UNIT III – APPLICATION OF MICROBES METHODS IN MICROBIOLOGY

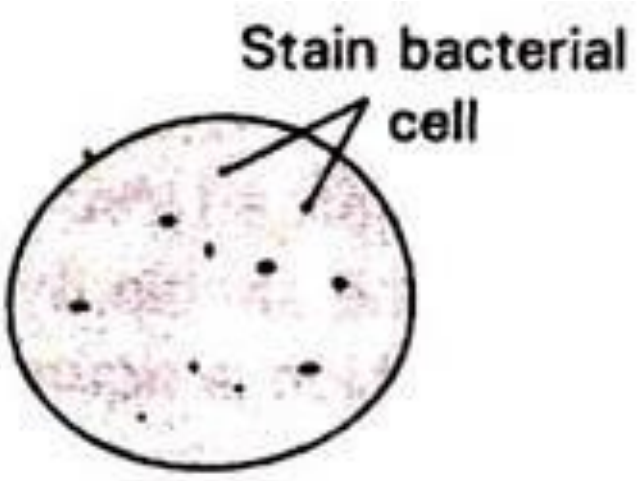
TYPES OF STAINING MICROBIOLOGY

Types of Staining.

1. **Simple Staining 2. Differential Staining 3. Gram Staining 4. Acid Fast Staining 5. Endospore Staining.**

1. Simple Staining:

Colouration of microorganisms by applying single dye to a fixed smear is termed simple staining. One covers the fixed smear with stain for specific period, after which this solution is washed off with water and slide blotted dry. Basic dyes like crystal violet, methylene blue and carbolfuchsin are frequently used in simple staining to determine the size, shape and arrangement of prokaryotic cells.



2. Differential Staining:

These staining procedures are used to distinguish organisms based on staining properties. They are slightly more elaborate than simple staining techniques that the cells may be exposed to more than one dye or stain, for instance use of Gram staining which divides bacteria into two classes-Gram negative and Gram positive.

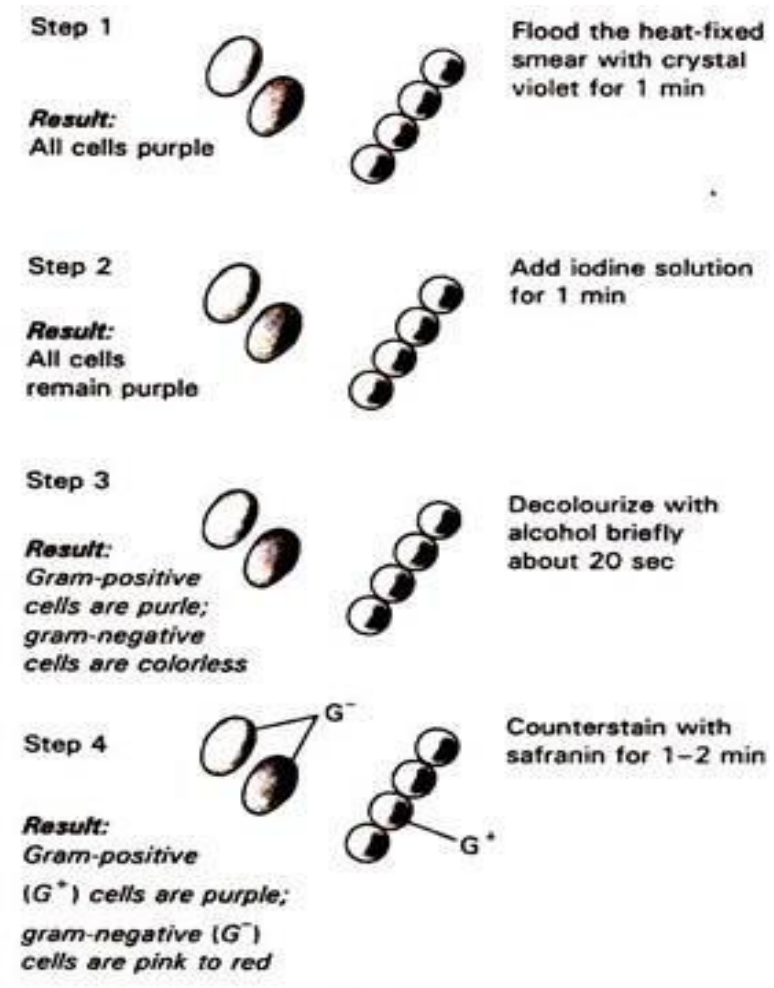
3. Gram Staining:

It is one of the most important and widely used differential staining techniques in microbiology. This technique was introduced in 1884 by Danish Physician Christian Gram. Gram staining procedure is illustrated in fig 5.2.

In the first step the smear is stained with basic dye crystal violet (Primary stain) followed by treatment with iodine solution functioning as mordant.

Iodine increases the interaction between cell & dye so that cell stains strongly. The smear is next decolourized by washing with ethanol or acetone. This step generates the differential aspect of Gram stains. Gram positive bacteria retain crystal violet and become colourless.

Finally smear is counter-stained with a simple basic dye different in color from Crystal violet. Safranin is the most common counter stain which colours Gram negative bacteria pink to red and leaves Gram positive bacteria dark purple.



The differences in staining responses to the Gram stain can be related to chemical and physical difference of cell walls. The Gram-negative bacterial cell wall is thin, complex multilayered structure and contains relatively high lipid contents in addition to protein and mucopeptide.

The higher amount of lipid is readily dissolved by alcohol, resulting information of large pore in the cell wall, thus facilitate leakage of crystal- violet – iodine (CV-I) complex which results in decolorization of the bacterial cell.

Which later take counter stain and appears red. In contrast the cell wall of gram+ve bacteria is thick and chemically simple, composed mainly of mucopeptides. When treated with alcohol, it causes dehydration and closure of cell wall pore, thereby does not allow the loss of (CV-I) complex and cell remain purple.

4. Acid Fast Staining:

It is another important differential staining procedure. It is most commonly used to identify Mycobacterium spp. These bacteria have cell wall with high lipid content such as mycolic acid -a group of branched chain hydroxy lipids, which prevent dyes from readily binding to cells.

They can be stained by Ziehl-Nulsen method, which uses heat and phenol to derive basic fuchsin into the cells. Mycrobacterium spp. were penetrated with basic fuchsin, not easily decolourized by acidified alcohol (acid alcohol) and thus are said to be acid fast.

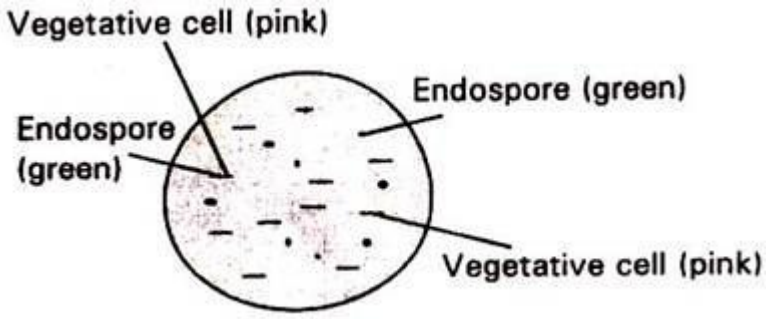
Non arid fast bacteria are decolourized by arid alcohol and thus are stained blue by methylene blue counter stain.

5. Endospore Staining:

Spore formation takes place in some bacterial genera to withstand unfavourable conditions. All bacteria cannot form spores, only few bacterial genera including Bacillus, Clostridium, Desulfotomaculum produce sporulating structure inside vegetative cells called endospore.

Endospore morphology and location vary with species and are valuable for identification Endospores are not stained well by most dyes, but once stained, they strongly resist decolorization.

In the Schaffer-Fulton procedure, endospores are first stained by heating bacteria with malachite green, which is very strong stain that can penetrate endospores. After malachite green treatment, the rest of the cell is washed free of dye with water and is counter-stained with safranin. This technique yields a green endospore with red vegetative cell.



METHODS USED FOR STERILIZATION |

The process that effectively kills or eliminates transmissible agents (such as fungi, bacteria, viruses, spore forms, etc.) from the surface of equipment, article, food, medication, or biological culture medium is called sterilization. The Sterilization is carried out by the methods according to requirement. The methods are: 1. Moist Heat Sterilization 2. Dry Heat Sterilization 3. Gas Sterilization and Others.

1. Moist Heat Sterilization:

Moderate pressure is used in steam sterilization. Steam is used under pressure as a means of achieving an elevated temperature. It is important to ensure the correct quality of steam is used in order to avoid the problems which follow, superheating of the steam, failure of steam penetration into porous loads, incorrect removal of air, etc.

For aqueous preparations and for surgical dressing, heating in saturated steam under pressure is carried out. A number of time-temperature combinations have been proposed.

The British and European Pharmacopoeia (2001) recommended 121°C temperature and 15 $Ib/inch^2$ maintained throughout the load for 15 minutes as the preferred combination for this method of terminal sterilization. It is used to sterilize aqueous parenteral solutions and suspensions, surgical dressing and fabrics, plastic and rubbers closures, metal instruments, glass apparatus etc.

2. Dry Heat Sterilization:

Dry heat sterilization is used for heat-stable non-aqueous preparations, powders and certain impregnated dressings. It may also be used for sterilization of some types of container. Sterilization by dry heat is usually carried out in a hot-air oven. Heat is transferred from its source to load by radiation, convention and to a small extent by conduction.

Temperature-time exposures necessary to kill pathogen by dry heat indicates that a period of 90 minutes at 100°C destroys all vegetative bacteria but a period of 3 h at 140″C kills the spores.

Mould spores are of intermediate resistance and are killed in 90 minutes at 115°C. Most viruses have resistance similar to vegetative bacteria but some viruses are as resistant as bacterial spores e.g. virus that causes homologous serum jaundice.

The British Pharmacopoeia (2001) states a minimum temperature of 160°C for at least 2 h for dry heat sterilization. Other combinations of temperature and time are permissible subject to first demonstrating a reproducible level of lethality in routine operation. Dry heat treatment, greater than 220°C, provides a useful method for sterilization and dehydrogenation of glassware in a particular container intended for a large volume of parenteral dosage.

This process can remove heat-resistant endotoxin. In each cycle it is important to ensure that the whole content of each container is maintained for an effective combination of time and temperature especially to allow temperature variations in hot-air ovens, which may be considerable. Dry heat is used to sterilize glassware, porcelain and metal equipment, oils and fats and powders i.e. talc, etc.

3. Gas Sterilization:

Gaseous sterilizing agents are of two main types, oxidizing and alkylating agents. Vapour phase hydrogen peroxide is an example of the former. Ethylene oxide and formaldehyde are examples of the alkylating agents. However, the BP states that gaseous sterilization is used when there is no suitable alternative. The main advantage of ethylene oxide is that many types of materials, including thermolabile materials, can be sterilized without damage.

