult. Non destructive direct mass measurement is rarely feasible, in most cases due to the technical difficulty encountered in physically separating the mycelium from the substrum.

Describing hyphal branching:

A germ tube hypha will initially grow in length exponentially, at a rate that increases until a maximum, constant extension rate is reached, and that thereafter, it will increase in length linearly. The primary and subsequent branches will behave similarly. Thus, there develops a scenario in which individual hyphae extend linearly yet the biomass of the whole mycelium increases exponentially. It was due to the exponential increase in tips due to branching.

Hyphal branching is calculated by equation(1) $E = \mu_{max} G$

Where E is the mean tip extension rate, μ_{max} , is the maximum specific growth rate, and G is the hyphal growth unit. G is defined as the average length of a hypha supporting a growing tip according to equation(2)

$$G = \frac{L_t}{N_t}$$

Where L_t is total mycelial length, and N_t is the total number of tips. The hyphal growth unit is approximately equal to the width of the peripheral growth zone, which is a ring shaped peripheral area of the mycelium that contributes to radial expansion of the

colony. In a mycelium that is exploring the substratum, branching will be rare and thus G will be large. G is therefore an indicator of branching density.

Prosser and Trinchi (1979) studied for exponential growth and branching in fungal mycelia. The process was modeled in two steps:

- 1) Vesicles were produced in hyphal segments distal to the tip and were absorbed in tip segments.
- 2) Vesicles flowed from one segment to the next, towards the tip.

Apical branching initiated when the concentration of vesicles in the tip exceeded the maximum rate that the apex could absorb the new material. Varying the ratio of these steps produced different flow rates and branching patterns. The model also incorporated the concepts of the 'duplication cycle'. This was achieved by increasing the number of nuclei in the model mycelium at a rate proportional to the rate of biomass increase. Septa were then assumed to form in growing hyphae when the volume of the apical compartment per nucleus branched a threshold level. This provided for initiation of lateral branches by assuming that vesicles accumulated behind septa to a concentration comparable to that which initiated apical branching. This model achieved good agreement for total mycelial length, number of hyphal tips and hyphal growth unit length in *Geotrichum candidum*.

CHAPTER No 12 VIRUSES

CONTENTS

12.1 Viruses

12.2 Structure of virions

12.3 Morphology of well known viruses

12.4 Lytic and lysogenic cycle

12.5 Animal viruses

12.6 Types of infections

12.7 Diseases

12.8 Plant viruses

12.9 Diseases of plant viruses

12.9.1 Classification of virus

Virus can be defined as a genetic element containing either DNA or RNA that replicates in host cells as intracellular parasites but is characterized by having an extracellular state or are intracellular obligate parasites multiples in living host.

Virus can use the metabolic machinery of the host cell and can modify the genetics of the host cell.

- ✚ **Extracellular state:** Outside the host cell, the virus is a minute particle containing nucleic acid surrounded by protein, which is referred as virions or virus particles. Virions are inert and have no biosynthetic and metabolic functions.
- ✚ **Intracellular state:** The active state in which the virus replicate in the host cell. When the virus nucleic acid is introduced to host cell and replication starts, the process referred as infection.
- ✚ **Chemistry of virus:** All the living cells have double strand DNA as their genetic material. In contrast viruses can either DNA or RNA as their genetic material and it can be either single strand or double strand. The third group of virus which contain RNA as genome but replicate by DNA intermediate, used both DNA and RNA as their genetic material in their reproductive stage.

The viruses can be divided into following groups based on their nucleic acid and their form (single/double strand)

I DNA containing virus:

Ss DNA virus

Ds DNA virus

II RNA containing virus:

Ss RNA virus

Ds RNA virus

III RNA – DNA virus

Virus are also divided into 3 major groups based on their host as 1) plant viruses 2) Animal viruses and 3) Bacteriophages.

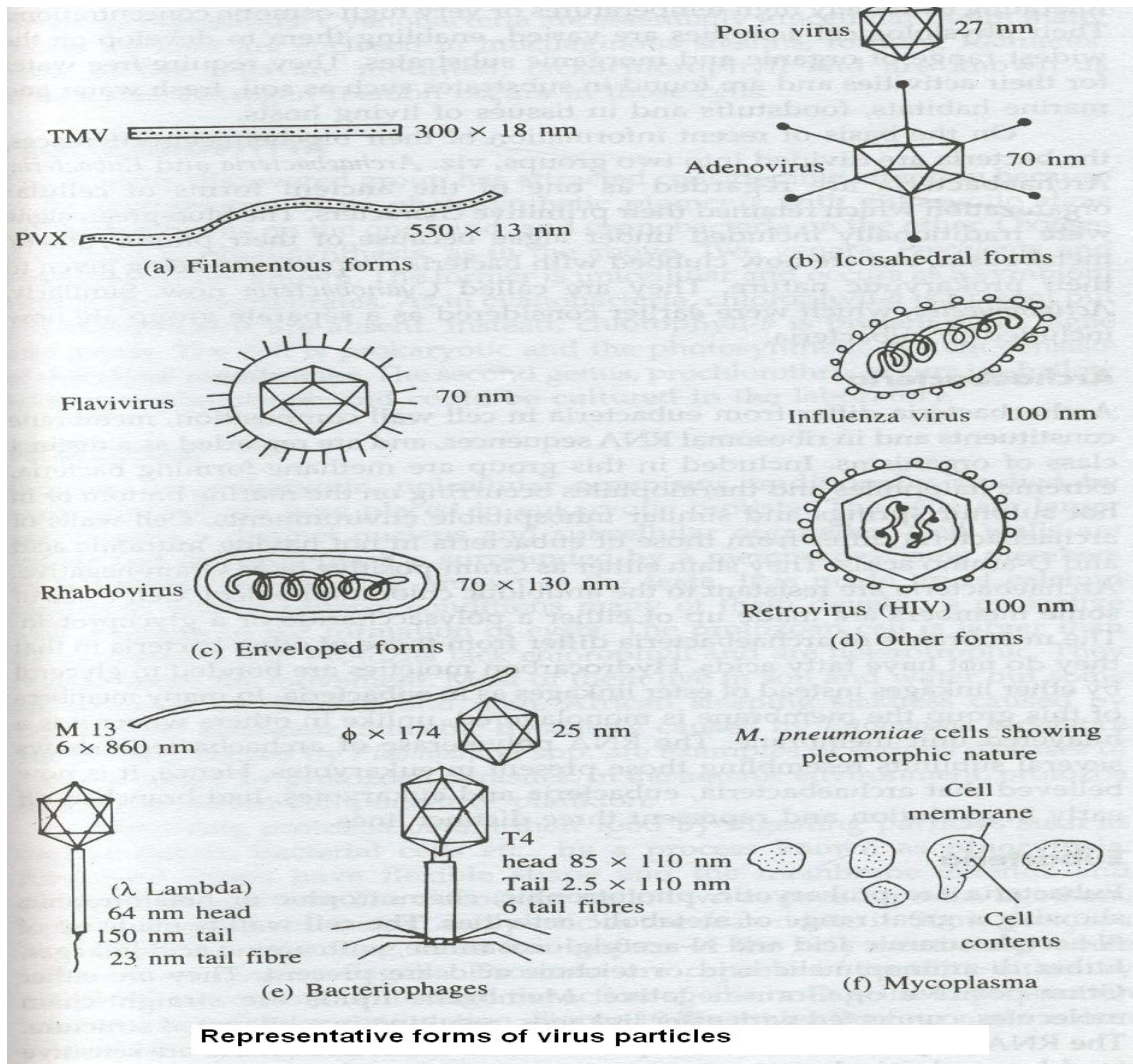
12.2 Structure of virions:

The structure of virus varies to spherical, rod, complex structures and the size of virus is in nanometers (nm). The size ranges from 20-200nm. Ex: small pox virus – 200nm, poliovirus – 28nm.

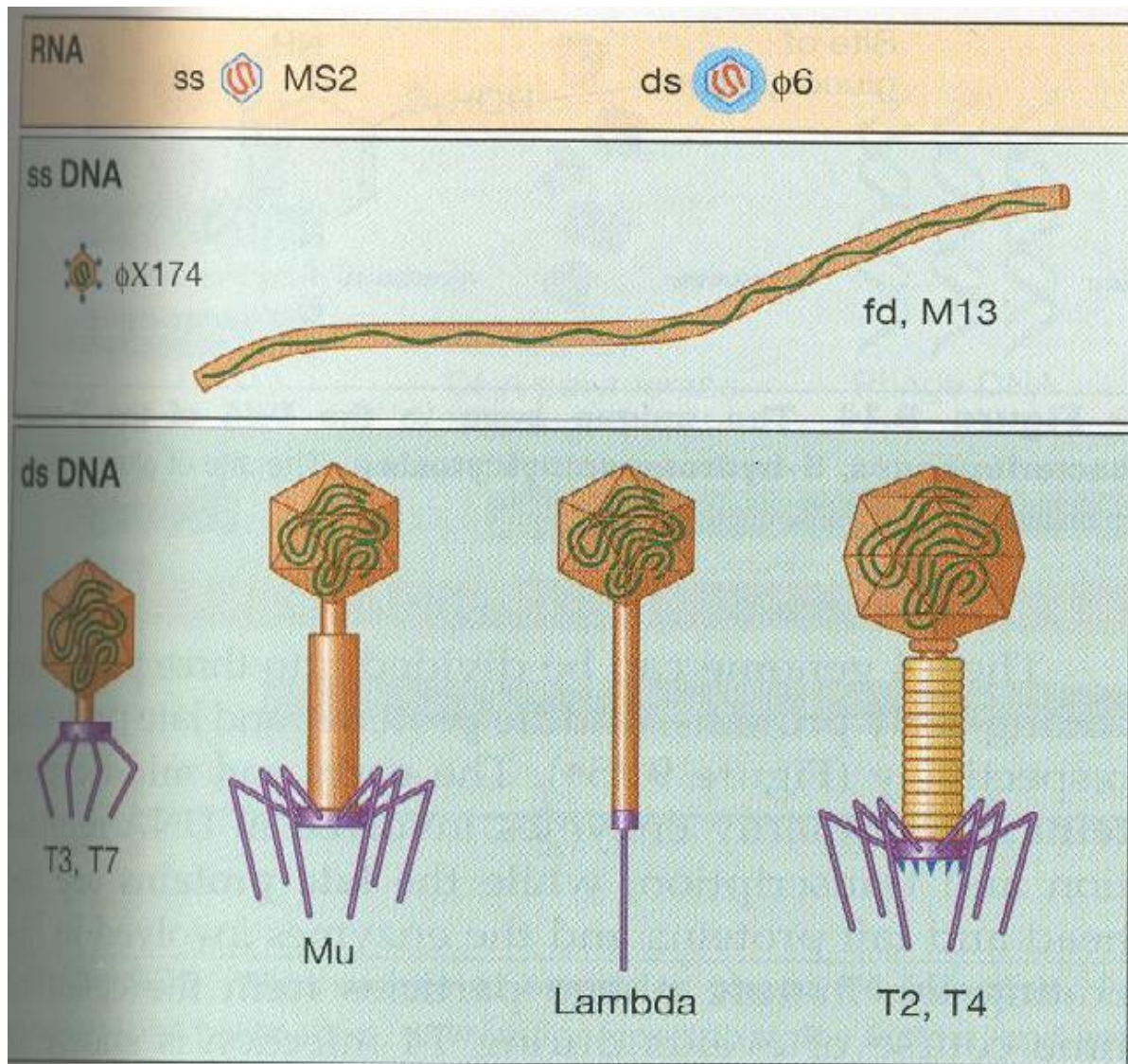
The genome size of each virus also varies. Herpes virus – Ds DNA-150 kbp, Polio virus – SsRNA-7kbp and Cowpea mosaic virus – ss RNA-9 kbp.

The nucleic acid of viron is always located within in the particle and surrounded by protein coat called capsid. The protein coat is always formed of a number of individual subunits referred as capsomers. Capsomers are arranged in a precise or highly respective pattern around the nucleic acid.

12.3 Morphology of Well known viruses:



The complete complex of nucleic acid and protein packed as virus particle is called as nucleocapsids. Some viruses are covered with lipid bilayer which are referred as envelopes. Such viruses are referred as enveloped viruses. Some groups of viruses have complex structures with several shapes. They provide head, tail and end plates and tail fibres. The maximum of 20 different proteins will be present in these viruses.



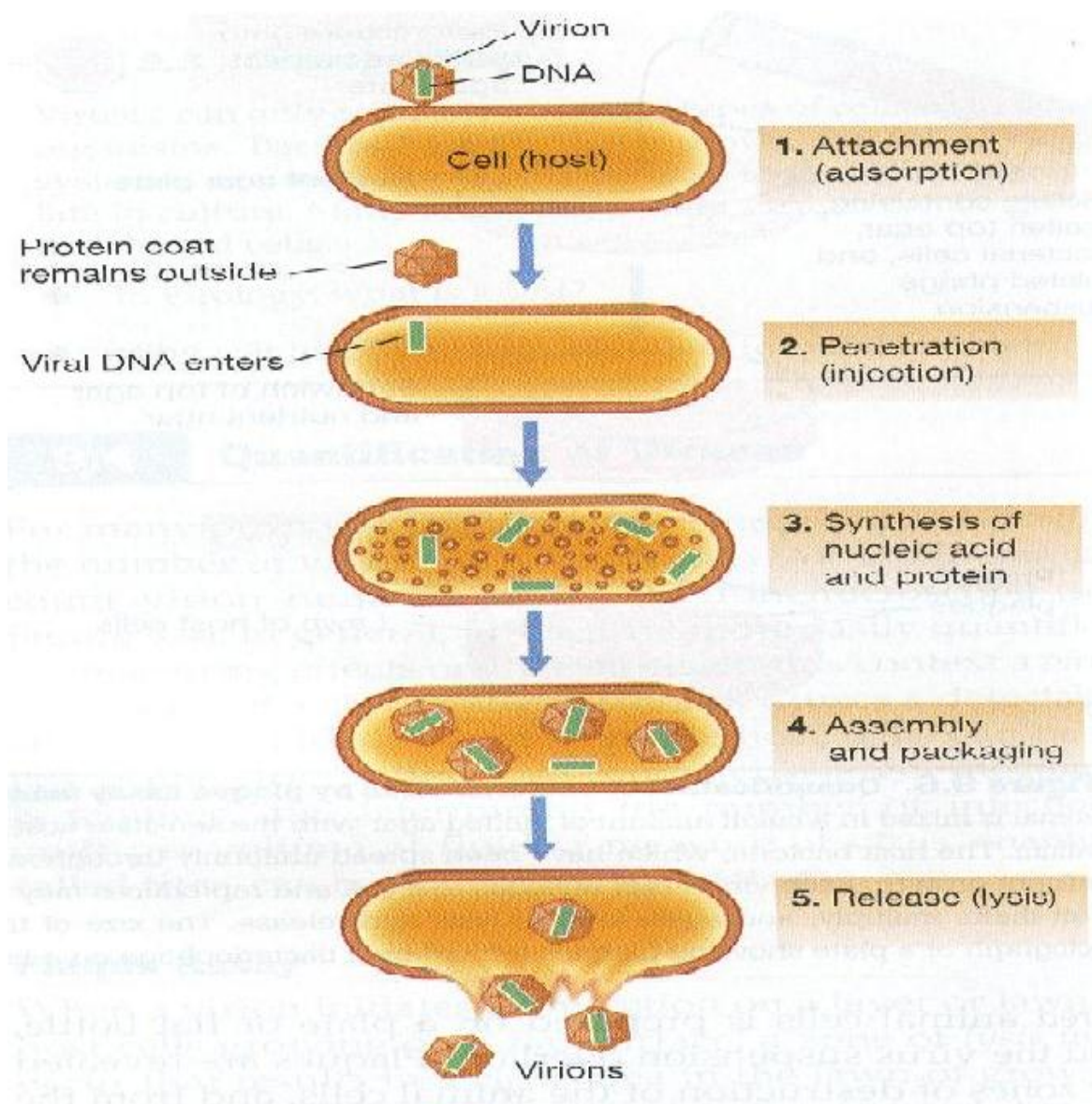
Types of Bacterial Viruses

12.4 Bacteriophages: A large number of viruses infecting various bacteria are called bacteriophages.

The bacteriophages can exist in three phases:

- 1) as a free particle-virion
- 2) in a lysogenic state as a prophage and
- 3) in the vegetative state-lytic cycle.