The gas can diffuse through packaging materials and rubber, and diffuse out after sterilization. It has the disadvantages of being toxic and combustible and also requires the correct humidity. In practice, the relative humidity in the chamber atmosphere is usually between 40 and 50% with temperatures up to 6°C.

Low temperature steam with formaldehyde has been used as an option for sterilizing thermolabile substances. Both ethylene oxide and formaldehyde have health risks and strict monitoring of personnel exposed to the gases required to ensure protection from harmful effects.

4. Sterilization by Radiation:

Radiations can be divided into two groups: electromagnetic waves and streams of particulate matter. The former group includes infrared radiation, ultraviolet light, X-rays and gamma rays. The latter group includes alpha and beta radiations. Most commonly infrared radiation, ultraviolet light, gamma radiation and high-velocity electrons are used for sterilization.

(i) Ultraviolet Light:

A narrow range of UV wavelength (220-280 nm) is effective in killing the microorganism. The wavelength close to 265 nm and adjacent wavelengths are strongly absorbed by the nucleoproteins. The most serious disadvantage of UV radiation as a sterilizing agent is its poor penetrating power. This is the result of strong absorption by many substances. The application of UV radiation is limited.

(ii) Ionizing Radiations:

Ionizing radiations are suitable for commercial sterilization processes. It must have good penetrating power, high sterilizing efficiency, little or no damage effect on irradiated materials and are capable of being produced efficiently. The radiations that fulfill these four criteria are best high-speed electrons from machines and gamma rays from radioactive isotopes.

Sterilization by gamma rays is carried out using the radioactive isotope of [CO-60]. Articles for sterilization by radiations are packed in boxes of standard size which are sterilized by a series of slow passages around the gamma ray source. The absorbed dose for sterilization is 25 kGy.

The plastic syringes and catheters, hypodermic needles and scalped blades, adhesive dressings, single-application capsules of eye ointment, containers made of polythene and packaging materials using aluminium foil and plastic films are sterilized by gamma radiation.

5. Sterilization by Filtration:

Membrane filters are made from cellulose derivates or other polymers. There are no loose fibres or particles in membrane filters. They retain particles larger than the pore size on the filter surface such filters particularly useful in detection of small numbers of bacteria.

Sterilization by filtration is a method permitted by the British pharmacopoeia (2001) for heat sensitive solutions or liquids that are not sufficiently stable to withstand the process of heating in an autoclave.

Passage through a filter of appropriate pore size (e.g. 0.22 pm) can remove bacteria and moulds. Viruses and mycoplasma may not be retained. After filtration the liquid is aseptically distributed into previously sterilized containers which are later sealed. This method has disadvantages that specialized facilities and skilled operations are required.

The final preparations cannot be released until the manufacturing batch has passed the appropriate test for sterility. Validation of autoclave has been described in detail in Practical Microbiology by Dubey and Maheshwari (2007).

TYPES OF CULTURE MEDIA

1. Physical Type of Culture Media 2. Chemical Types of Culture Media 3. Function Types if Culture Media!

1. Physical Type of Culture Media:
Liquid, semisolid and solid media are routinely used for growth of micro-organisms.

(i) Liquid Media:
It is also called as broth and contains only dissolved nutrients in water. Liquid media are used for growth of pure batch cultures for fermentation studies and various other purposes while solidified media can be used widely for isolation of pure cultures for estimating viable microbial population and a variety of other purposes.

The usual gelling agent for solid or semisolid medium is agar, a hydro colloid derived from red algae. Agar is used because of its unique physical properties. One is that it melts at about 96°C and remains liquid until cooled to 40 to 45°C. Thus after being melted in boiling water, it can be cooled to a temperature that is tolerated by human hands as well as microbes.

Finally agar is excellent hardening agent because most microorganisms cannot metabolize it. It can be aided in light medial at consequence 1.5 to 2.0 permuted. Silca gel sometimes can also be substituted for agar as solidifying agent. Silca gel is made from silic acid and gel formed from this acid is completely resistant to microbial breakdown

2. Chemical Type of Culture Media:

A medium in which the exact chemical composition is known is called chemically defined (synthetic) medium. It can be in liquid form (broth) or solidified by agents such as agar. Chemically defined media are often used for cultivation of autotrophs and are also useful.

Simple peptone water, medium, 1% peptone with 0.5% NaCI in water may be considered semisynthetic medium since its composition is approximately known.

For defining the nutritional requirement of heterotrophs. Typically they contain simple sugar as the carbon and energy source, an inorganic Nitrogen source, various mineral salts and if necessary growth factors (purified amino acids vitamins, purine and pyrimidines).

A media in which exact chemical composition are not known is called complex (non-synthetic medium).

It usually contains complex material of biological origin etc. such as blood or milk or peptone yeast extract and beef extract. Complex media provide full range of growth factor that may be required by an organism so they may be used to cultivate unknown microorganisms or whose nutritional requirement is complex (i.e., organism that requires lot of growth factor).

This is the situation with fastidious organisms that have complex nutrition requirement; they may even require a medium containing blood or serum. Potato extract agar, Sail extract agar, Oatmeal agar, nutrient broth and tryptic soya broth are commonly used complex media after cultivation of heterothophic microorganism (table).

3. Functional Type of Culture Media:

No single medium or set of conditions can support the growth of all the different types of organisms that occur in nature. To cultivate, recognize, enumerate and isolate certain types of microorganism many special purpose media are needed.

On the basis of their application and functions these media are classified into following types:

Selective media provide nutrients that enhance growth and predominance of particular type of microorganism and suppresses all other microorganisms that are present in culture. This medium is best used for isolating specific organism from a mixed natural population.

For instance, cellulose utilizing micro-organism alone will grow in medium containing only cellulose as a carbon and energy source. Endo agar, eosin methylene blue agar and

Mac Conkey agars are three media widely used for detection of E. coli and related bacteria in water supply.

These media contain dyes that inhibit the growth of gram +ve bacteria but allow gram negative bacteria to grow. Physical condition such as pH and temperature also used to render its selectivity for growth of microorganism in media. As an example, gonorrhea causing Nosseria

gononhoeae, can be grown and isolated from medium containing certain antibiotics. Thus antibiotics exhibit growth of contaminating bacteria.

(i) Differential Media:

Differential media is used for the differentiation of various kinds of microorganisms on the basis of appearance on the medium and even permit tentative identification of microorganism. These media allow certain microorganisms to produce macroscopically distinct colonies or characteristic zone around colonies, which are differentiating these organisms from others in the samples.

This medium contains certain indicator or reagent or supplement which may allow such differentiation. For example, if a mixture of bacteria is inoculated on to a blood-containing agar mediums (blood agar), some of bacteria may hemolyze (destroy) the red blood cells, others do not. Thus we can distinguish between hemolytic and non-hemolytic bacteria on the same medium.

Mac Conkey's agar, contains lactose and a dye which turns when pH drops below. Any microorganism that can ferment lactose produces an acid end product that Lowers the pH and causes colony to turn red. Microorganism that fails to ferment lactose produces colour-less colonies. Dye in Mac Conkye's agar also inhibits the growth of gram +ve bacteria. This medium is, therefore, both selective and differential.

(ii) Enrichment Medium:

Enrichment medium is that in which nutritional environment is adjusted in such a manner as to enhance selectively the growth of certain bacterial type with in a gives mixed inoculum. For instance addition of extract of plant and animal tissue to nutrient broth and nutrient agar media provides additional nutrient and media starts favouring the growth of fasticious herotropic bacteria.

In the case of delicate micro-organisms like goner which may not survive the time taken for transporting the specimen to the laboratory or may be overgrown by non-pathogens (such as dysentery or cholera organism in feces), special media are devised for transporation of the specimens.

These are termed transport media for example, Stuart's medium a non-nutrient soft agar gel containing a reducing agent to prevent oxidation, and charcoal to neutralize acetone bacterial inhibitors for gonococci and buffer acid glycol saline for enteric bacilli.

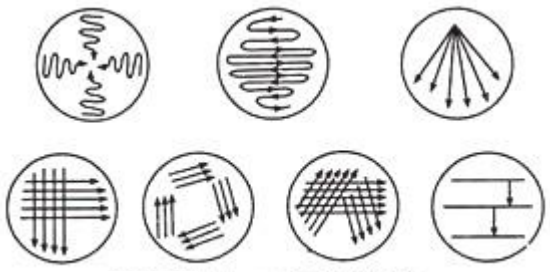
PURE CULTURE METHODS

1. Streak Plate Method:

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

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

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

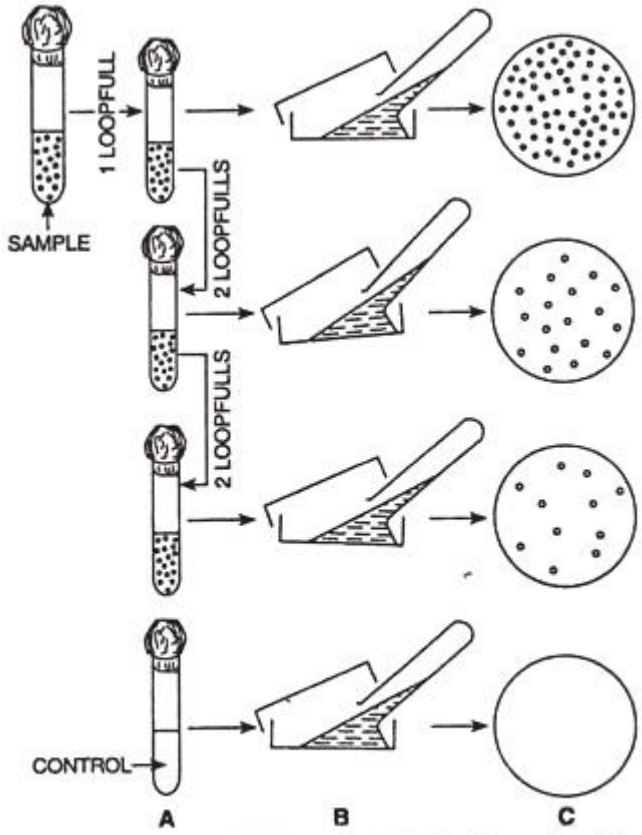


Various methods of streaking.