As a virion it is inert and cannot reproduce. In the lysogenic state, the DNA of its phage is integrated within the bacterial DNA and exists in a non-infectious form (prophage) and replicates in synchrony with the bacterial DNA. In the lytic cycle, the phage particle infects the susceptible host, multiplies and causes the lysis of the bacterial cell with concomitant release of progeny viral particles. Also, when the integrated phage is induced to become vegetative phage, the lytic cycle follows. Phage that cause lysis are called virulent phages as opposed to those which can exist in a lysogenic state which are called as temperate phages. Bacteria which carry temperate phages are called lysogenic bacteria, and such bacteria are immune to super infection by the same phage.



The Replication cycle of Bacterial virus

12.5 Lytic and lysogenic cycle :

In a typical lytic virus it is not the presence of viral DNA that leads to the production of new virions and host cell death. Rather it is expression of the viral genome that is deleterious. Host cells can harbor viral genomes without harm if the expression of the viral genes can be controlled. This is the situation found in lysogens. However if this control is lost, the virus enters the lytic pathway and produces new virions, eventually lysing the host cell lysogeny can thus be considered a genetic trait of a bacterial strain.

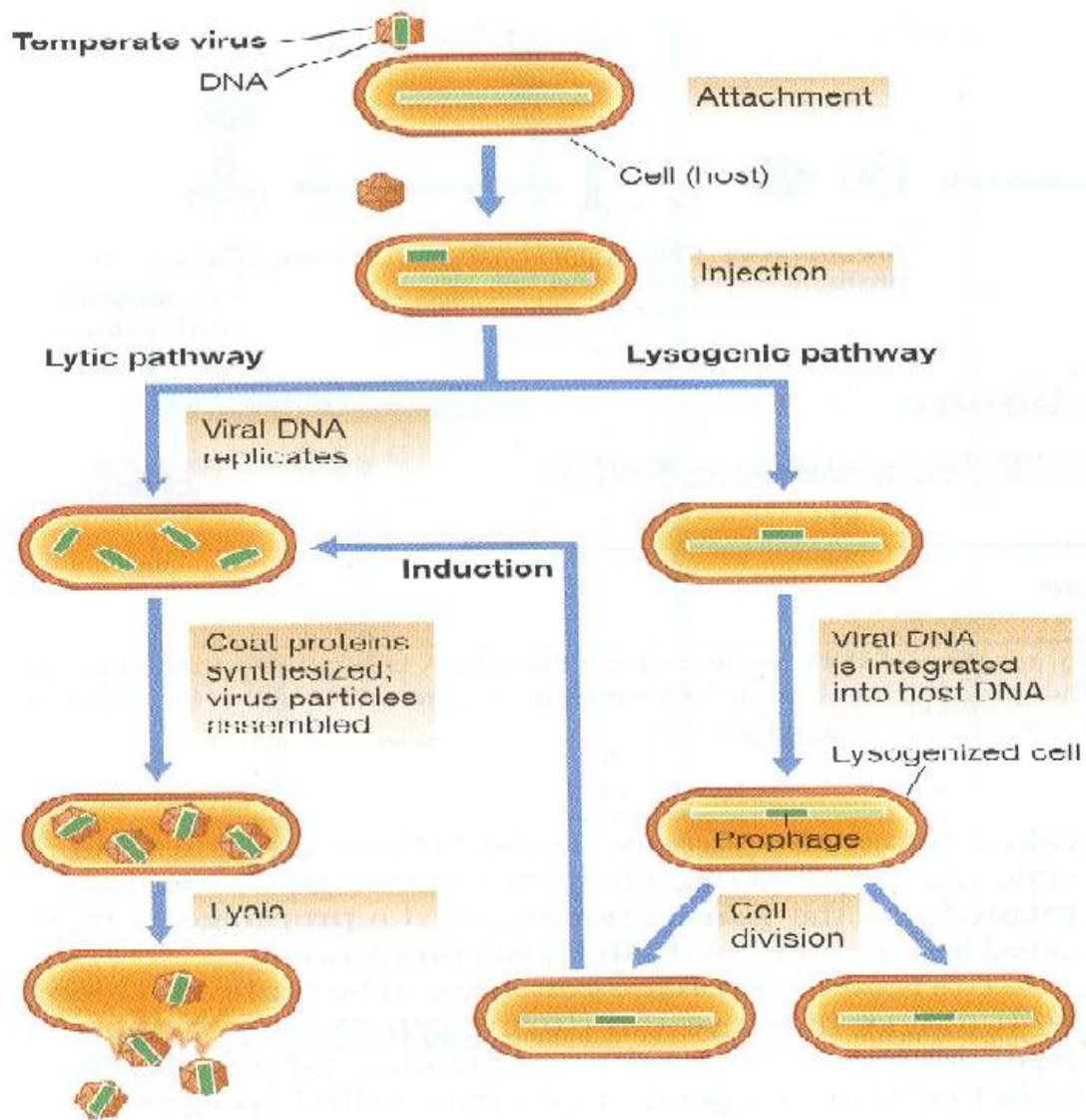
The temperate virus does not exist in its extracellular form inside the cell. Instead, the prophage is integrated into the bacterial chromosome and replicates along with the host cell as long as the genes controlling its lytic pathway are not expressed. Typically this control is maintained by a phage encoded repressor protein. The virus repressor protein not only control the lytic genes on the prophage but also prevents the expression of any incoming genes of the same virus. This results in the lysogens having immunity to infection by the same type of virus.

If the phage repressor is inactivated or if its synthesis is prevented, the prophage is induced. Induction results in the production of new virions and the lysis of the host cell. In some cases, induction can be brought about by environmental conditions. If the virus loses the ability to leave the host genome, becomes a cryptic virus. Genomic studies have shown that many bacterial chromosomes contain DNA sequences that were clearly one part of a viral genome. Thus the establishment and breakdown of the lysogenic state is likely a dynamic process in prokaryotes.

Replication of bacteriophage involves four main phases:

- 1) the adsorption of the phage to the bacterium
- 2) injection of nucleic acid into the host cytoplasm
- 3) the replication of the phage nucleic acid and synthesis of phage specific proteins
- 4) assembly, maturation and release of the bacteriophage.

Generally, viruses which kill the host cell during release are referred as lytic viruses and the cycle is referred as lytic cycle. But some bacteriophages will multiply inside the host cell and release to the environment after assembly without killing the host cell are referred as temperate phages and the cycle is referred as lysogenic cycle.



Infection by a Temperate Bacteriophage

12.5.1 Animal viruses:

- * Animal viruses are both DNA and RNA viruses. Retrovirus is a RNA virus but can have DNA as an intermediate.
- * The animal viruses enter into host cell as whose viron through phagocytosis.
- * The viral nucleic acid will be produced at the nucleus of host cell and the protein coat will be produced at cytoplasm and assembly takes place.
- * Most of the animal viruses are enveloped.

12.5.2 Types of infections:

- * lytic infection- destroys host cells.
- * Persistent infection- host cell continues to shed viruses over long time.
- * oncogens – infection by certain viruses causes cells to change, become cancerous.
- * latent infection – virus genes may not be expressed for long time.

12.6 Diseases:

Mice minute virus,

Hepatitis B virus,

Smallpox virus,

Chickenpox virus,

Poliovirus,

Hepatitis A virus,

Blue tongue virus,

Rabies virus,

Influenza virus,

SARs virus, HIV and Measle virus.

12.6.Plant viruses:

- * most of the plant viruses are naked viruses
- * they don't have specific mechanism for entering the host plants. The injuries, insect vectors are the means of entry.
- * the cell wall and cuticle layers of plant are the basis for the entry of virus.
- * the virus diseases of plants are very rare because infection is not strong enough to kill the plant.
- * mostly the virus infection can be recognized by mosaic like leaf pattern, in which loss of chlorophyll leads to yellow in color.
- * some viruses multiply in the host plant, without showing the symptom referred as latent infection.

12.6.1 Diseases of plant viruses:

Tobacco mosaic (TMV), Turnip crinkle virus, cucumber mosaic virus, wound tumour virus, maize streak virus, tomato golden mosaic virus, cauliflower mosaic virus.

Viroids: Viroids are the smallest infectious agents which are having a circular RNA alone as genome. They use DNA dependent RNA polymerase of host plant for their multiplication.

Prions: Prions are infectious protein particles that cause several diseases in animals and humans. Prions are the only infectious agents that do not contain any nucleic acids and only proteins are present. Prion diseases are often called spongiform encephalopathies because of the post mortem appearance of the brain with large vacuoles. They cause brain diseases in sheep (scrapie disease), cows (mad cow disease) humans (kuru and creutzfeldt jakob-disease) and other animals.

12.7 Classification of virus:

Phylum- vira

Subphylum – Deoxyvira (DNA virus):

Class :1) Deoxy helica (helical symmetry)

2) Deoxycubi (cubical symmetry)

3) Deoxybinala (viruses with head and tail)

4) Ribohelica (helical symmetry)

5) Ribocubia (cuboidal symmetry)

I. ss RNA viruses:

1) Helical (plant viruses)

2) Icosa hedral (plant and animal viruses)

3) Enveloped

II. DNA viruses:

1) Segmented genome (animal and plant viruses)

2) Enveloped

III. ss DNA viruses:

1) Icosa hedral – Bacteriophages and parvo viruses

2) Helical – Bacteriophages

IV. Ds DNA viruses:

1) Icosahedral complex (Tailed)

2) Enveloped

3) Nuclear assembly

3) Cytoplasmic assembly

**CHAPTER NO 14
MICROBIAL GENETICS**

CONTENTS

14.1 Microbial Genetics – Basic Concepts

14.2 Genotype and phenotype

14.3 DNA and chromosomes

14.4 Enzymes that associated with DNA

14.5 DNA replication

14.6 Transcription

14.7 Translation

14.8 Bacterial genome organization

14.9 Plasmids

15.0 Gene regulation and expression

15 .1The operon model of gene expression

15.2Importance of microbial genetics

15.3 Genetic Recombination

15.4Artificial transformation

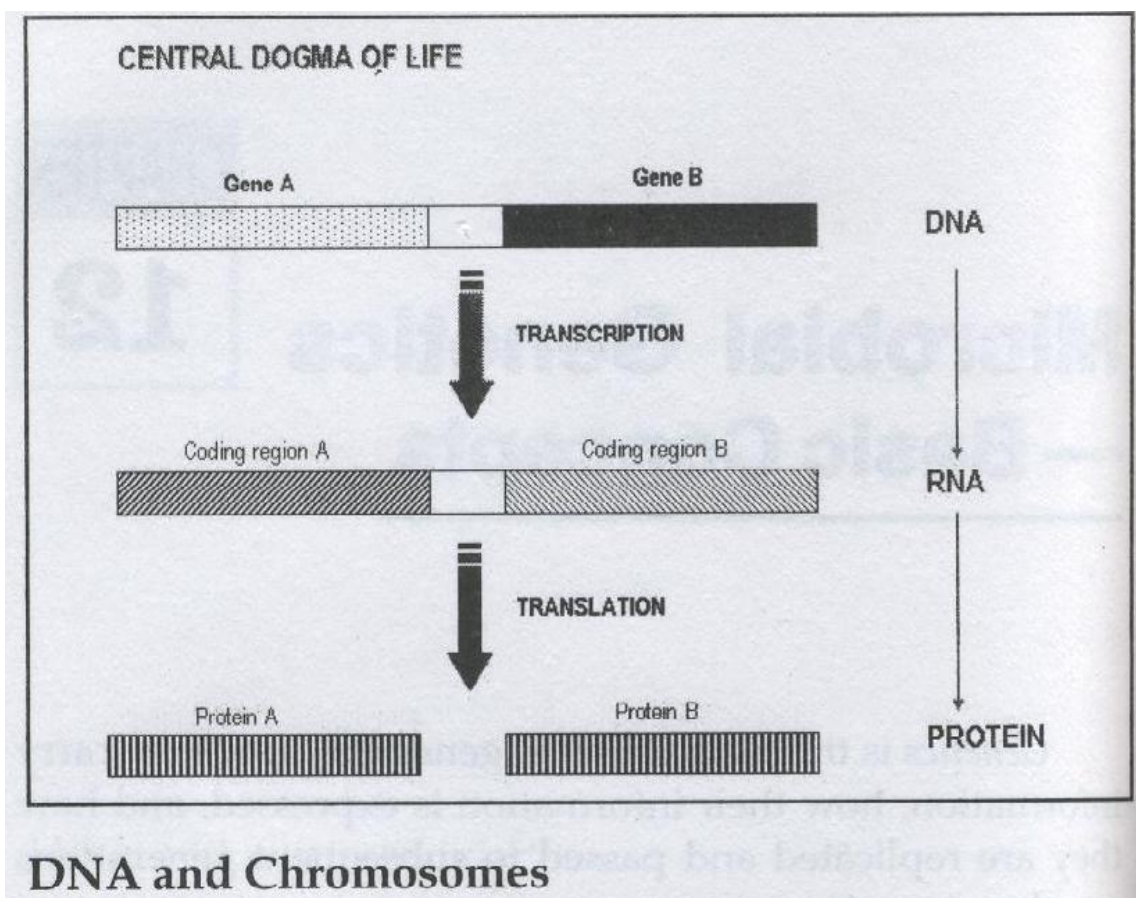
15.5 Genetic Engineering:

14.1 Microbial Genetics – Basic Concepts:

Genetics is the study of what genes are, how they carry information, how their information is expressed, and how they are replicated and passed to subsequent generations or other organisms.

Deoxy-ribose nucleic acid (DNA) in cells exists as a double stranded helix; the two strands are held together by hydrogen bonds between specific nitrogenous base pairs; Adenine-Thiamine (A-T) and cytosine guanine (C-G).

A gene is a segment of DNA, a sequence of nucleotides that code for a functional product, usually a protein. When a gene is expressed, DNA is transcribed to produce ribose nucleic acid (RNA); mRNA is then translated into proteins. This is also referred as central Dogma of life. The DNA in a cell is replicated before the cell divides. so each daughter cell receives the same genetic information.



14.2 Genotype and phenotype:

Genotype is the genetic composition of an organism its entire DNA. Phenotype is the expression of the genes-the proteins of the cell and the properties they confer on the organism.

14.3 DNA and chromosomes:

The DNA in a chromosome exists as one long double helix associated with various proteins that regulate genetic activity. Bacterial DNA is circular, the chromosome of *E. coli*, for

example, contains about 4 million base pairs and is approximately 1000 times longer than the cell.