2. Pour Plate Method:

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

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



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

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

Pour plate method has certain disadvantages as follows:

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

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

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

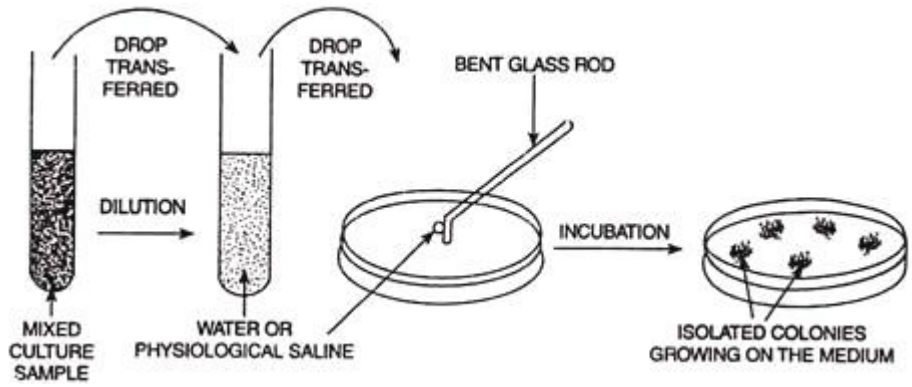
3. Spread Plate Method:

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

A drop of so diluted liquid from each tube is placed on the center of an agar plate and spread evenly over the surface by means of a sterilized bent-glass-rod. The medium is now incubated.

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

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



BACTERIAL MOTILITY

The below mentioned article provides a study note on bacterial motility.

Some bacteria are self-motile. They are able to swim through the liquid in which they live. They cannot crawl over dry surfaces or fly through the air. Motility is universal among the spirilla, common among the bacilli but lacking or rare in coccal forms.

The organs of locomotion are small whips or hair-like appendages called the flagella. These fine filaments are of uniform thickness. They are about 120A°-150A° thick and 4 or 5μ long.

They are cytoplasmic nature. Each flagellum arises within the cytoplasmic membrane but very in nature. Each flagellum arises within the cytoplasmic membrane but very close to it from a granule called a blepharoplast and passes out through the cell wall.

1. Flagellation (Distribution of Flagella):

The flagella are distributed over the surface of the bacterial cell in a characteristic manner. Their number, position and arrangement varies with the species.

They may be restricted to one or both the ends of the bacterium cell (polar flagellation) or may be distributed uniformly all over the body surface (non-polar flagellation). Bipolar flagellation is of rare occurrence.

On the basis of number and arrangement of flagella, bacteria are classified into the following categories:

A. Polar Flagellation :

This type of flagellation is restricted to a rather homogenous group of bacilli and spirilla. They are all gram-negative.

Polar flagellation is of the following four types:

(a) Monotrichous:

In this case the bacterium cell bears only single flagellum which is inserted at or near one pole of the cell (A). The flagellum has more than two curves. All vibrios (Vibrio cholerae and V. metschnikovii) are monotrichous.

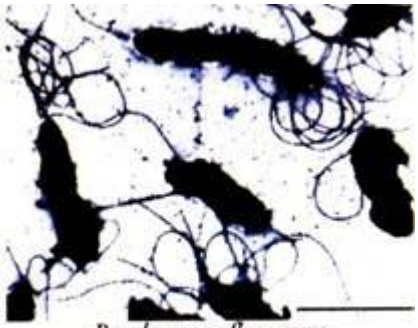
Vibrio cholerae (Monotrichous flagella)

(b) Amphitrichous:

There is one flagellum at each pole of the bacterium cell (B). This type of flagellation is of are occurrence.

(c) Cephalotrichous (Thimman, 1959):

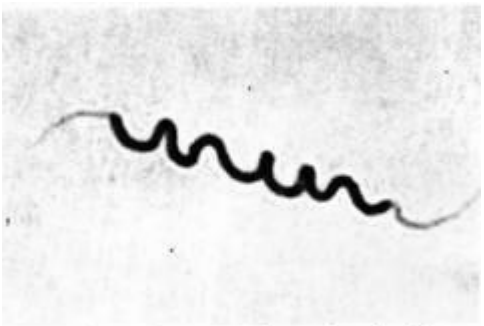
There are two or more flagella in a bunch at one pole of the bacterium cell (C). Rod-shaped bacterium, Pseudomonas fluoresces is an example of this category. The flagellum shows more than two curves.



Pseudomonas fluorescens (Cephalotrichous flagella)

(d) Lophotrichous (Thimman, 1959):

There are two or more flagella at both the poles of the bacterium cell (D). The flagella show one or two curves. The spirilla are Pseudomonas fluoresces the common examples (Spirillum volutans).



Spirillum volutans (Lophotrichous flagella)

B. Non-polar Flagellation :

In this case the flagella are found all over the cell surface. It is of one type only.

(e) Peritrichous:

The bacterial cell bears a large number of flagella which are distributed evenly all over the cell surface. Proteus vulgaris and Bacillus typhosus are the common examples. In fact, peritrichous flagellation is more commonly found among the bacteria than polar flagellation.

(f) Atrichous:

All bacteria which lack flagella are called atrichous. Diptheria bacilli and Lactobacillus—a milk souring group of bacteria—are entirely without flagella and thus non- motile. The passive type of movement is exhibited by most of the bacteria.

It is purely physical and is also known as Brownian movement. This type of movement is shown by all small particles suspended in a liquid. The movement of bacterial flagella is screw-type motion in three dimensions.

2. Structure of Bacterial Flagellum:

The bacterial flagella are the most primitive of all motile organs. Structurally they are unique. Each is composed of a single thin fibril as against the 9+2 pattern of fibrillar structure characteristic of eucaryotic motile cell types.

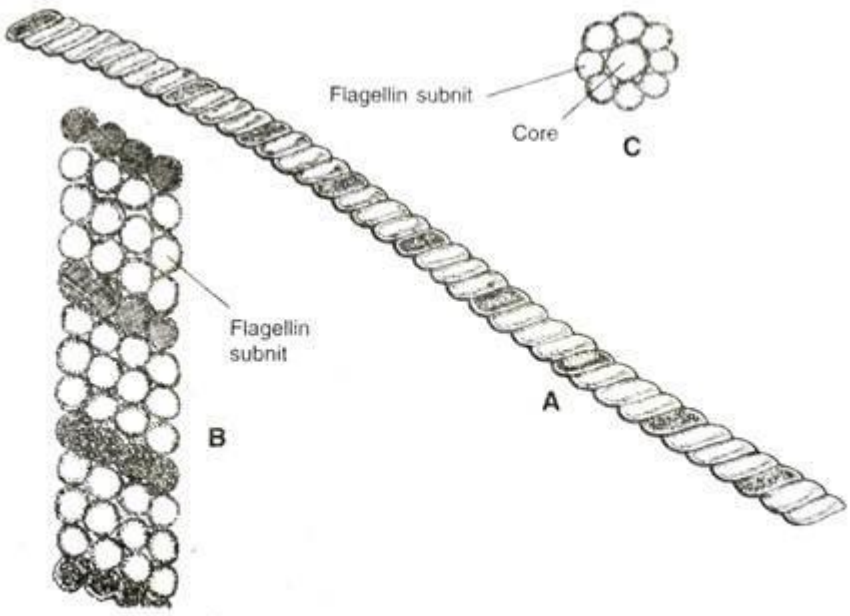
The bacterial flagellum is thus equivalent to one of the peripheral half fibrils (microfibrils) of the flagellae of all motile eukaryotic organisms. Electron microcopy reveals that the bacterial flagellum is helical in shape.

Because of its spiral patterned surface it apparently seems to consist of a few fine fibrils twised tightly into a rope-like structure coiled in an open wave form (A). The flagellum is composed entirely of protein which is Flageliin.

It gives a typical helical diffraction pattern in the electron microscope. Its molecular weight is about 40,000. According to Lowy and Hanson (1965), the bacterial flagellum, under high resolution of electron microscope, is seen to be composed of globular subunits arranged in helices of various kinds.

The diameter of each subunit is approximately 40-50A (Angstrom units). The flagellum is composed of several chains of subunits (flagellin molecules) forming a hollow cylindrical thread.

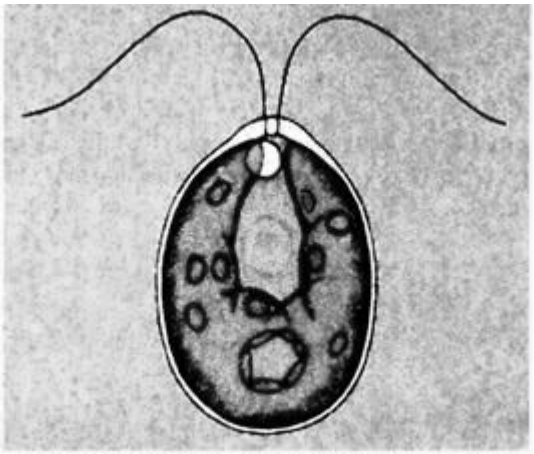
There is no axoneme. Whether the hollow core is empty or filled with a similar or different protein is not known. The number and orientation of chains in the flagellum varies with the species. It is not a uniform feature of all bacterial flagella.



Bacteria. Molecular structure of flagellum. A, Flagellum as seen in electron microscope; B, Portion of flagellum under high resolution of electron microscope; C, end view of flagellum.

In Salmonella, for example, the flagellum consists of four helices or spiral chains of subunits as shown in figure 18.6 B. One of the chains is shown by black globules. Ringo (1967) investigated the molecular structure of the flagellum of a species of Chlamydomonas.

He reported that in a cross section each of the peripheral half fibrils (microfibril) consists of a ring of 13 subunits surrounding a homogeneous core. The diameter of each subunit is 40A which corresponds to a protein with a molecular weight of approximately 40,000.

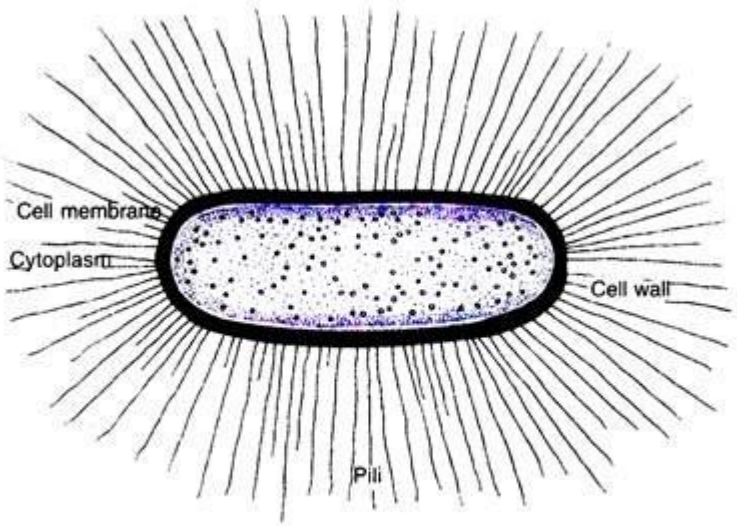


Chlamydomonas (Flagellum microfibrila comprise of 13 subunits

Pili :

These are minute, straight, hair-like, non-flagellar appendages found extending out from the cell wall of many gram- negative bacteria. Like flagella they can only be seen in electron micrographs. They are somewhat rigid and composed entirely of a protein known as pilin.

They may be several microns long but are smaller in diameter which is around 30-50A. There may be one hundred to four hundred pili or fimbriae distributed over the surface of the bacterium cell. Pili are considered as the organelles of attachment.



Bacteria. Cell showing the distribution of pili.

ENUMERATION OF MICROBES

Measurement of Cell Numbers and Cell Mass

Measurement (Count) of Cell Numbers:

1. Breed Method:

A known volume of microbial cell suspension (0.01 ml) is spread uniformly over a glass slide covering a specific area (1 sq. cm). The smear is then fixed by heating, stained, examined under oil immersion lens, and the cells are counted.

Customarily, cells in a few microscopic fields are counted because it is not possible to scan the entire area of smear. The counting of total number of cells is determined by calculating the total number of microscopic fields per one square cm. area of the smear.

The total number of cells can be counted with the help of following calculations:

(a) Area of microscopic field = πr^2

r (oil immersion lens) = 0.08 mm.

Area of the microscopic field under the oil-immersion lens

= πr^2 = 3.14 x $(0.08 \text{ mm})^2$ = 0.02 sq. mm.

(b) Area of the smear one sq. cm. = 100sq. mm.

Then, the no. of microscopic fields = 100/0.02=5000

(c) No. of cells 1 sq. cm. (or per 0.01 ml microbial cell suspension)

= Average no. of microbes per microscopic field x 5000

2. Courting Chamber Technique or Direct Microscopic Count (DMC):

The number of cells in a population can be measured by taking direct microscopic count using Petroff-Hausser counting chamber (for prokaryotic microorganisms) or hemocytometers (to larger eukaryotic microorganisms). Prokaryotic microorganisms are more easily counted if they are stained or, if not stained, phase contrast of florescence microscope is employed.

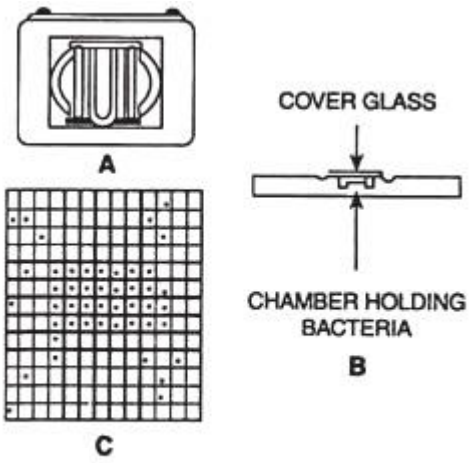
These are specially designed slides that have chambers of known depth with an etched grid on the chamber bottom. Each square on the grid has definite depth and volume . Total number of micro-organisms in a sample can be calculated taking the count of number of bacteria per unit

area of grid and multiplying it by a conversion factor (depending on chamber volume and sample dilution used).

More specifically, for convenience, the Petroff-Hausser counting chamber is a specially designed slide accurately ruled out into squares that are 1/400 mm^2 in area; a glass coverslip rests 1/50 mm above the slide, so that the volume over a square is 1/20,000 mm^3 (i.e., 1/20,000,000 cm^3).

A suspension of unstained bacteria can be counted in the chamber employing a phase contrast microscope. If, for example, an average of five bacterial cells occurs in cached ruled square, there is 5 x 20,000,000 or 10^8 bacterial cells per millimeter.

The direct microscopic method is easy, inexpensive and relatively quick to count bacterial cell number. However, using this method dead cells are not distinguished from living cells and also very small cells are usually missed.



Petroff-Hausser counting chamber used for counting of bacteria.

3. Viable Count—Standard Plate Count (SPC) Method:

A bacterial culture need not contain all living cells; there might be some dead cells as well. The culture when grown in proper medium and under standard set or growth conditions, only living cells grow and form colony.

This fact is used to estimate number of living bacterial cells; the estimation of number of living bacterial cells is called viable count. Standard Plate Count (SPC) method is the most commonly used laboratory technique for viable count of bacterial cells in milk, food, water, and many other materials.

Various aspects of SPC are the following:

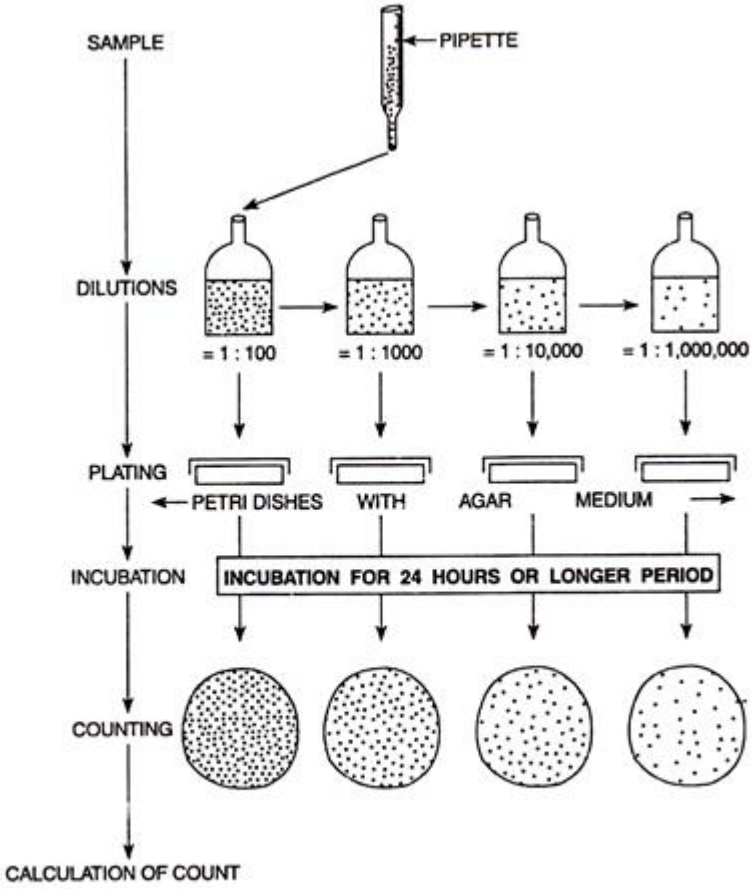
1. Procedure:

To estimate the number of living bacterial cells in milk, for convenience, the sample of well mixed milk is taken into a pipette. 1 ml of milk dropped and mixed in 99 ml of sterile dilute solution (may be water or nutrient broth or saline solution) taken into a flask.

This results in a dilution of 1: 100 into the flask. Other flaks each containing 99 ml of sterile dilute solution are taken and dilutions of 1: 1000, 1: 10,000, and: 1,000,000 are prepared into them.

Now, 1 ml of each dilution is transferred into separate Petri dishes containing pre-solidified agar medium. The Petri dishes are incubated for 24 hours or more. Each living bacterial cell in dilutions grow in respective Petri dishes reproducing itself until a visible mass of bacterial cells, a colony, develops, i.e., one bacterial cell gives rise to one colony.

The original sample is subsequently diluted till the number of colonies developing on Petri dish fall in the range of 30-300 because the count is almost accurate, and the possibility of interference of one colony with that of another is minimized.



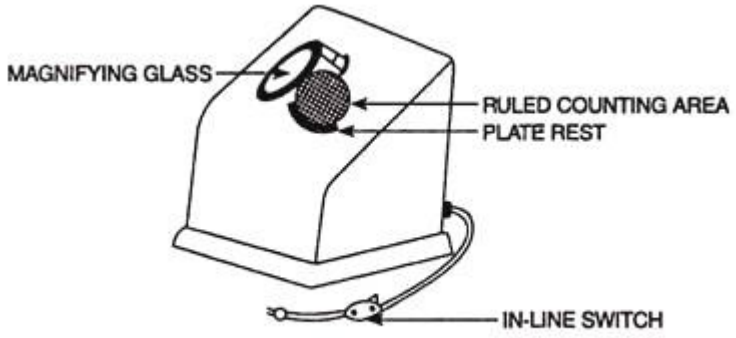
Standard Plate Count (SPC) method. In this technique the sample is diluted quantitatively and measured amounts of the dilutions are cultured in Petri dishes containing agar medium. A Petri dish is selected which contains from 30 to 300 colony forming units (CFUs) and then the probable number of bacteria per ml in original sample is calculated using formula as given in the text.

2. Counting of colonies:

Each Petri dish is taken for counting of colonies. Colonies are usually counted by illuminating them from below (dark field illumination) so that they are easily visible, and a large magnifying lens is often used. For this purpose, various instruments such as Quebec colony counter and electronic colony counter are used. Quebec colony counter is one of the simplest colony counters used in small laboratories.

In this, the Petri dish containing bacterial colonies is mounted on a platform. When the Petri dish is illuminated from beneath, the visible colonies can be counted with the help of its lens that provides X1.5 magnification.

Electronic colony counter is highly improved device. The Petri dish is placed on its illuminated stage, the count bar is depressed, and the precise number of colonies is instantly displayed on a digital readout.



Quebec colony counter.

3. Calculation of count:

The probable number of bacteria per ml in original sample can be estimated by multiplying bacterial colony count by the reciprocal of the dilution and of the volume used.

For example, if bacterial colony count is 50 for 1 : 10,000 dilution when volume used is 1 ml, then The number of colony forming bacterial cells = 50 x 10,000 x 1 = 5 x 10^5

4. Limitations of SPC:

(i) Only bacteria that will be counted are those which can grow on the medium used and under the conditions of incubation provided.

(ii) Each viable bacterial cell that is capable of growing under the culture conditions provided may not necessarily result in one colony. The development of one colony from one bacterial cell can only take place when the bacterial suspension is homogenous and no aggregates of cells are present in it.

However, if the bacterial cells possess the tendency to aggregate, e.g., cocci in clusters (staphylococci), chains (streptococci), or pairs (diplococci), the resulting counts will be lower than the number of actual bacterial cells. For this reason the counts are often reported as colony forming units (CFU) per millilitre rather than number of bacterial cells per millilitre.

5. Advantages of SPC:

SPC is easy to perform and can be used to measure bacterial populations of any magnitude. It is very sensitive technique and even very small number of bacterial cells can be counted using it. Theoretically, if 1 ml sample contains as few as one bacterial cell, the latter develops one colony upon transferring the sample into medium containing Petri dish.

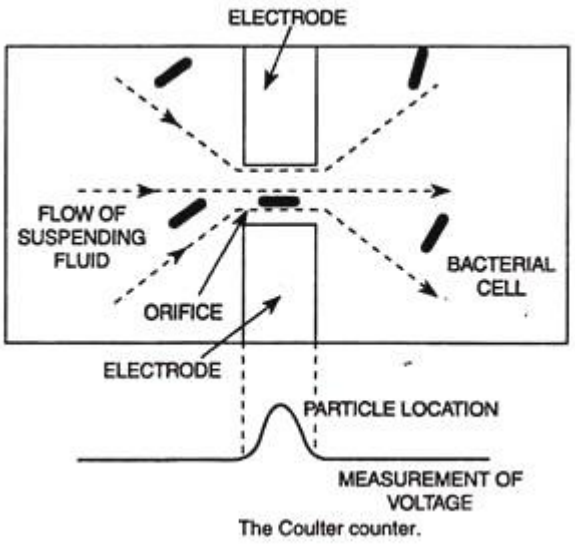
4. Coulter Counter:

Coulter counter is an electronic device used to count number of bacteria and other micro-organisms such as protozoa, microalgae and yeasts. This device is provided with a tiny orifice 10-30 pm in diameter. This orifice connects the two compartments of the counter which contain an electrically conductive solution (electrodes).