Genomics is the molecular characterization of genomes. Information contained in the DNA is transcribed into RNA and translated into proteins.

14.4 Enzymes that associated with DNA:

- 1) **DNA polymerase:** An enzyme that synthesize a new strand of DNA in **5'-3'** direction using the anti parallel strand as template.
- 2) **Restriction endonuclease:** An enzyme which recognizes and makes double strand DNA breaks at specific sequence of DNA.
- 3) **RNA polymerase:** An enzyme that synthesize RNA in **5'-3'** direction using antiparallel DNA strand as template.
- 4) **DNA ligase:** An enzyme that lygate or joint double strand DNA fragments.
- 5) **DNA gyrase:** This enzyme introduce super coiling of DNA in prokaryotes (also referred as topoisomerase-II).
- 6) **Topoimerase-I:** An enzyme which removes the super coiling of DNA (super coiling refers the highly twisted form of DNA).

14.5 DNA replication:

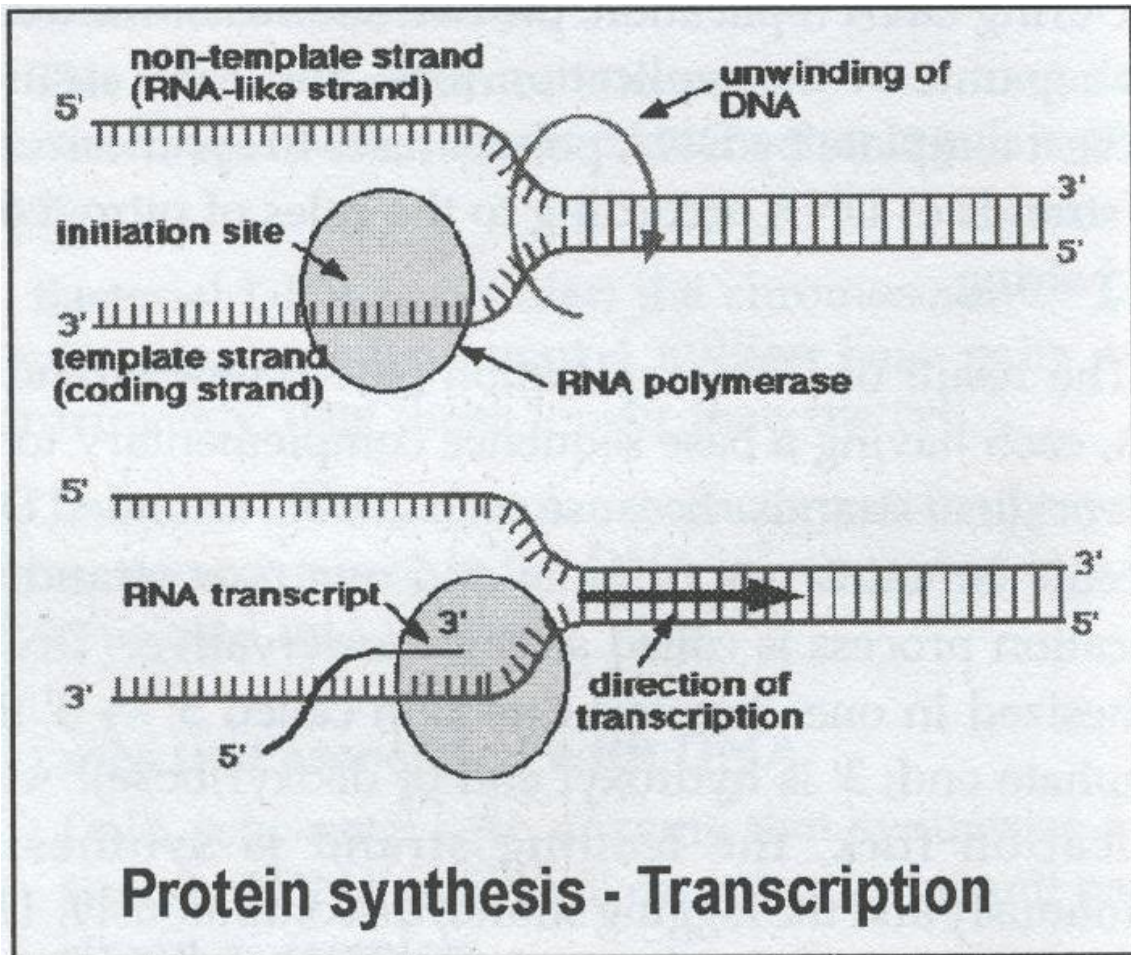
During DNA replication, the two strands of the double helix separate as the replication fork, and each strand is used as a template by DNA polymerases to synthesize two new strands of DNA according to the rules of nitrogenous base pairing.

The result of DNA replication is two new strands of DNA, each having a base sequence complementary to one of the original strands. Because each double stranded DNA molecules contains one original and one new strand, the replication process is called as semi-conservative method. DNA is synthesized in one chemical direction called **5'-3'** (5' is phosphate ends, 3' is hydroxyl end of deoxyribose). At the replication fork, the leading strand is synthesized continuously and the lagging strand discontinuously. DNA polymerase proof reads new molecules of DNA and removes mismatched bases before continuing DNA synthesis.

14.6 Transcription:

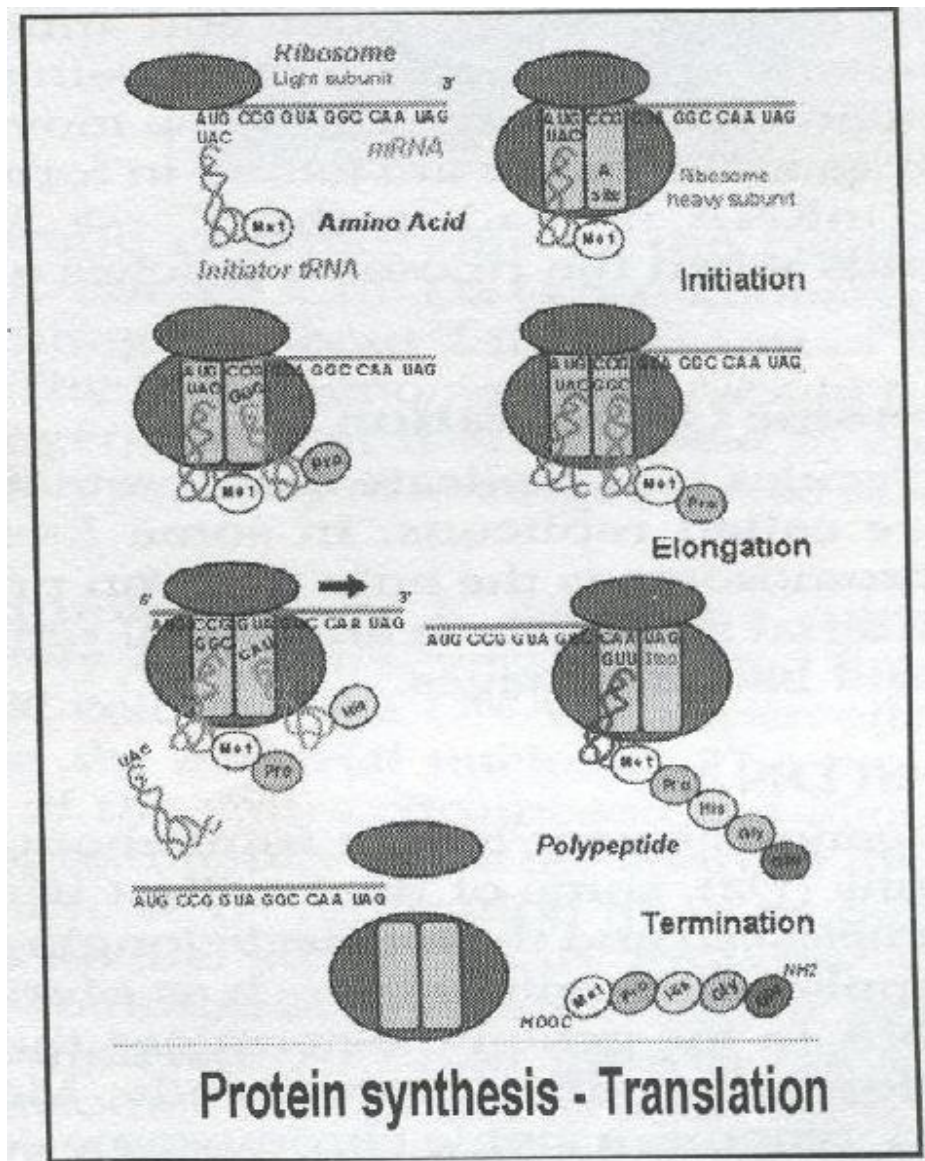
During transcription, the enzyme RNA polymerase synthesizes a strand of RNA from one strand of double stranded DNA, which serves as a template. RNA is synthesized from nucleotides containing the bases A, C, G and U which pair with the bases of the DNA sense

strand. The starting point for transcription, where RNA polymerase binds to DNA, is the promoter site; the region of DNA that is the end point of transcription, is the terminator site, RNA is synthesized in the 5'-3' direction.



14.7 Translation:

Translation is the process in which the information in the nucleotide base sequence of mRNA is used to dictate the amino acid sequence of a protein. The mRNA associates with ribosomes, which consist of rRNA and protein.



14.8 Bacterial genome organization:

DNA molecules that replicate as discrete genetic units in bacteria are called replicons. In some *Escherichia coli* strains the chromosome is the only replicon present in the cell. Other bacterial strains have additional replicons, such as plasmids and bacteriophages.

14.9 Plasmids:

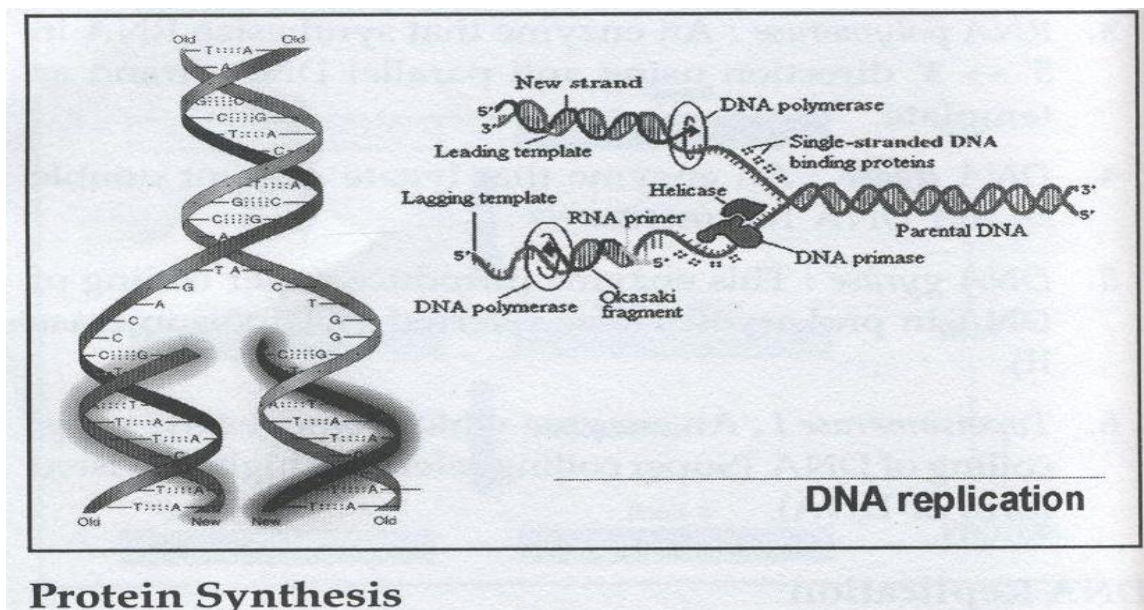
Plasmids are replicons that are maintained as discrete, extra chromosomal genetic elements in bacteria. They are usually much smaller than the bacterial chromosome, varying from less than 5 to more than several hundred Kbp (kilo base pairs), though plasmids as large as 2 Mbp occur in some bacteria. Plasmids usually encode traits that are not essential for bacterial viability,

and replicate independently of the chromosome. Most plasmids are super coiled, circular, double stranded DNA molecules, but linear plasmids have also been demonstrated in *Borrelia* and *Streptomyces*.

Many plasmids control medically important properties of pathogenic bacteria, including resistance to one or several antibiotics, production of toxins, and synthesis of cell surface structures required for adherence or colonization. Plasmids that determine resistance to antibiotics are often called R plasmids (or R factors) and the plasmids responsible for conjugation are F plasmids.

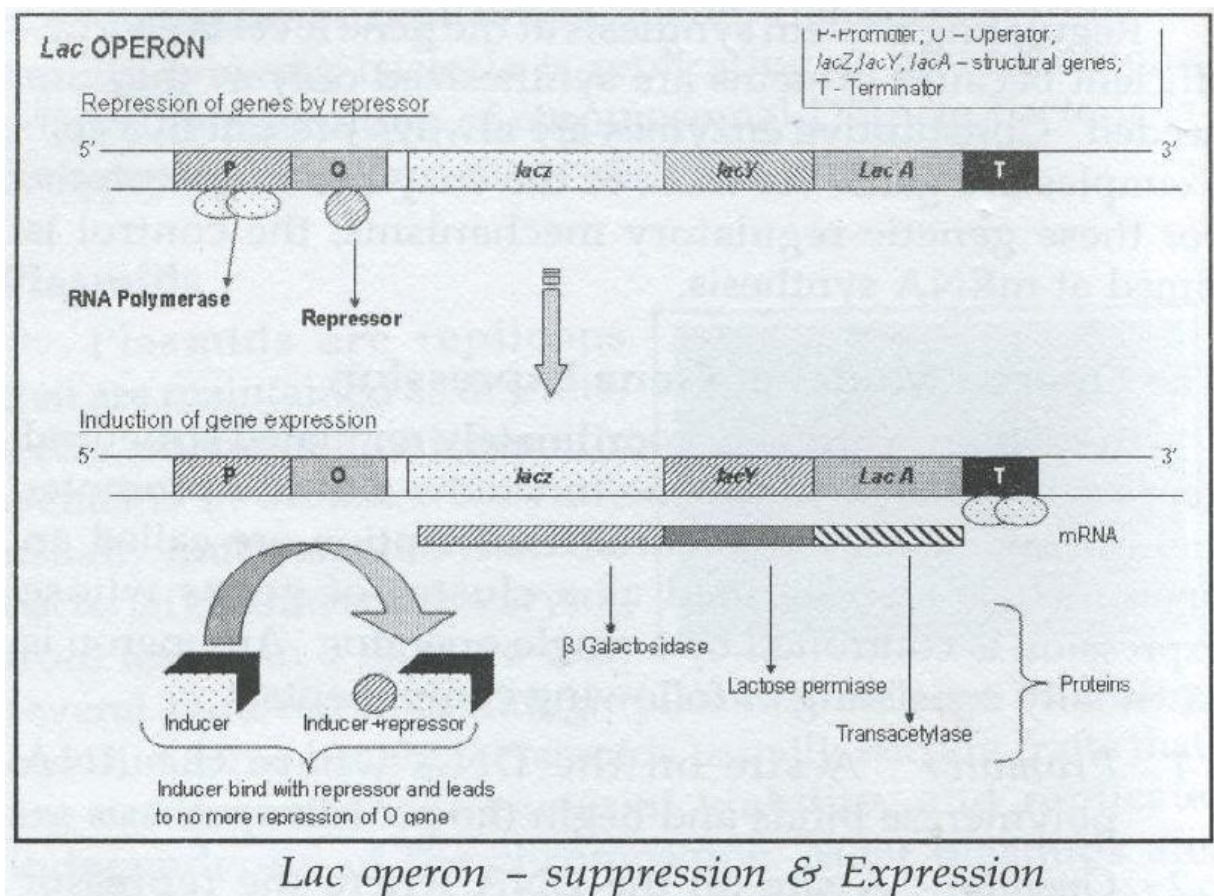
15. Gene regulation and expression:

The genes are always present in the bacterial chromosomes but their expression is always controlled. Here, the gene expression refers to the mRNA and protein synthesis. The expression of the gene was systematically controlled some proteins. Whenever needed, the genes will be expressed to get the protein (i.e. enzymes). Regulating protein synthesis at the gene level is energy efficient because proteins are synthesized only as they are needed. Constitutive enzymes are always present in a cell. Examples are genes for most of the enzymes in glycolysis. For these genetic regulatory mechanisms, the control is aimed at mRNA synthesis.



15.1 The operon model of gene expression:

In bacteria, a group of co-ordinately regulated structural genes with related metabolic functions and the promoter and operator sites that control transcription are called an operon. It is also defined as a cluster of genes whose expression is controlled by a single operator. An operon is essentially consisting of the following components.



- 1) **Promoter:** A site on the DNA where the RNA polymerase binds and begin the protein synthesize.
- 2) **Operator:** A site on the DNA where the repressor protein binds and blocks the mRNA synthesis .
- 3) **Repressor protein:** A protein bind in the operator region and block the mRNA synthesis.
- 4) **Structural genes:** They are the actual coding region of the proteins or enzymes. This protein of the gene is also referred as open reading frame (orf), because this protein alone is read by RNA polymerase to transcript the mRNA.
- 5) **Terminator:** This portion of DNA of the operan will terminate the mRNA synthesis by forming loop like arrangement in the same strand.
- 6) **Ribosome binding site:** Some 6 base pairs next to promoter, where ribosome bind with mRNA during translation process.

These are the components of any operon. As a model, the diagrammatic representation of lactose utilizing genes, referred as lac operon was given in the below diagram.

P denotes the promoter region, in which the RNA polymerase binds, O denotes the operator region, in which suppressor protein binds and blocks; Lac Z, Lac Y and Lac A are the structural genes which code the enzymes namely, β -galactosidase, lactose permease and lactose transactylase (which are essential for lactose utilization); T denotes the terminator which stops the mRNA synthesis.

Under normal conditions, meaning, no lactose in the medium, the repressor blocks the operator of the lac operon and thereby no production of these enzymes. When lactose was introduced to medium transport to inside of the cell, act as inducer. The inducer will bind with repressor protein and lead to no more blockage of operator region. Then RNA polymerase starts synthesizing mRNA upto the terminator region. The transcription will happen until the presence of inducer (lactose). When lactose was exhausted in the medium, the repressor protein will again block the operator and no more mRNA synthesis takes place. This kind of gene regulation is referred as **repression** and sometimes induction type gene regulations are also common one.

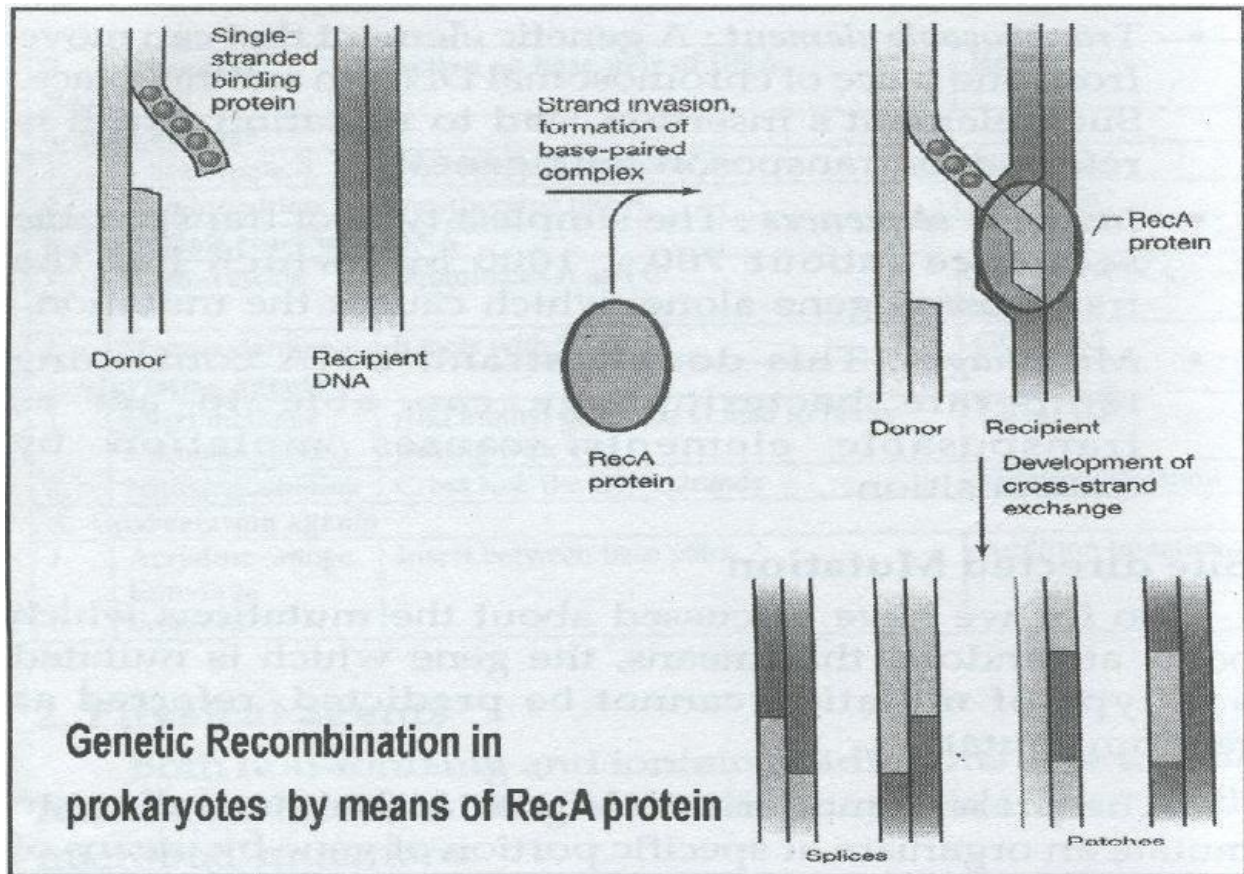
15.2 Importance of microbial genetics:

- 1) To understand the gene function of microorganisms
- 2) Microbes provide relatively simple system for studying genetic phenomenon and thus useful to other higher organisms.
- 3) Microorganisms are used for isolation and multiplication of specific genes of higher organisms which is referred as gene cloning.
- 4) Microbes provide many value added products like antibiotics, growth hormones etc. Microbial genetics will be helpful to increase these products productivity by microbial technology.
- 5) Understanding the genetics of disease causing microorganisms especially virus, will be useful to control diseases.
- 6) Gene transfer among the prokaryotes play major role in the spread of the genes in a particular environment. Microbial genetics will be useful to study the gene transfer from one organism to another.

15.3 Genetic Recombination:

Genetic recombination involves the physical exchange of genetic material between organisms. It involves the genetic exchange between homologous DNA sequences from two different organisms. In classical genetics, it is referred as **crossing over**. In prokaryotes, *RecA* protein is a DNA binding protein involved in the genetic recombination. The following sequence of steps involved in the genetic recombination of prokaryotes.

The cell which gives DNA is donor and which receives the DNA is recipient. In prokaryotes, the genetic recombination is observed because of homologous DNA from one cell to another by three processes.



- I. Transformation:** Donor DNA free in the environment (naked DNA) transferred to recipient cell.
- II. Transduction:** Transfer of donor DNA to recipient cell by means of virus vector.
- III. Conjugation:** Transfer of donor DNA to recipient through cell to cell contact.

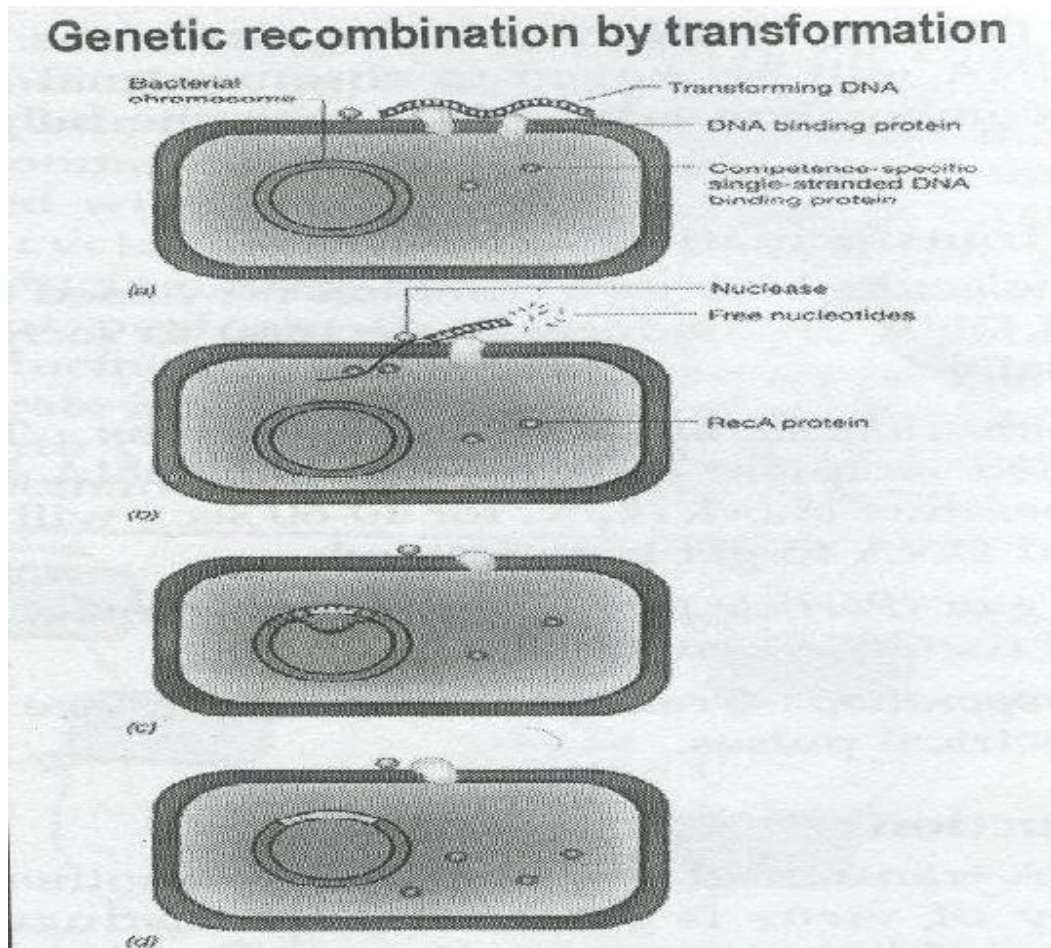
I. Transformation :

A naked DNA or cell free DNA incorporated into the recipient cells and brings about genetic recombination is referred as **transformation**. Most of the bacteria both G^+ and G^- are easily transformable but only a fragment of DNA can easily be transformed. The ability of an organism to take up the foreign DNA molecule is referred as **competence** and the organism is said to be competent. The competence of organisms varies among the organisms. *Bacillus* has 20% competence cells and *E. coli* and *Streptococcus*, 100% of cell become competence.

The gram -ve bacterium, *Haemophilus* takes only double strand DNA whereas the *Streptococcus* and *Bacillus* can take only single strand DNA, while the complementary strand is simultaneously degraded.

The naked DNA first bound on the surface of the recipient cell with the help of surface

proteins. A single strand of DNA is allowed to enter inside the cell. The entered DNA will be moved to chromosomal DNA and genetic recombination takes place with the help of *RecA* protein.



■ Artificial transformation

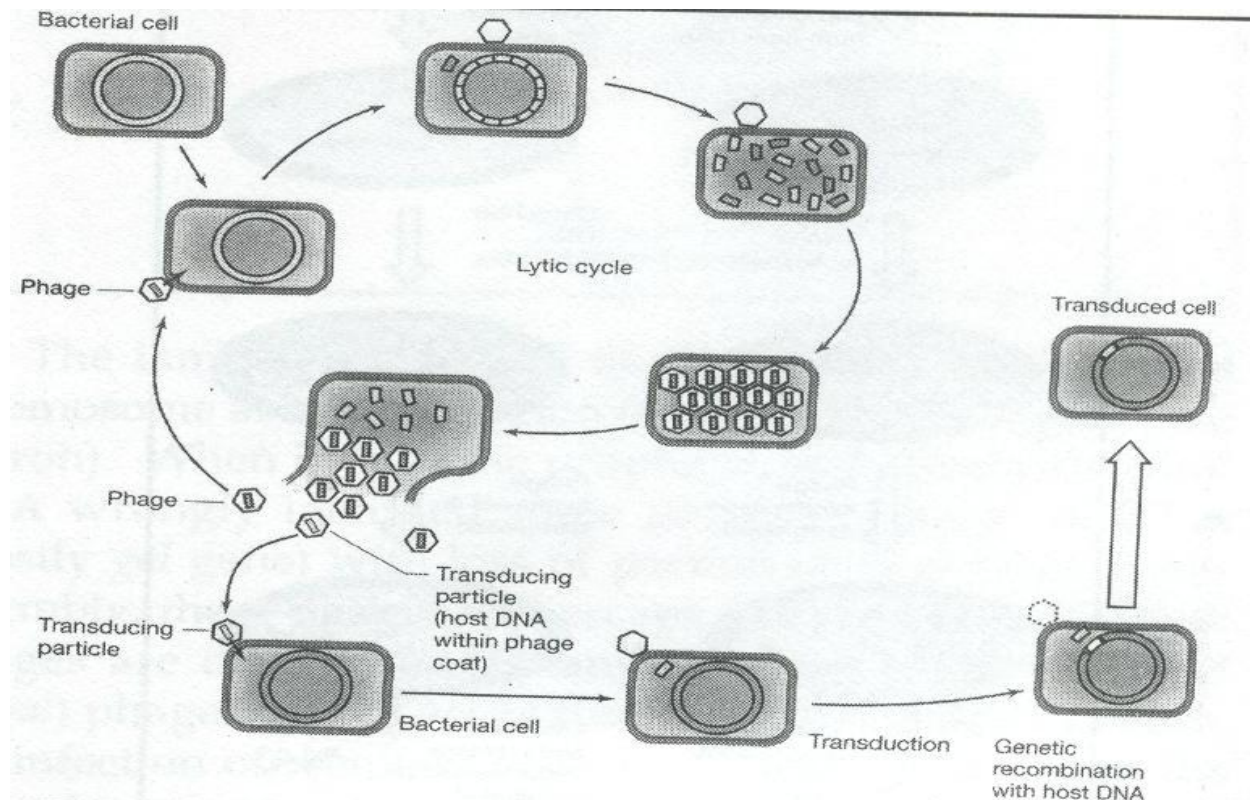
The molecular biology techniques now used to transfer a naked DNA to recipient cell artificially are;

- 1) **Calcium chloride mediated transformation:** The CaCl_2 washed recipient cells mixed with DNA allowed a temperature shock (42°C for 40-60 sec.) will allow the naked DNA to get transformed.
- 2) **Gene gun (particle gun):** will allow transformation of plant tissues, yeast, algae etc.
- 3) **Electroporation:** Transformation takes place by means of electrical pulses.