In this method, the sample of bacterial cells is forced through the small orifice (small hole). On the both sides of the orifice, electrodes are present to measure the electric resistance or conductivity when electric current is passed through the orifice.

Every time a bacterial cell passes through the orifice, electrical resistance between the two compartments (electrodes) increases momentarily or the conductivity drops. The generates an electrical signal which is automatically counted.

Each electrical signal represents the counting of one bacterial cell. The Coulter counter gives accurate results with larger cells. The precaution to be taken in this method is that the suspension of samples should be free of any cell debris or other extraneous matter.

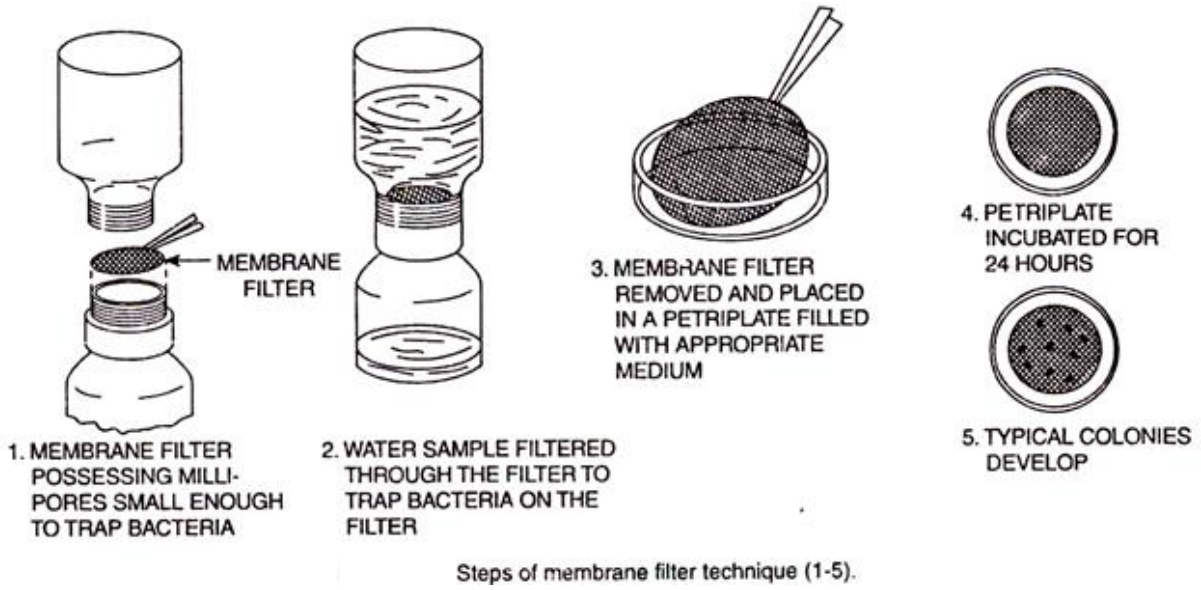


The Coulter counter.

5. Membrane-Filter Technique:

Microbial cell numbers are frequently determined using special membrane filters possessing millipores small enough to trap bacteria. In this technique, a water sample containing microbial cells is passed through the filter. The filter is then placed on solid agar medium or on a pad soaked with nutrient broth (liquid medium) and incubated until each cell develops into a separate colony.

Membranes with different pore sizes are used to trap different microorganisms. Incubation times for membranes also vary with the medium and the microorganism. A colony count gives the number of microorganisms in the filtered sample, and specific media can be used to select for specific microorganisms. This technique is especially useful in analysing aquatic samples.



Steps of membrane filter technique (1-5).

Measurement of Cell Mass:

1. Dry Weight Technique:

The cell mass of a very dense cell suspension can be determined by this technique. In this technique, the microorganisms are removed from the medium by filtration and the microorganisms on filters are washed to remove all extraneous matter, and dried in desiccator by putting in weighing bottle (previously weighed).

The dried microbial content is then weighed accurately. This technique is especially useful for measuring the growth of micro fungi. It is time consuming and not very sensitive. Since bacteria weigh so little, it becomes necessary to centrifuge several hundred millions of culture to find out a sufficient quantity to weigh.

2. Measurement of Nitrogen Content:

As the microbes (bacteria) grow, there is an increase in the protein concentration (i.e. nitrogen concentration) in the cell. Thus, cell mass can be subjected to quantitative chemical analysis methods to determine total nitrogen that can be correlated with growth. This method is useful in determining the effect of nutrients or antimetabolites upon the protein synthesis of growing culture.

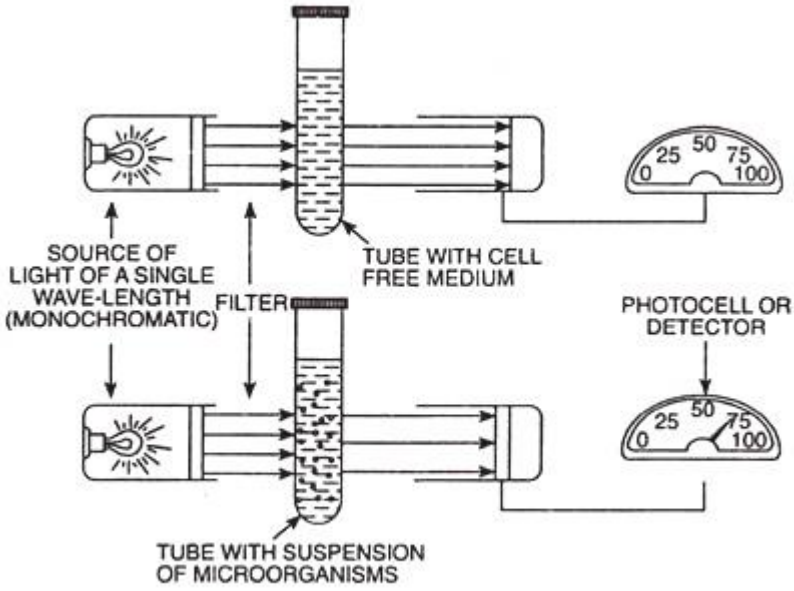
3. Turbidometric Estimation (Turbidometry):

Rapid cell mass determination is possible using turbidometry method. Turbidometry is based on the fact that microbial cells scatter light striking them. Since the microbial cells in a population are of roughly constant size, the amount of scattering is directly proportional to the biomass of cells present and indirectly related to cell number.

One visible characteristic of growing bacterial culture is the increase in cloudiness of the medium (turbidity). When the concentration of bacteria reaches about 10 million cells (10^7) per ml, the medium appears slightly cloudy or turbid.

Further increase in concentration results in greater turbidity. When a beam of light is passed through a turbid culture, the amount of light transmitted is measured.

Greater the turbidity, lesser would be the transmission of light through medium. Thus, light will be transmitted in inverse proportion to the number of bacteria. Turbidity can be measured using instruments like spectrophotometer and nephelometer.

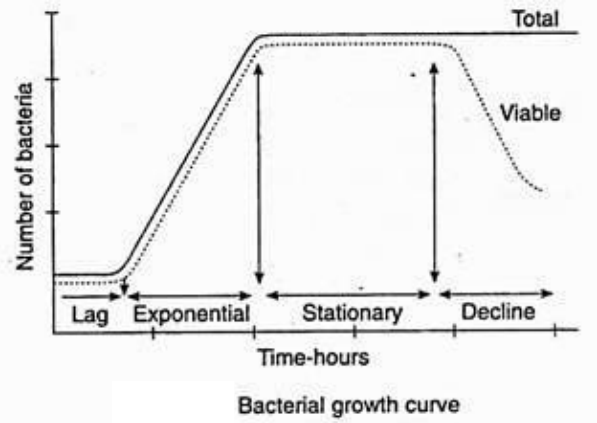


Determination of cell mass using turbidometry method.

GROWTH CURVE OF BACTERIA

The four main phases of growth curve in bacteria:

1. **Lag Phase 2. Log Phase or Exponential Phase 3. Stationary Phase 4. Decline Phase.**



Bacterial growth curve

1. Lag Phase:

After inoculation into the sterile nutrient medium, the bacterium first undergoes a period of acclimatisation. At that time, necessary enzymes and intermediate metabolites are synthesised, thereby bacterium reaches a critical stage before multiplication, multiplication takes place at this stage.

The duration of lag phase depends on the type of bacteria, quality of culture medium, size of inoculum and several environmental factors such as CO_2, temperature, pH, etc. The average time of lag phase is 2 hours, although it varies from species to species (1-4 hours).

2. Log Phase or Exponential Phase:

In this phase, the bacteria undergo cell division and their population (number) increase exponentially at a logarithmic rate. The number of viable count, when plotted against time, gives a straight line of inclined fashion. The average time of log phase is 8 hours, though it varies in different species.

3. Stationary Phase:

In this phase, the growth i.e., cell division, almost ceases due to exhaustion of nutrients and also the accumulation of toxic products. At this stage the cell death starts at a slow rate and is compensated by the formation of new cell through cell division.

The total cell number increases at a slow rate, but the viable count remains almost constant. The duration of this phase is variable which ranges from few days to few hours. Secondary metabolites like antibiotics, toxins etc. are produced in this phase.

4. Decline Phase:

In the phase of decline, the total number of cells remains constant, but the number of viable cells gradually decreases due to exhaustion of nutrients and also the accumulation of toxic products. In some cases a few- cells remain viable for long time, even after death of most of the cells. These viable cells probably grow by utilising nutrients released from dead cells.

The cells attain maximum size at the end of lag phase and become smaller in log phase (exponential phase). In spore forming species, the sporulation occurs at the end of log phase (exponential phase) or in the early part of stationary phase.

UNIT IV – PRINCIPLES OF PATHOLOGY PLANT PATHOLOGY

HISTORY AND IMPORTANCE OF PLANT DISEASES

Plant pathology is a branch of botany. It deals with the diseases of plants, helps to maintain good health of plants, and also take proper steps to increase the productivity. Plant diseases caused disasters like famine in Ireland (1845- 1846) and Bengal (1943) by late blight disease of potato (C.O. Phytophthora infestans) and brown spot of rice (C. O. Helminthosporium oryzae) respectively.

To overcome such problems, it is essential to carry out research on development of disease-tolerant varieties or on production of more effective pesticides in comparatively low cost or in inducing plant's own defense mechanism. Thus, plant pathologists are the plant doctors, responsible to maintain the good health of plants.