II. Transduction:

A DNA transferred from one cell to another through the agency of virus is referred as **transduction**. The transduction of bacteria occurs through bacteriophages.

Some phages are lytic, which kill the host cell during replication, called lytic cycle and virulent phages. Ex. T₂ phage, some phages replicate in the bacteria without killing the host cell, called as lysogenic cycle and temperate phage. The host cell is called as lysogen. Based on the behavior of virus, transduction occurs in two way. 1) Generalized transduction 2) Specialized transduction.



Generalized transduction

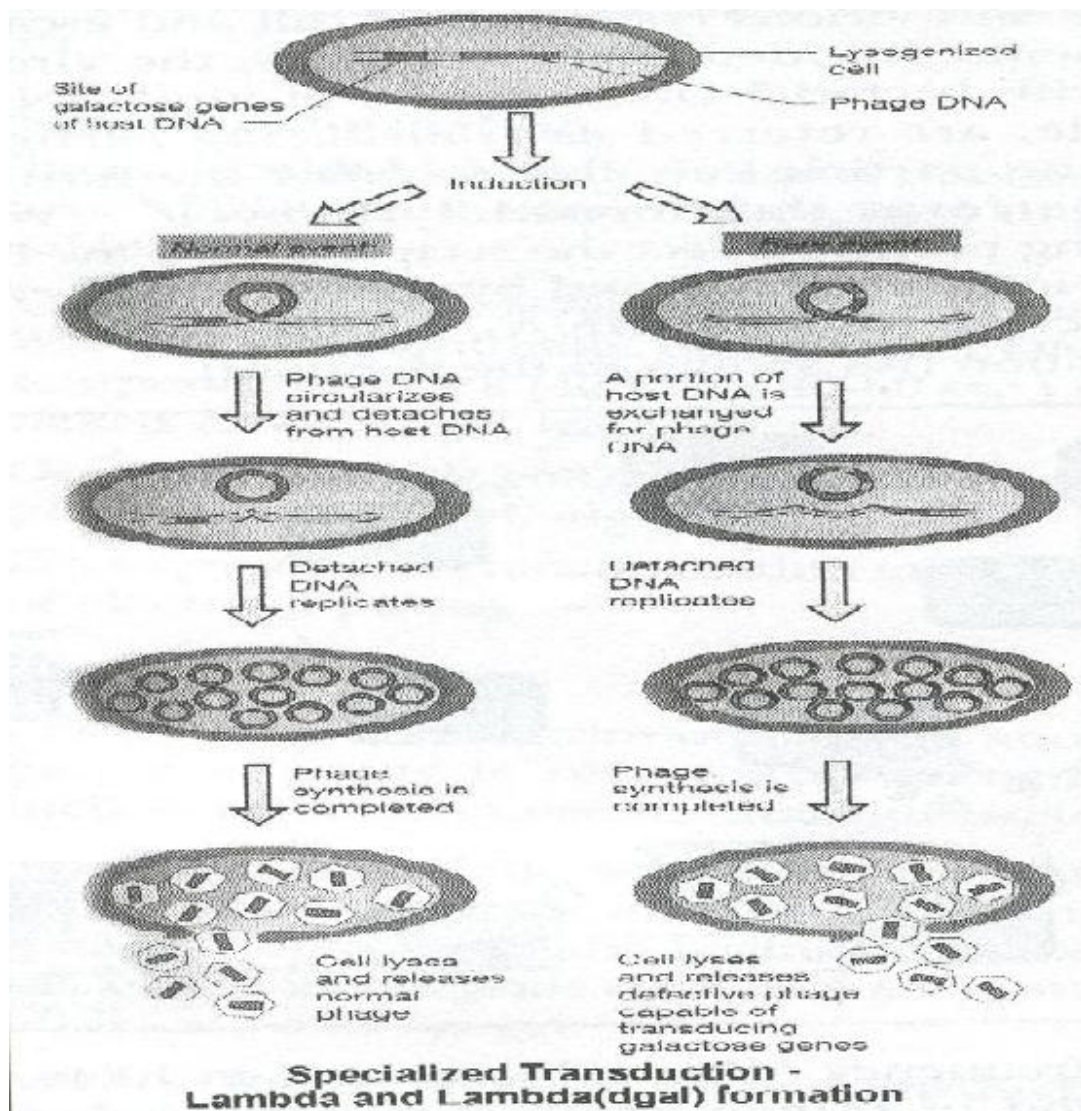
The generalized transduction occurs due to lytic phage. Normally, the virulent phage send its DNA after absorption into host cell and DNA will order the chromosomal DNA to synthesize viral DNA and viral proteins. Then packing takesplace and virion lyse the host cell and escape to the environment. Accidentally, sometimes viral protein packed with bacterial DNA instead of viral DNA during lytic cycle, are referred as **transducing particle**. This can able to infect the host cell, but cannot perform the normal lytic cycle. When the transducing particle infect the recipient cell, the DNA will penetrate into cytoplasm and by the help of *RecA* protein get into chromosomal DNA, which leads to genetic recombination as shown in the diagram.

The frequency of transduction is 1 in 10⁶ to 10⁸ cells. This method of transduction is generalized one. Any portion of donor DNA can become transducing particle and become transduced and transduction takes place; this type of transduction is referred as generalized transduction.

Specialized transduction:

The best studied specialized transducing phage is the phage lambda (λ) of *E. coli*. The location of the λ prophage in the bacterial chromosome is almost always between the bacterial genes gal and bio. Whenever the phage genome comes out of, or is excised from the bacterial chromosome, it sometimes takes with it gal or bio genes. When phages carrying gal or bio genes infect a new host, recombination with the gal or bio genes of the host can occur. It should be noted that almost all phages that carry some bacterial genes because of 'incorrect' excision are defective in certain viral functions because they are missing a piece of phage genetic information taken up by the bacterial genes. They cannot proceed through their entire replicative cycle, but the cell will yield phages if it is also infected with a complete phage that can code for the missing functions of the defective phages.

On the other hand, some temperate phages can be able to take up a specific portion of donor DNA as their part and transduction takes place, which is referred to as **specialized transduction**. The temperate phage (Lambda phage) sends its DNA after absorption. The penetrated viral DNA will get integrated into bacterial chromosomal DNA and persist for ever. This stage of phage is referred to as prophage. The cycle is lysogenic cycle. Due to chemical or physical induction, the prophage gets activated, replicates its viral DNA and protein coat followed by assembly and lysis of the cell.

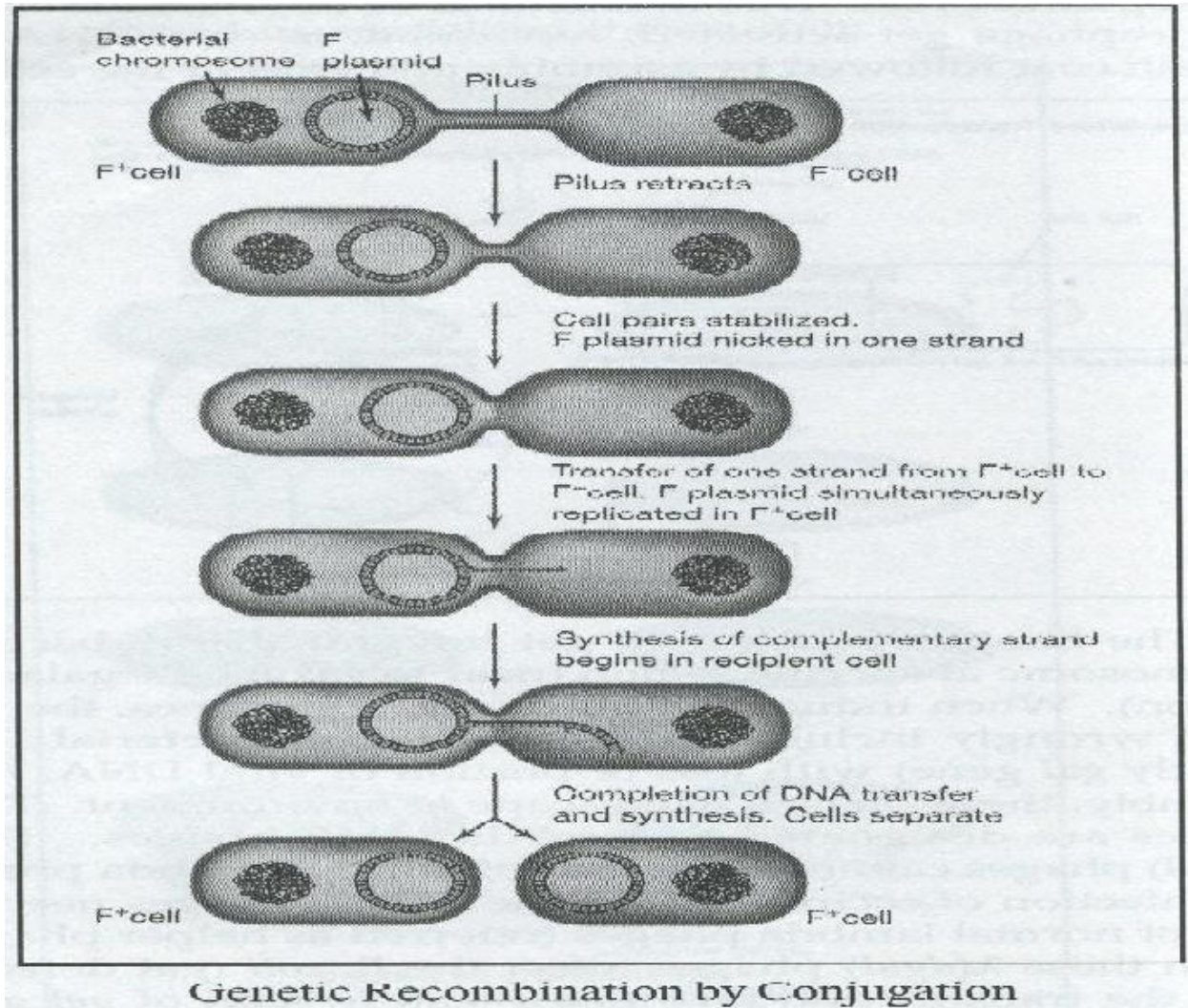


III. Conjugation:

Bacterial conjugation (mating) is a process of genetic recombination that involves cell to cell contact. The gene for conjugation (ability to mate) is a plasmid borne character. The gene involved in the conjugation is designated as F factor (fertility factor) which is located in the plasmid (referred to as F plasmid). The cell which has the F plasmid is the donor (male) designated as F^+ cells and the cell which does not have the F plasmid is the recipient (female) designated as F^- cells. The DNA will be transferred from donor to recipient through physical contact. So another requirement for cell to cell contact is sex pilus through which the donor DNA will be transferred to the recipient cell.

The diagrammatic representation shows how conjugation takes place. The sex pilus makes a connection between the donor and recipient. A nick (split) in the complementary strand of the F plasmid takes place. The single strand plasmid is transferred to the F^- cell and becomes double stranded. The F^+ cell also synthesizes its cDNA and becomes double stranded. After conjugation, both the

cells become F^+ cells.



Hfr cells:

Some times, F plasmid transferred from F^+ cell to F^- cell get integrated into recipient cell chromosomal DNA. Such conjugates possess the F factor in the chromosomal DNA instead of plasmids. They are referred as HFR cells (high frequency recombination). They have very high frequency of conjugations.

15.4 Other Genetic Recombinations:

The process by which a gene moves from one place of chromosome to another in a genome is called as **transposition**. The genes involved are transposon genes or transposable elements.

Insertion sequences are also a type of transposable element (of about 700 bp size) which also can cause the genetic recombinations. These DNA are used to insert a foreign DNA to an organism and also cause mutagenesis.

Genetic Engineering:

Development of sophisticated procedures for isolation, manipulation and expression of genetic materials in an organism, a field is called as **genetic engineering**. Isolation, purification and replication of fragment of DNA through some vectors are referred as **gene cloning**. The plasmids and bacteriophages are used as **vectors** for cloning.

CHAPTER NO 16 MUTATION

CONTENTS

16.1 Mutation:

16.2 Mutant identification

16.3 Replica plating technique:

16.4 Spontaneous and induced mutation

16.5 Point mutation

16.6 Mutagens

16.6.1 Chemical mutagens:

16.6.2 Physical agents:

16.6.3 Biological agents:

16.1 Mutation:

Mutation is an inherited change in the base sequence of the nucleic acid comprising the genome of an organism. A strain carrying such change is called as **mutant strain** and non-mutated which was isolated from natural environment is called as **wild type** strain.

Genotype of an organism for particular character can be written as three lettered lower case with italic letters following a capital letter. Ex. lac Z refers the gene which codes the enzyme β -galactosidase. The protein transcribed from this can be written as normal letters starting with capital letter. For the above gene, the protein may be written as *Lac Z*. The phenotype of the

organism for this gene can be written as Lac Z⁺ for presence and Lac Z⁻ for absence.

16.2 Mutant identification

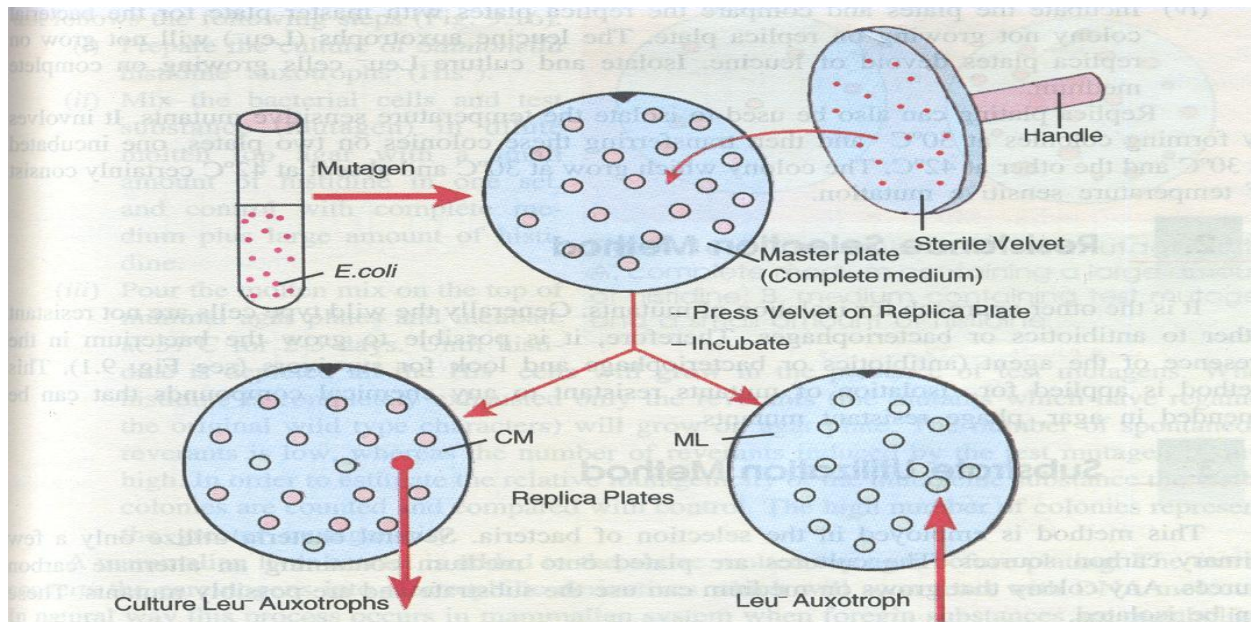
In a wild type organism very rarely the mutation will occur with very low mutation frequency. So identifying the mutant from the wild type is a key phenomenon. The mutants can be identified from a population either by screening or selection. Screening refers the procedure that permit the sorting of organism by phenotype or genotype selection refers the procedure in which pressure will be given to grow a particular genotype.

For example, in a mixed population, or particular mutant lost its color (pigmentation), we have to go for screening procedure to identify the mutant. On the other hand, in a mixed population, a particular mutant gain an antibiotic resistance, we can use selection procedure to identify the mutant by growing the mixed population over the medium incorporated with the particular antibiotic which will allow mutant alone to grow on that. This type of mutant identification is a powerful tool to identify the mutant.

16.3 Replica plating technique:

This is also a selection type of mutant identification procedure which will be useful to identify auxotroph mutants. Auxotrophic mutant is a metabolic defective mutant. The mutant which cannot synthesize a particular nutrient, required the nutrient through externally is referred as **auxotrophic mutant**. The strains which can synthesize the nutrients by themselves are referred as **prototrophs**. The auxotrophic mutants are very common for amino acids. Normally the wild strains can able to synthesize all the essential amino acids by themselves. By mutation if a strain lost its ability to synthesize particular amino acids (Ex. leucine), the mutant is referred as **leu-** auxotroph mutant. This mutant can grow only the medium supplemented with leucine,. Whereas the prototroph can grow in the medium without leucine. This type of auxotrophic mutant can be selected by replica plating technique.

The procedure of replica plating technique is as follows; the master plate (mixture of prototroph and auxotroph) will be duplicated to medium with and without leucine by means of rubber stamp like device (called as replica plate). A clone which grows in medium with leucine and which cannot show growth on medium without leucine is an auxotroph.



Replica plating technique for isolation of leucine auxotrophs (leu⁻); CM, complete medium;, ML, medium devoid of leucine.

16.4 Spontaneous and induced mutation

The natural radiation (cosmic rays, U.V. rays etc) alter the genotype of an organism which is called spontaneous mutation. Induction of mutation by some agents artificially is referred as induced mutation.

16.5 Point mutation

Mutation occurs in one or two base pairs of a gene is referred as point mutation. The other mutation is change in the segment of chromosome, referred as chromosomal aberrations. Since bacteria are having single filamentous double strand DNA as their genome, the point mutation is a common one.

16.6 Mutagens

The chemical, physical and biological agents that can induce the mutation are referred as mutagens.

16.6.1) Chemical mutagens:

The chemicals can be divided into four groups based on their action namely.

- * **Base analogs** – incorporate in the DNA instead of particular N base.
- * **DNA reacting chemicals** – react with DNA nucleotide.
- * **Alkylating agents** – change the structure of DNA and,
- * **Intercalating dyes** – these chemicals will insert between base pairs of DNA.

The most revealing findings about mutation in recent years have come from studies on the mutagenic effects of various chemicals. There are three main types of mutagenic chemicals. The first consists of compounds that can react chemically with DNA. Since the specificity of DNA replication depends upon purine-pyrimidine binding which results from hydrogen bonding between the amino and hydroxyl groups of the purines and pyrimidines, chemical modification of these amino and hydroxyl groups can cause mutation. Nitrous acid, which can remove amino groups from purines and pyrimidines is such a mutagen. The second type of mutagenic chemicals consists of base analogs. There are chemicals sufficiently similar in structure to normal DNA bases to be substituted for them during DNA replication. Although similar in structure, base analogs don't have the same hydrogen bonding properties as the normal bases. They can therefore introduce errors in replication which results in mutation. A third type of mutagenic chemicals is intercalating agents. These are flat molecules that can intercalate between base pairs in the central stack of the DNA helix. By this means they distort the structures and cause subsequent replication errors. Examples of such agents are acridine orange proflavin and nitrogen mustards.

16.6.2) Physical agents:

Ionizing and non-ionizing radiation:

Mutations most commonly occur during DNA replication. Some mutations occur as the result of damages inflicted by ultraviolet (UV) light or x-rays. Since these agents are an inescapable part of the environment (for eg, UV light is a component of sunlight), they probably account for many spontaneous mutations. However, mutation rates can be increased substantially by deliberately exposing a culture to such radiation. Any agent that increases the mutation rate is called a mutagen. Mutations obtained by use of a mutagen are said to be induced rather than spontaneous, though they may differ only in frequency not in kind. For example, UV light causes mutation under both natural and laboratory conditions. The number of mutants obtained by laboratory conditions is much higher, however, because of the high dosage of UV light used.

The major effect of UV light is to cause the formation of dimers by cross linking between adjacent pyrimidine, especially thymine, residues in DNA. These cross linked residues disrupt the normal process of replication by preventing the various polymerases from functioning. When x-rays interact with DNA, the result is usually a break in the phosphodiester backbone of the nucleic acid.

Both non-ionizing and ionizing radiations are able to cause mutation. Non-ionizing radiation has wider use in the microbial mutations.

- * **U.V. light:** This non ionizing radiation can be absorbed by purine and pyrimidine strongly at 260 nm. Two adjacent pyrimidines form a dimer, by which covalently joint. During replication, the DNA polymerase wrongly recognize the pyrimidine and add a wrong pair which leads to mutation called dimerization of thymine.
- * **Gamma rays:** This ionizing radiation at low concentrations react as free radical attack and DNA breakage which leads to mutation. Gamma rays are not commonly used for microbial mutations, instead highly useful for mutating plant materials.

16.6.3) Biological agents:

Transposable element, insertion sequences and Mu phage are some of the biological agents cause mutation.

- * **Transposable element:** A genetic element that can move from one place of chromosomal DNA to another place. Such elements insertion leads to mutation which is referred as transposon mutagenesis.
- * **Insertion sequences:** The simplest type of transposable sequence (about 700-1000 bp) which has the transposable gene alone, which causes the mutation.
- * **Mu-phage:** This double strand DNA containing temperate bacteriophage can able to act as transposable elements, causes mutation by transposition.

16.7 Site directed mutation:

The present modern molecular techniques allow to mutate on organism at specific portion of gene by means of special vectors (plasmids) with controlled system. This type of mutation is referred as site directed mutation.

CHAPTER No 17
INTERRELATIONSHIP BETWEEN MICROORGANISMS:

CONTENTS:

17.1 Interrelationship between microorganism: Beneficial and harmful relationships:

17.1.1 Plant microbe interaction:

17.1.2 Plant microbe – microbe interaction:

17.1.3 Microbe – microbe interaction;

17.2 Microbial interaction in soil:

17.3 Interrelationship between microorganisms : Beneficial and harmful relationship

17.4 Symbiosis:

17.5 Commensalisms

17.6 Negative / harmful / deleterious interactions

17.7 Antagonism

17.8 Predation

17.9 Parasitism

17.1 Interrelationship between microorganism: Beneficial and harmful relationships:

Interrelationships in soils are of 3 types

1. Plant-microbe interaction

2. Microbe – microbe interaction and
3. Plant- microbe – microbe interaction

17.1.1 Plant microbe interaction:

It mainly constitutes the association of microorganism with plants either in a positive way or in a negative way. The positive approach is mainly the symbiotic relationships and the negative approach constituents mainly pathogen plant interactions.

17.1.2 Plant-microbe – microbe interaction: Also called tripartite symbiosis

E.g., Alnus – Frankia – Mycorrhiza

Casuarina – Frankia – Mycorrhiza

17.1.3 Microbe – microbe interaction;

17.2 Microbial interaction in soil:

17.3 Interrelationship between microorganisms : Beneficial and harmful relationship

Microorganisms live in the soil, not in the form of pure culture, but as complex populations. Each particle of soil, contains more than one type of organisms. So, microbial ecosystem of soil is the sum of the biotic and the abiotic components of soil. Many of these organisms depend upon one another for direct and indirect nutrients. Some compete with one another for energy sources and for the elements and components used as nutrients. This results in the formation of numerous associations among the soil micro organisms. The composition of the microflora of any habitat is governed by the biological equilibrium created by the associations and interactions of all individual found in the community.

The microorganisms that inhabit the soil exhibited many different types of associations or interactions. Some of the associations are indifferent or neutral, some are beneficial type of interactions and others are detrimental or negative.

I. Beneficial / positive interactions

- a. Neutralism
- b. Symbiosis / mutualism
- c. Protoco-operation

d. Commensalism

a. Neutralism

It is a type of neutral association, in which two microorganisms behave entirely independently. e.g., Each could utilize different nutrients without producing metabolic end products that are inhibitory. This might be transitory as the condition change in the environment, particularly the availability of nutrients, the relationship might change.

b. Symbiosis / Mutualism

It is the type of association in which two symbionts relying on one another and both are benefited by the relationship. The manner in which benefit derived varies.

The living together of two or more organisms; Symbiotic association is evident in soil among several groups of organisms. Algae and fungi in lichens, bacteria residing within protozoan cells, bacteria and roots in the *Rhizobium*-legume symbiosis, fungi and roots in mycorrhizae. In lichens, the algae and fungi are in such an intimate physical and physiological relationship that the lichens they make are classified as distinct organism. The alga benefits in part because of the protection afforded to it by the hyphae that envelop and protect it from environmental stresses. While the fungi gains by making use of the CO₂ fixed by its photosynthetic partner. Symbiotic relationship exist between micro and macro organisms. In *Rhizobium*-legume symbiosis, *Rhizobium* fixes N₂ and transferred to host, inturn *Rhizobium* nutrients from the host.

c. Proto co-operation

One type of mutualistic association is that involving the exchange of nutrients between two species, a phenomenon called synergism. Many microorganisms synthesize the vitamins and organic acids in excess of their nutritional requirements. Others have a requirement of one or more of these nutrient. Hence certain combinations of species will grow together but not apart when nutrient levels are very low.

As association of mutual benefit to the two species, but without the co-operation being obligatory for their existence or for their performance of some reaction.

Nutritional proto co-operation has been demonstrated in cultures. Eg: In a medium deficient in nicotinic acid and biotin, neither *Proteus vulgaris* nor *Bacillus polymyxa* will multiply as the former (B) requires nicotinic acid and the latter requires biotin. In mixed culture, in the same medium however both have grown since the partner bacterium synthesizes the missing vitamins.

e. Commensalisms

It is the type of beneficial association, in which only one species derives benefit while the other is unaffected. This occurs commonly in soil with respect to degradation of complex molecules like cellulose and lignin. One patter can attack a substrate not available to the second organism, but the decomposition results in the formation of products utilized by the second. The one which offer benefit is called commensalism eg: (1) Many fungi able to degrade cellulose and yield glucose and organic acids. This can serve as a which source for many bacteria and fungi, which are non cellulolytic (2) The second type of commensal association arises from the need of many microorganism for growth factors. These compounds are synthesized by many micro organism and their exertion permits the proliferation of nutritionally fastidious soil inhabitants.

17.6 Negative / harmful / deleterious interactions

Detrimental effects of one species on its neighbours are quite common in soil, and they are dictated by the decreases in abundance or metabolic activities of the susceptible organisms.

This include;

- a) Competition
- b) Amensalism
- c) Parasitism and predation

a) Competition

The rivalry for limiting nutrients or other common needs. In such situations the best adapted microbial species will predominate or infact, eliminate other species which are dependent upon the same limited nutrient substances. Eg: Competition between strains of *Rhizobium* derived from soil and those applied with legume seeds at the time of sowing. The better competitor enters the root hairs more frequently and it is responsible for a high % of nodules.

b) Amensalism

It is a negative interaction, in which the release of products by one species is toxic to its neighbours. Antagonism is a type of amensalism.

17.7 Antagonism

The killing, injury or inhibition of growth of one species of microorganisms by another when one organism adversely affects the environmental of the other. The toxic compounds are antibiotic. An antibiotic is a substance formed by one organism that in low concentrations, inhibits the growth of another organism. Antibiotics is common among *Streptomyces* isolates, but numerous strains of *Micromonospora* and *Nocardia* are also active. The most common frequently

encountered bacterium synthesize antibiotics are species of *Bacillus*, strains of *Pseudomonas*, species of *Penicillium*, *Trichoderma*, *Aspergillus*, *Fusarium* are also excrete antibiotic substance. Antimicrobial compounds against fungi are present in the soil, which inhibit the germination of fungal spores. This phenomenon is termed as fungistasis. Cyanide is produced by certain fungi in concentrations toxic to other microorganisms, and algae elaborate fatty acids which exhibit a marked antibacterial activity. Other metabolic products that may result from microbial activity in soil, which are likely to be inhibiting to other species are CH₄, sulfides and other volatile S compounds. *B. t* toxic to lepidopteran insects. *Myxobacteria* (slime (B)) and *streptomyces* are antagonistic because they secrete potent lytic enzymes which destroy other cells by digesting their cell wall. The degraded cellular material, as well as the released protoplasmic material, serve as nutrients.

17.8 Predation

Direct attack of one organism on another is called predation. It is one of the most dramatic interrelationships among the micro organism in nature. Of the many microscopic inhabitants of soil, the bacteria stand out as particularly prone to the attack of predators. The most numerous predators on bacteria are protozoans, which by feeding on the billions of bacteria undisputedly affect their populations. Protozoans are a key factor in limiting the size of bacterial populations. Probably reducing the abundance of cells and serving to maintain a diverse community.

- *Myxobacteria* and cellular slime molds also affect by feeding directly on them
- Bacteria of the diverse genera are attacked by bacteriophages.
- *Bdellovibrio* is ubiquitous, capable of attacking a number of bacterial genera.

17.9 Parasitism

Is defined as a relationship between organism in which one organism lives in or on another organism. The parasite feeds on the cell, tissues or fluids of another organism the host, which is commonly harmed in the process. The parasite depends upon the light and lives in intimate physical and metabolic contact with the host. All major groups of plants, animals and micro organisms are susceptible to attack by microbial parasites. There are many strains of fungi which are parasitic on algae and other fungi by penetration into the host.

Viruses which attack bacteria, fungi and actinomycetes are strictly intercellular parasites. Protozoans are prone to parasitic attack as well specialized fungi penetrate the protozoans and destroy the cell. In addition to lysis associated with the actions of neighbouring heterotrophs, microorganism may autolyse merely because of nutrient deficiency.

Antagonistic interrelationships are also very common among the soil population where by one organisms directly or indirectly affects the activities of another organisms.

1. Competition among micro organism for available nutrition. This may occur between the same group, as between two types of (B), or between different organic (F, B, A).
2. Creation of one organic of conditions which are unfavourable for the growth of another (change in pH).
3. Production by one organic for specific substances which are injurious to growth of other organic alcohols, quinones and antibiotics.
4. Direct parasitism of one organic upon another various effects of (F) upon (B), and (B) upon (F).