Plant diseases are known from times preceding the earliest writings. Fossil evidence indicates that plants were affected by disease 250 million years ago. The Bible and other early writings mention diseases, such as rusts, mildews, and blights, that have caused famine and other drastic changes in the economy of nations since the dawn of recorded history. Other plant disease outbreaks with similar far-reaching effects in more recent times include late blight of potato in Ireland (1845–60); powdery and downy mildews of grape in France (1851 and 1878); coffee rust in Ceylon (now Sri Lanka; starting in the 1870s); *Fusarium* wilts of cotton and flax; southern bacterial wilt of tobacco (early 1900s); Sigatoka leaf spot and Panama disease of banana in Central America (1900–65); black stem rust of wheat (1916, 1935, 1953–54); southern corn leaf blight (1970) in the United States; Panama disease of banana in Asia, Australia, and Africa (1990 to present); and coffee rust in Central and South America (1960, 2012 to present). Such losses from plant diseases can have a significant economic impact, causing a reduction in income for crop producers and distributors and higher prices for consumers.

Loss of crops from plant diseases may also result in hunger and starvation, especially in less-developed countries where access to disease-control methods is limited and annual losses of 30 to 50 percent are not uncommon for major crops. In some years, losses are much greater, producing catastrophic results for those who depend on the crop for food. Major

disease outbreaks among food crops have led to famines and mass migrations throughout history. The devastating outbreak of late blight of potato (caused by the water mold *Phytophthora infestans*) that began in Europe in 1845 brought about the Great Famine that caused starvation, death, and mass migration of the Irish. Of Ireland's population of more than eight million, approximately one million (about 12.5 percent) died of starvation or famine-related illness, and 1.5 million (almost 19 percent) emigrated, mostly to the United States, as refugees from the destructive blight. This water mold thus had a tremendous influence on economic, political, and cultural development in Europe and the United States. During World War I, late blight damage to the potato crop in Germany may have helped end the war.

Definitions of plant disease

In general, a plant becomes diseased when it is continuously disturbed by some causal agent that results in an abnormal physiological process that disrupts the plant's normal structure, growth, function, or other activities. This interference with one or more of a plant's essential physiological or biochemical systems elicits characteristic pathological conditions or symptoms.

Plant diseases can be broadly classified according to the nature of their primary causal agent, either infectious or noninfectious. Infectious plant diseases are caused by a pathogenic organism such as a fungus, bacterium, mycoplasma, virus, viroid, nematode, or parasitic flowering plant. An infectious agent is capable of reproducing within or on its host and spreading from one susceptible host to another. Noninfectious plant diseases are caused by unfavourable growing conditions, including extremes of temperature, disadvantageous relationships between moisture and oxygen, toxic substances in the soil or atmosphere, and an excess or deficiency of an essential mineral. Because noninfectious causal agents are not organisms capable of reproducing within a host, they are not transmissible.

In nature, plants may be affected by more than one disease-causing agent at a time. A plant that must contend with a nutrient deficiency or an imbalance between soil moisture and oxygen is often more susceptible to infection by a pathogen, and a plant infected by one pathogen is often prone to invasion by secondary pathogens. The combination of all disease-causing agents that affect a plant make up the disease complex. Knowledge of normal growth

habits, varietal characteristics, and normal variability of plants within a species—as these relate to the conditions under which the plants are growing—is required for a disease to be recognized.

The study of plant diseases is called plant pathology. Pathology is derived from the two Greek words *pathos* (suffering, disease) and *logos* (discourse, study). Plant pathology thus means a study of plant diseases.

THE IDENTIFICATION OF PLANT DISEASES.

One of the important aspects of study of plant diseases is how to either cure the disease or prevent its recurrence. Before any cure can be recommended, the cause or in other words, the causal agency of the disease should be known—whether parasitic or non-parasitic.

Plant diseases can be studied in extremely variable methods which depend on the nature of disease, the causal agency, and the host involved. But the initial study of symptoms, periodicity, incidence, and extent of damage done to the host—all these aspects are to be studied irrespective of the nature of disease, causal agency, and host infected.

This initial study often produces information to identify the disease under study either as a parasitic or a non-parasitic disease. If the disease is a parasitic one, the next step will be to determine the nature of causal agency.

This area of study extends:

(i) A closer examination of disease infected host tissue in the laboratory in naked eye and under microscope,

(ii) Isolation of pathogen from the disease infected tissue, and

(iii) Growing of pathogen in pure culture.

The presence of a pathogen can frequently be determined by naked-eye examinations of lesions.

But in many cases the pathogen is too small to be seen without magnification, or may be internal within the tissues programs. Microscopic examination of surface scrapped mounts or

dissected mounts of lesions without staining in water or staining in lactophenol cotton blue or potassium hydroxide and phloxine may also reveal much information about the pathogen.

Often microscopic examination of thin free hand or serial microtome sections stained in suitable dye combinations is necessary to work out the details of the pathogen.

The microscopic examination is expected to reveal the nature of pathogen, at least, the group to which it belongs and the details of mode of growth in the host tissue, nutrition, and damage done to the host cells by the pathogen.

Isolation is a technique by which a pathogen is transferred from its natural habitat (infected host tissue) to a suitable artificial medium. Isolation of pathogen should be done from vigorously infected areas. Small beats of infected tissue should be placed on sterilized nutritive agar medium in Petri dishes or culture tubes.

Earlier to of is the selected infected host tissue bits should be cleaned from dirt with water and surface sterilized either by applying mercuric chloride or silver nitrate and sodium chloride technique. For isolation work two per cent, potato dextrose agar medium has been found very suitable.

The entire isolation process should be done aseptically in an isolation room or chamber especially built for the purpose. The Petri dishes or culture tubes, whatever used, should then be put in an incubator having incubation temperature range of 20°C. to 25°C. In a few days-time the pathogen grows out on the agar surface from the infected host tissue.

The pathogen is then transferred to fresh nutritive agar medium and grown in pure culture under the same cultural condition. The pathogen should then be accurately described. The procedures described above is, however, very suitable for the study of disease caused by fungi.

But just because a fungus is present in a disease infected tissue does not really prove that this particular fungus is responsible for inducing this particular disease. The pathogenicity or causal relationship of the fungus concerned has to be established. The pathogenicity tests of a pathogen are also designated as Koch’s postulates or Koch’s Rules of Proof.

KOCH'S POSTULATES

They comprise of certain postulates or conditions that are to be fulfilled, only when the causal relationship of a pathogen is established.

They are as follows:

1. The causal organism must be constantly associated with the disease in question.

2. The causal organism must be isolated from the diseased plant and grown in pure culture and accurately described.

3. Inoculations with inocula from pure culture of the causal organism must reproduce the disease in question in the same species or variety of plant from which the causal organism was isolated.

4. The causal organism must be re-isolated from the plant in which disease has been produced by inoculating with inocula from pure culture.

These postulates cannot, however, be fulfilled with organisms which cannot be grown on artificial media. In that case constant accompaniment of the organism with the diseased plant may also be taken as proof of causal relationship.

Procedures for the first and second postulates have been discussed above. As to the third and fourth postulates, the inoculum should be a suspension of spores or mycelium of the fungus isolated from the infected host tissue.

Suspension of spores or mycelium should be prepared by following standard technique. The suspension so prepared should be sprayed with an atomizer on the surface of the host plant which has been grown in a pot in the greenhouse.

The variety of host plant must be the same as the one on which the disease had first appeared. The sprayed plant should then be covered with a bell-jar or polythene covering. The soil of the pot in which the host plant is grown should be kept moist. This enables to maintain humid condition around the plant.

The sprayed potted plant with covering is then placed for twenty-four to forty-eight hours in a place where the temperature and humidity both are favourable for the appearance of the disease. The sprayed plant should not be exposed to direct sunlight, as the spore germination and mycelial growth may be hampered. Usually lesions start appearing after twenty-four to forty-eight hours.

Ultimately typical disease symptoms that have been recorded earlier will be visible. The fungus is then re-isolated from the lesions produced by artificial inoculation. Its character is then studied and compared with what has been recorded earlier. When all the stages produce expected results, then only the pathogenicity of a particular fungus is established.

The procedure described above is, however, suitable for diseases where the infection is not soil borne. But in soil borne plant diseases the isolation of pathogen should be done from soil around the diseased plant.

These isolations will form the inocula and they are aseptically mixed with sterilized potted soil in which the seeds of the exactly same variety of plant should be sown after surface sterilization by suitable fungicides.

Under suitable temperature and humidity the seeds develop into seedlings and receive infection through roots from the pathogen that has been growing in the soil and ultimately the disease appears as expected. In all the above experiments good number of un-inoculated controls should be kept side by side to make a comparative study.

While growing in pure culture, the fungus concerned often produces spores.

The study of sporulation and spores in details is also essential for identifying the fungus. The mode of formation of spores and their nature and function, all these aspects besides being useful in the identification of the fungus also yield clue about the mode of dissemination of the disease and perpetuation of its inoculum. All these data may also be utilized in connection with recommending control measures of the disease.

HOST-PARASITE INTERACTION

Parasitism is an association or a situation in which two organisms of different taxonomic positions live together where one enjoys all sorts of benefits (like derivation of nourishment, reproduction etc. which are basic requirements for existence) at the expense of the other. The benefited organism is called the parasite and the organism harbouring the parasite is called the host.

Hosts are not hospitable to parasites. Instead they consider parasites as foreign bodies and want to exterminate or overpower them by operating various devices like: producing antibodies, increased peristalsis, diarrhoea, mucus secretion, encystation by host tissues etc. Parasites to avoid host's reaction for existence develop many specialities like increased fecundity, polyembryony, safe-habitat, production of special enzymes, a good deal of transmission etc.

Definition:

Due to close contact/intimate association, the responsive reactions and resistance displayed by a host to its parasite and the protective devices adopted by a parasite in response to its host's reactions in order to establish them in their respective environments are called host-parasite-interactions. Parasitism is a very broad term and different types of parasites are recognised on different basis.

In the course of their life cycle, parasite may become associated with more than one host. In many cases the life cycle is characterised by numerous very rigid requirements. Whenever a parasite is able to live and reproduce within a host—the result is an elaborate host- parasite interactions.

Host Specificity of Parasites:

In mature condition a given parasite is quite often found in limited number of hosts. In extreme condition, distribution of a parasite may be restricted to a single host—mono-specific parasite. Even when poly-specific the different hosts are phylogenetically related. This host specificity is a function of physiological specialization and evolutionary age.

It is broadly divided into two parts:

(a) Ecological specificity:

The parasites are capable of making room in a foreign host but normally never reach another host due to ecological barriers. Such parasites are able to develop in more host-species under laboratory conditions than in nature.

(b) Physiological specificity:

The parasites are physiologically incapable of surviving and reproducing in a foreign host, e.g., Taenia solium in dog survives but never achieves reproductive ability. If the parasites find the conditions suitable for their development—then it is said to be compatible with that of the host. If not, it is said to be incompatible.