Course Outline-INTRODUCTORY MICROBIOLOGY
PRACTICAL

Sl. No. Lectures	Title of Experiments
1-2.	Study of compound microscope/bright field microscope A. Study of parts of microscope B. Examination of animate and inanimate objects C. Working of microscope
3.	Microscopic examination of living microorganisms by Temporary Wet Mount (TWM) technique .
4.	Hanging drop technique for demonstrating motility of living bacteria.
5.	Simple Staining technique
6.	Differential staining technique-Gram stain
7.	Study of structural staining technique
8.	Sterilization techniques
9.	Preparation of culture media
10.	Preparation of nutrient agar
11.	Preparation of nutrient broth
12.	Preparation of nutrient agar slants/slopes
13-14	Isolation and culture of microorganisms by streak plate method
15-16	Isolation and culture of microorganisms by serial dilution technique and pour-plate method
17-18	Determination of rate of microbial growth by Turbidometric technique.

STUDY OF COMPOUND MICROSCOPE / BRIGHT FIELD MICROSCOPE

- A. STUDY OF PARTS OF MICROSCOPE
- B. EXAMINATION OF ANIMATE AND INANIMATE OBJECTS
- C. WORKING OF MICROSCOPE

A microscope may be defined as an optical instrument consisting of lens or combination of lenses for enlarging or magnifying images of minute objects.

Compound microscope differs from a simple one. In that it consists of two sets of lenses – one known as objectives and other as ocular or eye – piece.

A. STUDY OF PARTS OF MICROSCOPE

Mechanical parts:

1. **The body with its draw tube:** It is a metallic part and draw tube is provided with a millimeter scale, which shows the total length of the working tube.
2. **Stage:** It is a platform which accommodates the microscopic glass slide on which the object to be examined is mounted. It has an aperture in the center to permit light. The stage may be fitted with two metal clips, which are of mechanical type where by the slides can be moved by rack and pinion adjustment.
3. **Coarse adjustment:** It is a metallic part of the microscope, which is big circular screw, it moves the nose - piece up and down rapidly for approximate focusing.
4. **Fine adjustment:** It is the metallic part of the microscope (small screw) which move the nose - piece very slowly for definite sharp focusing.
5. **Nose-piece:** It bears different objective lenses and can be rotated to change from one objective to another according to the requirement.
6. **Arm:** It resembles English letter 'C', supports the upper half of the microscope.
7. **Base:** This is horse – shoe shaped metallic part and supports the entire microscope.
8. **Irish diaphragm:** This is made of metal and controls the amount of light striking the object. It can be opened and closed with the lever.
9. **Eye-piece:** It is an optical part of the microscope and also called a ocular. It has two lenses.
 - 1) Field lens (upper small lens)
 - 2) Eye lens (lower larger lens)
10. **Objectives:** This is an optical part of the microscope, which is nearest to the stage. Originally microscopes have three objectives, which are fixed in a revolving nose-piece.
 - 1) **Low power objective (or 10x):** It is an objective where the working distance is more. If the ocular is 10x, then the magnification is 100 times.
 - 2) **High – power objective (40x or 45x):** Here the working distance is less as compared to low power. If the ocular is 10x, then the magnification is 400 or 450 times.
 - 3) **Oil immersion objective: (100x):** This requires the use of a drop of either cedar wood oil or liquid paraffin between the lens and the object. The oil serves to prevent loss of light rays due to refraction. It has a refractive index (R.I.) 1.51. Here the working distance is very small. If the ocular is 10x, then the magnification is 1000 times.
11. **Condenser:** It is an optical part, which is made of lenses. It condenses the light rays there by preventing the escape of light rays. It also controls light intensity.
12. **Mirror:** It has two reflecting surfaces (1) plane and (2) concave. The plane mirror reflects the light rays parallel to one another and the light source may be artificial like

tube lights, while the concave mirror concentrates the light and the light source may be visible natural diffused light rays.

PRINCIPLE OF MICROSCOPE:

The light rays reflected from mirror pass through the diaphragm then condenser were the rays are condensed pass through the specimen and reach the objective. The first real and inverted image is formed within body tube, which again serves for further magnification, by ocular system, which forms virtual image.

DEFINITIONS:

1. **Resolving power** : The resolving power of a lens is its ability to show two closely adjacent points as distinct and separate

$$\text{Resolving power (RP)} = \frac{\text{Wavelength}}{2 \times \text{Numerical aperture}}$$

2. **Numerical aperture**: It is the function of the effective diameter of an objective in relation to its focal length and refractive index of the medium between the specimen and the objective.
3. **Magnification**: It is the ratio between size of the image and size of the object.
4. **Working distance**. It is distance between the specimen and the objective lens.

B. EXAMINATION OF ANIMATE AND INANIMATE OBJECTS

MATERIALS REQUIRED:

Pond water sample, cover slips, slides, filter paper strips, distilled water and microscope.

Procedure:

1. Place a small piece of filter paper on clean grease free slide and pond water on another slide.
2. Use a drop of distilled water to fix the filter paper (small pieces) to slide and if necessary use the cover slip.
3. Mount the slide on stage, adjust and focus with low power and high power objectives.

OBSERVATIONS:

1. Observe cellulose fibers in low power and high power objectives. Take neat diagram of fibers.
2. Observe different microorganisms namely algae, protozoa from pond water sample in low power and high power objectives. Take neat diagram of the specimens.

CONCLUSION

WORKING OF A MICROSCOPE:

MATERIALS REQUIRED

A slide with a smear, lens cleaning paper, cedar wood oil or liquid paraffin and microscope.

1. Place the clean grease free slide on the stage, specimen side up and center the specimen as accurately as possible over the hole in the center of the stage.
2. Adjust the light source until it posse's maximum amount of light through the specimen. With the low power objective in position, lower the body tube by means of coarse adjustment until the objective is about $\frac{1}{4}$ inch from the slide.
3. Look through the eye-piece; adjust with coarse adjustment knob until the specimen is the approximate focus. Bring the specimen to sharp focus with the fine adjustment knob.
4. After examining with low-power objective change to the thigh power objective by rotating the nose-piece without changing the position of the slide.
5. Look though eye-piece and bring the image into final accurate focus by using fine adjustment.
6. Focus the specimen by the oil immersion objective with a small drop of cedar wood oil on the object/specimen. Raise the condenser, open the Irish diaphragm fully and turn the mirror to get maximum amount of light. Use fine adjustment knob to get clear sharp image of the specimen.

Precautions to betaken while using a microscope.

1. Open both the eyes while looking into a microscope.
2. Never touch the lenses. If the lenses are dirty, wipe them gently with lens cleaning paper
3. Always remove oil from the oil- immersion objective after use.
4. Keep the stage of the microscope clean and dry,
5. Never remove any parts of the microscope.
6. Always use both hands while carrying the microscope
7. When the microscope is not is use keep it covered in the microscope compartment.

Exercise No.3

Microscopic examination of living microorganisms by Temporary Wet Mount (TWM) technique.

Normally two techniques are employed to study microbial cells. First method employs examination of living cells and the second employs the study of stained cells. Living organisms can be studied to determine the natural size and shape of cells, cellular arrangement, motility, reactions to various chemicals or immune sera and response to environmental factors. But the

observation of cells in their natural or unstained state is sometimes difficult because of their semi-transparency.

Direct examination of living microorganisms (*viz.*, bacteria protozoa, algae) can be studied by two methods: Wet mount and Hanging drop technique. Both the techniques are very useful in determining size, shape and movement.

Materials required:

12-18 hour old broth culture of *Bacillus cereus*
Spirogyra sp. (culture or in pond water)
Paramecium sp.(in pond water)
Glass slides
Cover slips
Dropping pipettes

Protocol:

1. Transfer a small drop each of the *Bacillus* culture and pond water containing the algae and protozoa, using a fresh pipette for each organism, on the centre of clean glass slides.
2. Handle the cover slip by its edges and place it on the drop.
3. Press the cover slip gently with the end of a pencil.
4. Observe the slide under low and high power objective.

Observations

Note the size, shape and characteristics of motility of bacteria and other microorganisms observed in the preparations.

Exercise No. :4
Hanging drop technique for demonstrating motility of bacteria.

Hanging drop preparation is useful for microscopic examination of living microorganisms, especially bacteria without staining them and to see their motility due to flagella.

Materials required:

12 hour old broth culture of *Proteus vulgaris*
Hanging drop (cavity) slide
Cover slips
Vaseline or petroleum jelly
Match sticks
Wax marking pencil

Protocol:

1. Clean and flame a hanging drop slide and place it on the table with the depression uppermost.
2. Spread a little Vaseline or petroleum jelly around the cavity of the slide.
3. Clean a cover slip and apply a petroleum jelly on each of the four corners of the cover slip, using a match stick.
4. Place the cover slip on a clean paper with the petroleum jelly side up.
5. Transfer one loopful of culture in the centre of the cover slip.
6. Place the depression slide on to the cover slip , with the cavity facing down so that the depression covers the suspension.
7. Press the slide gently to form a seal between the cover slip and the slide.

8. Lift the preparation and quickly turn the hanging drop preparation cover slip up so that the culture drop is suspended.
9. Examine the preparation under low power objective with reduced light.
10. Switch to the high power objective and examine the preparation again.
11. Place a drop of oil on the cover slip and examine the preparation under oil immersion objective.

Observations

Record your findings as to the size, shape, color and motility of the bacteria.

Exercise No. 5 SIMPLE STAINING TECHNIQUE

Bacterial morphology may be examined in two ways (1) By observing the living, unstained organisms, as is done in demonstrating bacterial motility. (2) By observing dead cells stained with dyes. Bacteria differ chemically. It is this chemical difference that enables to distinguish bacteria by staining, the stain or dye generally reacting with the bacterial cell but not with the background.

Stains or dyes are generally salts in which one of the ion is colored, and colored ion referred to as chromophore and the other ion is called as auxochrome. Dyes are divided into two groups as basic and acidic. If the colour is in the positive ion of the dye, it is a basic stain eg. Crystal violet and carbol fuchsin. And if the color is in the negatively charged ion then it is acidic stain e.g. Eosin Y.

MATERIALS REQUIRED:

Slides
Methylene blue
Crystal violet
Bacterial cultures
Microscope

PROTOCOL:

1. Place a drop of distilled water on a clean glass slide and take a loopful of the pure culture and make thin smear on the slide.
2. Fix the smear by passing it over the flame or air dry.
3. Flood the fixed smears with 3-5 drops of methylene blue or Crystal violet and allow to act for 30 seconds.
4. Wash the stained preparation with water and blot dry.
5. Examine under low power and afterwards oil immersion objective and make sketches.

OBSERVATIONS:

Observe the difference in cell size, shape and arrangements. Make sketches of variety of cells as seen under the microscope.

CONCLUSION:

STUDY QUESTIONS:

Exercise No.6

DIFFERENTIAL STAINING TECHNIQUE – GRAM STAIN

All bacteria will not stain by a particular stain. Microorganisms also differ from one another, chemically and physiologically and thus may react differentially to a given staining procedure. This is the basic principle involved in differential staining. Thus, we may define differential staining as a method of differentiating many different types of bacteria.

Gram stain, the most useful staining procedure used in bacteriology, is a differential stain. In this procedure bacteria are divided to two groups based on cell wall composition (Murein complex in Gram – positive bacteria murein complex is thick and single layered while, in Gram – negative bacteria is thin and multi-layered. The first of these groups is stained purple by the Gram stain, while second group is stained pink colour. The organisms stained purple are known as Gram-positive and organisms stained pink are known as Gram-negative.

The Gram stain required four different reagents:

1. Primary stain
2. Mordant
3. Decolorizing agent
4. Counter stain

1. **Primary Stain:** It is the stain used at the first step to colour the bacterial cells e.g. : crystal violet.
2. **Mordant:** It is a substance, which increases the attraction between the cell wall and the dye helps to fix the dye on the cell. Under the action of a mordant, cell is more strongly stained is much more difficult to washout the stain after the application of a mordant. Eg: Acid, Basic metallic salts and Iodine.
3. **Decolorizing agent:** It is a substance, Which removes the dye from a stained cells decolorize much more easily than others. In the Gram's stain and other differential staining in this variation which helps in the rate of decolorization e. g. 95% alcohol, Acetone.
4. **Counter stain:** It is also a stain of different color from the initial one. The purpose of counter stain is to give color to the decolorized cells. Those organisms, which are not readily decolorized, retain the color of the primary stain. Those, which are readily, decolourized retain the color of the counter stain e.g. Safranin.

MATERIALS REQUIRED:

Clean slides
Inoculation needle,
Bacterial cultures,
Stains,
Mordents,
Decolorizing agent
Microscope,
Immersion oil.

PROTOCOL:

1. Take a clean slide and place one drop of distilled water.
2. Transfer a small quantity of bacterial culture.
3. Thoroughly mix the culture and spread the drop on the slide to form a thin film of smear.
4. Air dry the slide or fix the cells by holding the slide above the flame.
5. Flood the slide with crystal violet, and allow it to react for one minute.
6. Drain off the excess stain into staining sink and immediately rinse it thoroughly with the gentle stream of running water or tap water.
7. Spread the film or smear with Gram's iodine. Allow the iodine to react for one minute.
8. Drain off the iodine and immediately rinse with running water or tap water.
9. Decolorize with 95% alcohol/acetone. Allow alcohol to react for 30 seconds.
10. Rinse with the tap water
11. Flood the slide with safranin. Allow the safranin to react for one minute.
12. Rinse with tap water and air dry the slide or blot dry.
13. Observe in 10x, select the area and place one drop of immersion oil.
14. Adjust for oil immersion objective or 100 X.
15. Take the diagrams with proper labeling.

OBSERVATIONS:

Culture	Gram reaction	Arrangement of cells
---------	---------------	----------------------

Yeast cells

Bacterial cells

Gram positive = +Ve, Gram negative = -ve

CONCLUSION:

STUDY QUESTIONS:

Exercise No. 7

STUDY OF STRUCTURAL STAINING TECHNIQUE

- 1) CAPSULE STAIN
- 2) ENDOSPORE STAIN

Many bacteria have a capsule, which apparently originates from the outer layer of the cell membrane. The chemical nature of the capsular material is slimy polysaccharide, like glycol-protein material. The capsules are often one or two times as the diameter of the cell and may appear to extending over a chain of organisms and completely surround paired organisms such as Diplococci. The capsule acts as a defensive structure against the activity of Phagocytic cells of the body.

MATERIALS REQUIRED:

Crystal violet

Slides

Cultures of *Klebsiella pneumoniae* or *Aerobacter aerogenes*

Aqueous copper sulphate solution (20%)

PROTOCOL:

1. Prepare a smear of the given organism and allow to air dry.
2. Do not heat fix
3. Stain with crystal violet for 5 minutes.
4. Wash with copper sulphate solution.

OBSERVATIONS:

The capsules appear blue violet and cell is stained dark blue, sketch a few cells to show the size of the capsule relative to the remainder of the cell.

CONCLUSION:**STUDY QUESTIONS:****2) ENDOSPORE STAINING:**

Species of the genera *Bacillus* and *Clostridium* produce a structure referred to as an endospore. Unlike the vegetative cell producing it, the endospore is a highly resistant body, capable of surviving for long periods even under unfavourable conditions as it possess calcium and dipicolonic acid in the cortex region.

MATERIALS AND METHODS:

Safranin
Malachite green
Bacterial culture
Slides
Microscope.

PROTOCOL:

1. Prepare a smear of the given culture, dry in air and fix it with heat.
2. Place the slide above the beaker containing boiling water.
3. Cover the smear with pieces of blotting paper, keep saturated with malachite green and continue heating for 5 minutes.
4. Wash gently with water.
5. Counter stain with safranin for 30 sec.
6. Wash with water and blot dry.
7. Observe under oil immersion objective and sketch.