1. **Commensalism:**

Commensalism represents a relationship between two microbial populations in which one is benefited and the other remains unaffected (i.e., neither benefited nor harmed). Thus the commensalism is an unidirectional relationship between two microbial populations. It is quite common, frequently based on physical or chemical modifications of the habitat, and is usually not 'obligatory' for the two populations involved.

Commensalistic association is often established when one microbial population, during the course of its normal growth and metabolism, modifies the habitat in such a way that the other population is benefited.

Following are some examples:

(i) A disease causing microbial population when opens a lesion on the host surface, if creates an entry- passage for other microbial population that otherwise could not enter and grow in the host tissues. For convenience, Mycobacterium leprae, the causative agent of leprosy, opens lesions on the body- surface and thus allows other pathogens to establish secondary infections.

(ii) When facultative anaerobes utilize oxygen and lower the oxygen content, they create anaerobic habitat which suits the growth of obligate anaerobes because the latter benefit from the metabolic activities of the facultative anaerobes in such a habitat.

On the contrary, the facultative anaerobes remain unaffected. The occurrence of obligate anaerobes within habitats of predominantly aerobic character, such as the oral cavity, is dependent on such commensal relationship.

(iii) Population of Mycobacterium vaccae, while growing on propane cometabolizes (gratuitously oxidizes) cyclohexane to cyclohexanone which is then used by other bacterial population, e.g., Pseudomonas (Fig. 33.11). The latter population is thus benefited since it is unable to oxidise cyclohexane to cyclohexanone. Mycobacterium remains unaffected since it does not assimilate the cyclohexanone.

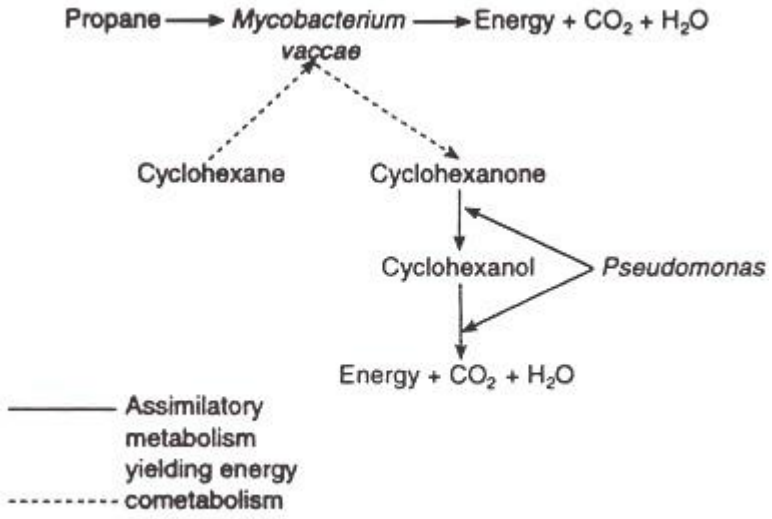


FIG. 33.11. An example of commensalism based on cometabolism.

(iv) Some microbial populations create commensalistic habitat by detoxifying compounds by immobilization. Leptothrix bacteria deposite manganese on their surface. In this way, they reduce manganese concentration in the habitat thus permitting the growth of other microbial populations. If Leptothrix do not act so, the manganese concentration would be toxic to other microbial populations.

2. Amensalism:

Amensalism (from the Latin for not at the same table) refers to such an interaction in which one microorganism releases a specific compound which has a negative effect on another microorganism. That is, the amensalism is a negative microbe-microbe interaction.

Some important examples are the following:

(i) Antibiotic production by a microorganism and inhibiting or killing of other microorganism susceptible to that antibiotic is the most important example of amensalism. Concentrations of such antibiotics in the bulk of soil or water are certainly small, though there could be a large enough quantity on a micro-habitat scale to give inhibition of nearby microorganisms.

The antibiotics reduce the saprophytic survival ability of pathogenic microorganisms in soil. The attini ant- fungal mutualistic relationship is promoted by antibiotic producing bacteria (e.g., Streptomyces) that are maintained in the fungal gardens (see box). In this case, Streptomyces produces an antibiotic which controls Escovopsis, a persistent parasitic fungus, which can destroy the ant's fungal garden (Fig. 33.9).

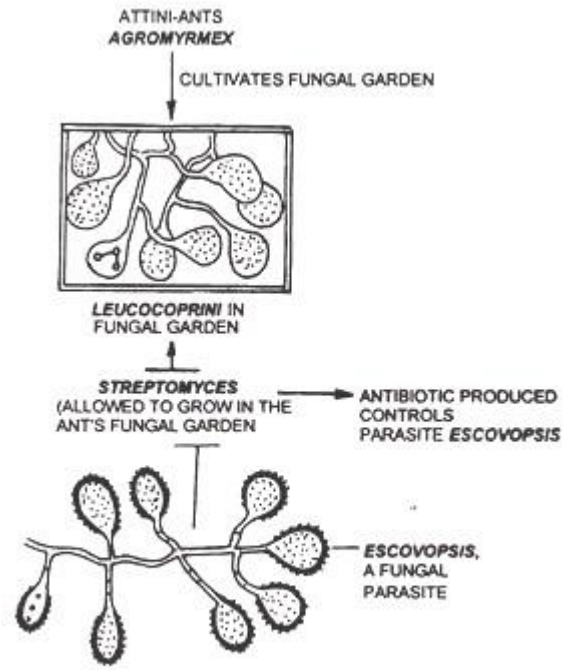


FIG. 33.9. A schematic diagram showing the use of antibiotic producing *Streptomyces* by Attini ants to control the growth of fungal parasite in their fungal garden.

(ii) Production of ammonia by some microbial population is deleterious to other microbial populations. Ammonia is produced during the decomposition of proteins and amino acids. A high concentration of ammonia is inhibitory to nitrite oxidizing populations of Nitrobacter.

3. Mutualism:

Mutualism describes a relationship in which both associated partners derive some benefit, often a vital one, from their living together.

Table 33.1 is an attempt to summarise the main kinds of mutualistic associations; some of which are trivial and of scientific interest only but others such as Rhizobium-legume association, mycorrhizae, coral-microbial association, herbivore-microbial association and lichens are very important, or indispensible, both to the local ecosystem and on a world scale.

Rhizobium-Legume Association:

The most important and the best studied mutualistic association between microorganisms and plants is undoubtedly that between Rhizobium spp. and various legumes.

Mycorrhizae (Sing. Mycorrhiza):

Mycorrhizae represent a mutualistic symbiosis between the root system of higher plants and fungal hyphae. Frank, who first noted the existence of such a characteristic association in the roots of Cupulifereae in 1885, coined the term ‘mycorrhiza’. Over the last 20 years, basic works conducted by hundreds of researchers from different countries has shown that this association is fundamental and universally occurring.

Among the different symbiotic associations between the soil microorganisms and root of plants, mycorrhizae are the most prevalent as they occur on more than 90% of the vascular plants.

However, Kumar and Mahadevan (1984) have studied a large number of mycorrhizal associations and found that they are highly influenced by the toxic substances that, when present, are essentially concentrated in the root of plants. Such substances may be alkaloids, phenolics, terpenoids, tannis, stilbenes, etc.

The mycorrhizae are advantageous because:

(i) The fungus derives nutrients via the root of the plant. Sugars formed in the leaves move down the stem as sucrose. Sucrose itself never accumulates in the fungus; it is converted into isomers such as ‘trehalose’ thus resulting in the low sugar concentration,

(ii) The fungal hyphae act like a massive root hair system, scavenging minerals from the soil and supplying them to the plant, and

(iii) Due to this associationship the plant partner, in addition to the nutritional benefits, develops drought resistance, tolerance to pH and temperature extremes, and greater resistance to pathogens due to ‘phytoalexins’ released by the fungus.

Mycorrhizae are generally classified into two types, although a third type that is more or less a combination of the first two is recognized by some. The two major types are termed Ectomycorrhizae and Endomycorrhizae while the third one, however, is referred to as Ectendomycorrhizae.

Ectomycorrhizae (Ectotrophic mycorrhizae):

Ectomycorrhizae (Fig. 33.2) are common on many forest trees, particularly pines, beech and birch which are of much economic value. The fungal hyphae form a sheath over the outside of the roots which is generally called ‘mantle of hyphae’. From this mantle, a hyphal network called hartignet extends into the first few layers of the cortex or rarely deeper and then reaches the endodermis.

Root hair formation is suppressed in the infected root and the root morphology is changed by the repeated formation of short branches with blunt tips and limited growth. Common ectomycorrhizal genera are Basidiomycetes, particularly Agaricales such as Amanita, Tricholoma, Russula, Lactarious, Suillus, Leccinum and Cortinarius’, some Ascomycetes such as the truffles have also been reported.

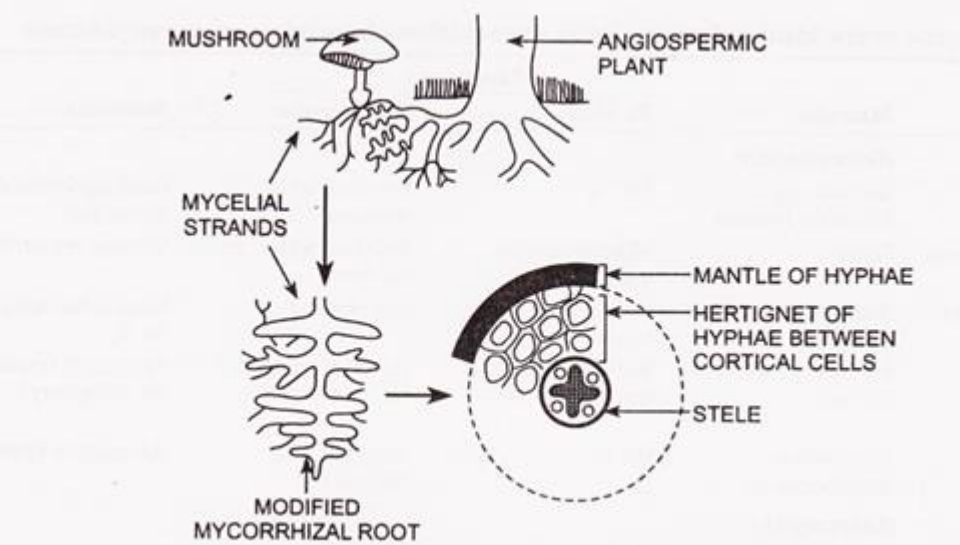


FIG. 33.2. Ectomycorrhiza (Ectotrophic mycorrhiza)

The fungi of ectomycorrhizae secrete various growth promoting substances such as auxins, cytokinins and gibberellic acids. Nevertheless, they produce some antimicrobial substances which protect the host plant against soil-borne pathogens.

Fungi derive their carbon from the host in the form of glucose, fructose or sucrose which is ultimately converted to manitol, trehalose, and glycogen. These mycorrhizae are known to stimulate plant growth and nutrient uptake in soils of low to moderate fertility.

Ectomycorrhiza (Ectotrophic mycorrhiza):

The mycorrhizae in which the fungal hyphae invade the root cells without forming any external sheath, mantle of hyphae, are called endotrophic mycorrhizae. Usually, some part of invading fungal hyphae lie externally as a loose mass of hyphae but they do not form mantle.

Three types of endomycorrhizae are recognised:

(i) Vasicular arbuscular (VA),

(ii) Orchidaceous, and

(iii) Ericaceous.

(i) Vesicular-arbuscular (VA) mycorrhizae:

Vesicular-arbuscular (VA) mycorrhizae (Fig. 33.3) represent associations between fungi, mostly the members of Zygomycetes, and a great number of angiosperms such as tropical forest

trees, almost all agricultural crops (except rice in paddy fields), and most of the herbs and grasses of tropical and temperate natural ecosystems.

Fungi forming VA mycorrhizae are restricted to only one family, Endogonaceae, of Zygomycetes with two genera, Endogone and Glomus, forming associations with a huge variety of distantly related plants. VA mycorrhizae are especially important because of their widespread occurrence and association with agricultural crops.

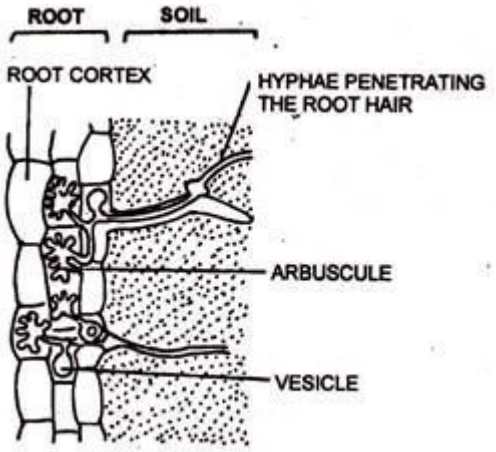


FIG. 33.3. VA mycorrhiza

In VA mycorrhizae the fungal hyphae develop some special organs, called vesicles and arbuscules, within the root cortical cells. Vesicles are thick-walled, spherical to oval in shape, borne on the tip of the hyphae either in intercellular spaces or in the cortical cells of the root. These vesicles are food storage organs of the fungus.

However, the arbuscules are brush-like dichotomously branched (extensively) haustoria developed within the cortical cells. Though widely distributed geographically, the VA mycorrhizae are not of usual occurrence in continuously flooded sites (Keeley, 1980).

The importance of VA mycorrhizae is in the effects that they have on plant nutrition, especially the immobile elements such as phosphorus. The external hyphae greatly increase the volume of soil and translocate the phosphorus to the roots. Plants are heavily infected with VA mycorrhizal fungi in phosphorus-deficient soils and mycorrhizae are poorly developed when the phosphorus supply is adequate.

It is thus a self-regulating system, increasing phosphorus uptake when this element is in short supply. The phosphorus so absorbed is converted into polyphosphate granules in the hyphae

and passed to the arbuscules for ultimate transfer to host plant. Gianinazzi et al. (1979) have demonstrated that transfer of polyphosphate occurs in the presence of acid phosphatase during the life span or scenescence of arbuscule.

In addition to stimulation of phosphorus uptake, mycorrhizal fungi stimulate rooting, growth, and survival of the transplant. Lambert et al. (1979) have studied that the VA mycorrhizae stimulate uptake of zinc, copper, sulfur, and potassium by the plant; enhances nodulation in legumes; decreases rots caused by fungal pathogen, and root penetration and larval development of nematodes.

Lichens:

Lichens are remarkable in that under natural conditions the algal-fungal or cyanobacterial-fungal association behaves as a single organism. The fungus (mycobiont) is usually an ascomycete and about 20,000 lichen fungi have been described which is approximately 25% of all known fungi. There are only some 30 genera of algae (photobiont; earlier called phycobiont) and cyanobacteria (cyanobiont) known to form lichens.

The relationship between the two associates of the lichen thallus is still not fully confirmed, though lichens have been the classic material for the study of microbial mutualistic symbiosis. The phycobiont/cyanobiont supplies carbohydrate to the mycobiont and the latter may supply minerals to the former.

We have no experimental confirmation that the mycobiont supplies minerals to its associates; also, the phycobiont may be able to absorb its own minerals from the substrate. ‘Good’ laboratory conditions cause the association to break down, whilst adverse conditions help to maintain it. This indicates that the association probably enables the associates to exploit habitat which would be unsuitable when they grow apart.

Lichens are considered the ‘pioneer organisms’ as they have been claimed to be important in increasing the rate of soil formation from bare rock. They may accelerate physical destruction of the rock by shrinkage and expansion of the thallus, may decompose the rock by wide range of chemical substances such as carbon dioxide (acting as H_2CO_3), various organic acids, and chelating agents.

Lichens may accumulate minerals and nitrogen which are eventually released to the primitive soil when the lichen thallus is decayed. Lichens are greatly effected (even killed) by the level of SO_2 present in the atmosphere; their abundance can be used as an indicator of atmospheric pollution. They or their products may be used as food dyes, and indicators (litmus).

METHODS OF CONTROLLING PLANT DISEASES.

The methods are: 1. Cultural Methods 2. Physical Methods 3. Chemical Methods 4. Plant Quarantine.

1.**Cultural Methods:**

(a) Selection of Geographical area which on the basis of the favourable temperature and humidity requirement for a particular crop but unflavored to the fungi and bacteria.

(b) Selection of field:

Many soil borne diseases are controlled by proper selection of the field. It is quite possible that a particular field soil contains a pathogen species. In that case the particular crop is not sown in that field for several years. The causal organism of Red rot of sugarcane Colletotrichum falcatum survives in the soil for several months. Water drainage is also taken care of while selecting the field.

(c) Choice of the time of sowing:

The susceptible stage of plant growth and the favourable environment for pathogen should not match at the same time.

(d) Disease escaping varieties:

Certain varieties of crop due to their growth characteristics are able to escape from disease. This disease escaping characteristics of the crop is not genetic rather it is due to growth habits and time of maturation. Early maturing variety of pea is capable of escaping powdery mildew and rust.

(e) Selection of seed:

To avoid seed borne diseases, healthy and disease free seeds are essential.

(f) Crop rotation:

Crop rotation is essential for controlling soil borne diseases and pathogens.

(g) Roguing:

Removal and Destruction of Diseased Plant Organs, eradication of alternate and collateral hosts and sanitation of Fields.

(h) Modification of cultural Practices:

Cultural practices such as – distance between the plants, time and frequency of irrigation, transplantation time and method, mixed cropping, amount and property of fertilizer and compost etc. can be changed to reduce losses caused by the disease.

(i) Eradication of Insect Vectors:

Insects serve as vectors for many diseases. Eradication of such insect vectors is essential for the control of pathogens.

2.**Physical Methods:**

(a) The hot water treatment method of Jensen was developed in 1887 which was used to control loose smut disease of wheat, barley and Oats. Until the development of systemic fungicide hot water treatment was the only method to control loose smut. Hot water treatment is also effective in the control of nematodes.

(b) Solar energy treatment to control loose smut was first developed by Lutlzra. In this method seeds are first rinsed or soaked in water for 4-5 hrs. before drying them in scorching sun.

(c) Hot air treatment for the control of virus in propagating stocks was first developed by Kunkal in Peach yellow.

3.**Chemical Methods:**

(a) Seed treatment with fungicide before transplanting.

i. Soil treating chemicals:

It is used for controlling such soil borne diseases which attack on seeds or seedlings. The examples of such chemicals are – Formaldehyde, Captan, Thiram, Zineb, Organo-mercurials, PCNB, Ethylene dibromide, vapam etc.

ii. For Externally seed borne diseases, chemicals such as formalin, copper carbonate, captan, organo-mercurials (Agrosan GN and Ceresan) are used for seed treatment.

iii. For Internally seed borne diseases (i.e. loose smut), hot water treatment and solar treatment are used.

iv. Systemic Organic Compounds are effective chemicals for controlling both externally and internally seed borne diseases eg. Oxanthin derivatives (Plantvax and Vitavax), Benlate, Bavistin, Demosan.

v. For controlling air borne diseases, foliar application of chemicals is more effective.

vi. The common copper fungicides are: Perenox, Perelan, Blitox, Cuprokyt, Cuprosanand Fytolan. Its use is comparatively better than that of Bordeaux mixture.

(b) Seed dressing with organomercurials and systemic fungicides.

4.**Plant Quarantine:**

Plant quarantine can be defined as a legal restriction on the movement of agricultural commodities for the purpose of exclusion, prevention or delay m the establishment of plant pests and diseases in areas where they are not known to occur.

BIOLOGICAL CONTROL OF PLANT DISEASES

Biological control may be defined as the utilization of a pest's natural enemies in order to control that pest.

It is the control of pests and parasites by the use of other organisms, e.g., of mosquitoes by fishes which feed on their larvae.

In other words, it is a practice in which an organism is used against another organism.

Under this practice, there are four types of pest control:

(i) Classical biological control or importation, in which a natural enemy from another geographical area, often the area in which the pest originated from, is introduced to contain the pest below the economic injury level, EIL, the definition of EIL is the pest density at which the difference between ne curve showing value of the crop and the curve showing cost of achieving this pest density is neatest;

(ii) Inoculation, in which the periodic release of a control agent is required so that it is available throughout the year. Inoculation is widely practiced in the control of arthropod pests in glasshouses, where crops are removed, along with their pests and their natural enemies at the end of the growing season;

(iii) Augmentation, which involves the release of an indigenous natural enemy in order to supplement an existing population, and is therefore carried out repeatedly usually to coincide with a period of rapid growth of pest population; and

(iv) Inundation, which s the release of large numbers of natural enemy, with the aim of killing those pests present at the time. These are usually termed biological pesticides. However, insects have been main agents of biological control against both insect pests and weeds.

General Theory of Biological Control:

The classical theory of biological control based on the Nicholson-Bailey model is an equilibrium theory (Huffaker and Messenger, 1976). According to this theory, a successful biological control IS produced by the predator imposing low, stable host equilibrium (Fig. 3.9).

But a successful bio-control agent should be host-specific, synchronous with the pest, should have high intrinsic rate of increase (r), should be able to survive with few prey available, and should have high searching ability. All these properties are shown by insect parasitoids than predators. Successful bio-control agents cause density-dependent losses in the host population.

Spatial density dependence occurs when parasitoids or predators cause a higher fraction of losses in dense host patches than in sparse host patches (Hossell, 1977). If predators can aggregate in patches of high host density, then, according to this theory, biological control of the pest is much more likely. The theory has been challenged recently by Murdoch et al. (1985). They have based their view on a non-equilibrium