OBSERVATIONS:

(Spores are stained green and the vegetative cell red.)

CONCLUSION:

STUDY QUESTIONS:

Exercise No. 8 STERILIZATION TECHNIQUES

Sterilization is a method of freeing up of an article from all living organisms. The common methods of sterilization are as below.

A) **Dry heat:** Sterilization by dry heat is carried out in many ways.

1. **Red heat:** Inoculation loop: forceps and spatula are sterilized by heating them in flame up to red-hot.
2. **Flaming:** Stoppers, culture tubes, flasks etc., are sterilized by passing them through the flame
3. **Hot air oven:** It is electrically heated and fitted with thermostatic arrangement with blower for ensuring rapid and controlled heating of the materials, equipments such as Petri plates, pipettes, test tubes, conical flasks are sterilized at a temperature of 160°C for one hour. The time and temperature for sterilization of glass ware are as follows:
 120°C – for 8 hours.
 140°C – for or $2\frac{1}{2}$ hrs.
 160°C for 2 hr. is most commonly used.
 180°C – for 20 minutes.

B. **Moist heat:** For most types of media, cloth, rubber and other materials that would be destroyed by dry heat is sterilized by moist heat in an autoclave at 121°C for 20 minutes using steam under 15 pounds pressure.

Water boils when vapour pressure equals the surrounding atmospheric pressure. This occurs at 100°C , when water is boiled in a closed vessel at increased pressure. Thus the temperature at which it boils and that of the steam will also rise. The temperature boiling time and pressure used are as follows.

Steam Pressure	Temp.	Time in min
10 - lbs	115°C	45 min
15 - lbs	121°C	20 "
30 - lbs	134°C	3 "

At 15 lbs pressure most of the organisms including the spores of *Bacillus* and *Clostridium* are readily killed hence the sterilization in the autoclave is carried out under 15 lbs. Pressure.

WORKING:

All the air in the autoclave is removed by opening the air outlet since the admixture of air with the steam results in the lowering of the temperature. After the pressure reaches 15 lbs., it is maintained for 15-20 minutes and then the steam supply valve is closed until the pressure falls to 0. After ensuring that there is no more pressure inside, the material is removed.

Working procedure of autoclave

The culture media are wet (moist) sterilized because they are either liquids or semisolid at gels containing water. An autoclave which is a boiler with an inner chamber and a light fitting lid is employed for this. Water is taken at the bottom between the inner chamber and outer jacket and arrangement is made to heat the water to produce steam. The autoclave is provided with a safety valve and a pressure gauge. The principle of moist sterilization is to produce steam which raises the pressure in the inner chamber. The steam temperature goes up with increasing pressure. A constant pressure is maintained by adjusting the heating. The most frequently adopted wet sterilization temperature is 121⁰C, which is achieved by maintaining a pressure of 151bs/sq inch (1.05kg/sq.cm) for 15 minutes at this temperature are sufficient for sterilization.

- I. **Chemical methods:** Disinfectants like alcohols, phenol, mercuric chloride etc., are used to surface sterilize any materials so that bacteria present on the outer surface are killed.
- II. **Filtration:** Many materials for example sugars, blood sera, which are destroyed by heating at temperatures, which are normally used for sterilization. To sterilize such heat labile materials filters can be used. The filters remove bacteria by the sieve-like action of the pores in the filter and also by adsorption of microbes to the filters. The types of filters used are membrane filter, sintered glass filter, Seitz asbestos pad filter, the chamber land and sels candle type filter etc., All these filters are used by being attached to a suction flask to draw the liquid materials through them.
- III. **Gaseous sterilization:** The use of ethylene oxide vapours under pressure in special equipment is a common method of cold sterilization: ethylene oxide is highly toxic for viral particles, bacterial and fungal cells and the heat-resistant endospores.

OBSERVATIONS:

Observe different instruments like Autoclave, Pressure cooker, Hot air oven, Filters and draw a neat sketch of these instruments along with the proper label.

CONCLUSIONS:

STUDY QUESTIONS:

Exercise No. 9

PREPARATION OF CULTURE MEDIA (NUTRIENT AGAR)

It is not always possible to identify organisms by morphology alone. One of the most important methods for studying and identifying the organisms is by growing them on simple artificial food material called 'culture media'. The microbial growth on solid culture media itself is called 'culture'.

Some naturally occurring substances are used for the cultivation of bacteria. Notable among these is milk, usually skimmed rather than whole. Such natural materials are merely dispensed into tubes or flasks and sterilized before use. Media of the nutrient broth or nutrient agar type are prepared by compounding the required individual ingredients or, more conveniently, by adding water to a dehydrated product which contains all the ingredients. Practically all media are available commercially in powdered form.

Some of the common ingredients of culture media are :

- 1) **Carbohydrates:** These are the energy sources in the form of maltose, glucose etc., which serves as a carbon and energy source for microbial growth.
- 2) **Proteins:** In the form of peptone, tripeptone etc., which serves as nitrogen requirement of microbial cell.
- 3) **Beef extract and yeast extract:** They are in the form of reduced organic bases, which helps to supply nitrogen, vitamins, minerals etc.,
- 4) **Amino acids:** They are in the form of reduced organic bases, which helps to supply nitrogen, vitamins etc.,
- 5) **Growth factors:** In the form of vitamins like riboflavin, niacin etc.,
- 6) **Solidifying agents:** In the form of Agar, silica gel and Gelatin. The most common in use is agar, which has certain unique properties. It is obtained from a sea -weed called, *Geladium cornea*,. Its melting point is 100⁰ C and solidifies at a temperature of 42⁰C-45⁰C.
- 7) **Mineral salts:** In the form of chloride, sulphate, nitrite and nitrate etc.,

Types of culture media

A) Media can be classified on the basis of physical state, as

- 1) Liquid Media
- 2) Solid Media
- 3) Semi solid media

B) Another way of classifying the media is on the basis of their components.

1) **Synthetic Media (Reproducible media).** The synthetic media are made with pure chemical substances and the exact composition of the medium is known. It can be reproduced any number of items. Eg. Norris nitrogen free medium.

2) **Non-synthetic Media:** non-synthetic media are represented by an almost infinite variety of naturally occurring substances for growing microorganism Eg. Milk, Blood serum, Coconut milk, Eggs, Potatoes, etc.,

C) **Special Media:** Special media are further classified into:

- 1) **Enriched media:** In these media substances such as blood serum, and egg are added to basal medium (NA). These media are used to grow bacteria, which are more exacting in their nutritional needs. e. g. Blood agar.
- 2) **Selective media:** The incorporation of certain special chemicals to nutrient agar will prevent growth of one group of bacteria without inhibiting others. Eg: Crystal violet at a specific concentration in Macconkeys agar will prevent the growth of gm+ve bacteria without affecting the growth of gm-ve bacteria eg. Macconkey's Agar.
- 3) **Differential media:** It is used to bring out differentiating characters of bacteria and thus helping them to distinguish. Eg: Eosine methylene blue agar (EMB agar), Yeast extract mannitol agar with congoed (YEMA).

PREPARATION OF NUTRIENT AGAR

Materials required:

Peptone
Yeast extract
Distilled water
Autoclave
Conical flask with cotton plug

PROTOCOL:

- 1) Each ingredient, or the complete dehydrated medium, is dissolved in the appropriate volume of distilled water.
- 2) The pH of the fluid medium is determined with a pH meter and adjusted if necessary.
- 3) If a solid medium is desired, agar is added and the medium is boiled to dissolve the agar.
- 4) The medium is dispensed into conical flasks.
- 5) The medium is sterilized, generally by autoclaving. Some media that are heat labile are sterilized by filtration.

Exercise No:10 PREPARATION OF NUTRIENT BROTH

MATERIALS REQUIRED:

Peptone
Yeast extract

Distilled water
Autoclave
Test tubes with cotton plugs and
Culture of any organisms

PROCEDURE:

1. Slowly add 100 ml of distilled water to 0.5 gm of peptone and 0.3 gm of yeast extract in a conical flask. Swirl the flask gently to dissolve the ingredients. The final solution should be clear.
2. The media is dispensed into test tube and sterilized in the autoclave.
3. After the nutrient broth is sterilized the media is cooled to about 45⁰C.
4. After cooling inoculate the broth aseptically with the culture provided.
5. Incubate the labeled test tube for 24-48 hrs. at 30⁰C.
6. Observe for growth in broth, which may be in the form of turbidity, pellicle formation (a mass of cells floats on the top of broth) or as sediment.

OBSERVATIONS:

Draw a neat diagram of test tubes having different patterns of growth. And also prepare a thin smear using simple staining technique observe the microbial cell and draw a neat diagram for low power and oil immersion objective.

Exercise No. 11

PREPARATION OF NUTRIENT AGAR SLANT/SLOPES:

MATERIALS REQUIRED:

Peptone
Yeast extract Agar
Distilled water
Autoclave
Test tube with cotton plug and
Culture of any organism.

PROCEDURE:

1. Slowly add 100 ml of distilled water to 0.5 gms. of peptone and 0.3 gm. of yeast extract. Swirl the flask gently to dissolve the ingredients.
2. When the broth is completely dissolved add 2 gms. of agar. This is heated to dissolve the agar.
3. The media is then dispensed into test tube and sterilized in an autoclave.
4. The test tubes are then cooled in an inclined position.
5. The test tubes are then cooled, the surface is streaked by the given culture with an inoculation loop.
6. The test tube is incubated at room temperature (30⁰C) for 24-48 hrs.
7. After incubation examine the mass of surface growth that develops.

OBSERVATION

CONCLUSION

STUDY QUESTIONS:

Exercise No. 12-13

ISOLATION AND CULTURE OF MICROORGANISMS BY STREAK PLATE METHOD:

Adding a solidifying substance to broth media containing bacterial cells traps the individual cells in place. They produce a fixed colony of cells that grows to form a visible mass. Mixtures of different types of bacteria from a liquid medium can be separated out and isolated into pure cultures to study their morphology and identify them. This can be done by streak plate method or pour plate method. If the cells are separated by dilution, anchored in a solid medium and allowed to form colonies, they can be isolated into separate colonies and the number of bacteria present in the original sample can be calculated.

MATERIALS REQUIRED:

Sterile plates, Nutrient agar, Culture media, Culture, Inoculation loops.

PROCEDURE:

1. Melt the nutrient agar and allow it to cool to 45⁰C.
2. Pour this cooled nutrient agar into a sterile plate (when the cotton plug is removed to pour the agar, flame the mouth of the conical flask to kill the microorganisms on the outer surface. While pouring the agar to the plate raise the cover of the plate only on the side just sufficient to admit easily the mouth of the flask).
3. Allow the agar to solidify.
4. A drop of microbial culture is applied to the surface of agar and spread with a loop (This is streaking), streaking is done in two ways:
 - a) A drop of culture is placed at one edge of the agar. Flame the inoculation loop and streak the culture back and forth from edge to in parallel lines.
 - b) Starting again with a drop of culture make two or three parallel streaks from it, flame the loop, make two or three streaks at right angles to the first, flame the loop again and repeat the procedure.
5. Incubate the plates in an inverted position at 30⁰C. For until the next laboratory period.
6. After incubation study the growth on agar plates. The differences in colony size, shape and appearance are noted.

OBSERVATIONS:

After the incubation period take out the plates with microbial growth and observe the total number of colonies, colony morphology, microscopic observation and note down microbial morphology and cell arrangement.

CONCLUSION:

STUDY QUESTIONS:

Exercise No. 14-15

**ISOLATION AND CULTURE OF MICROORGANISMS BY SERIAL DILUTION
TECHNIQUE AND POUR PLATE METHOD**

The technique of pour is a way of obtaining pure cultures from a mixture of microbes. There is not accurate way of predicting the number of viable cells in a given sample, so several dilutions of the sample are made and poured into different plates.

MATERIALS REQUIRED:

Sterile plates, Sterile 90 ml blanks, Sterile 9 ml blanks, Sterile pipettes, Soil Samples, Nutrient agar.

PROCEDURE:

1. Dissolve 10 gms of soil sample in 90 ml sterile water blank. From this add 1 ml of it to 9 ml sterile blank. This gives a dilution of 10^{-2} . Again from this go on diluting in a series of 9 ml blanks to get dilutions of 10^{-3} , 10^{-4} etc.,
2. 1 ml from 10^{-4} , 10^{-5} , and 10^{-6} dilutions is poured into sterile plates and the plates are labeled according to the dilution they hold.
3. Pour 10-15 ml of melted, cooled nutrient agar in to these plates and allow it to solidify.
4. After solidification keep the plates for incubation in an inverted position.
5. After incubation count the number of colonies in each dilution and calculate approximately the number of colonies present in 10 gms of soil sample.

OBSERVATIONS:

Soil Sample	No. of Colonies	Average No. of Colonies
-------------	-----------------	-------------------------

10^{-5} 10^{-6}

10^{-5} 10^{-6}

$$\text{Total No. of organisms} = \frac{\text{Average colony count X dilution factor}}{\text{Weight of the soil sample.}}$$

CONCLUSION:

STUDY QUESTIONS:

Exercise No. 17-18
Determination of Rate of Microbial Growth by Turbidometric
Technique.

Estimation of the growth rate of a bacterial culture can be successfully done with the help of a Photocolorimeter. A bacterial culture in broth acts as a colloidal suspension which can block or reflect light passing through the culture. The light blocked or reflected by a culture of bacteria is proportional to the concentrations of the cells in the suspension. Thus, by measuring the reflection of light rays (nephelometry) or the percentage of light obstructed (turbidimetry) by bacterial cells in a suspension you can calculate the density of bacterial cells in a culture and there by you can estimate the growth rate of the bacteria.

Most estimation are done by photocolorimeter to determine turbidity. In turbidometry, the percentage of light transmitted through a bacterial suspension is inversely proportional to the cell concentration. You can however express turbidity as optical density (O.D.) also which is directly proportional to the cell concentration in a bacterial suspension and can be expressed as:

$$\text{O.D.} = \log 100 - \log \text{ of galvanometer reading.}$$

Convert the galvanometer reading into O.D.

Materials required:

Bacterial culture
Nutrient broth
Turbidometer
Distilled water
Tissue paper

Protocol:

- 1) Make 5 ml suspension of bacteria (e.g. E. coli) in sterile distilled water and transfer it to a flask containing 200ml of sterile nutrient broth. Mix well and allow to incubate at 37° C. Note the time of inoculation.
- 2) Turn the colorimeter on and allow a few minutes to warm up if needed. Select a wavelength of 600 millimicrons and bring the needle to zero on the percentage transmission scale.

- 3) Transfer 5ml of the sterile nutrient broth from another flask to a clean colorimeter tube. See that all liquid and finger prints are completely wiped off with the help of cleansing tissue. Close the cover of the sample holder. Rotate the light control until the meter reads 100 on the percentage transmission scale.
- 4) Shake the inoculated flask and transfer 5ml portion of it into another colorimeter tube and insert the unknown tube in the place of the standard and read the percentage of transmittance. Note the result obtained at zero hour. Discard the sample. Wash the tube with disinfectants followed by several changes of sterile water.
- 5) Incubate the inoculated flask at 37 °C.
- 6) Then on every 2,4,6,8,16 and 24 hours remove 5ml of the sample with sterile 5ml pipettes and determine the O.D.
- 7) Plot a graph showing O.D. against time in hour. The curve obtained is called a growth curve for that specific organism.

Observations:

Conclusion:

Study Questions:

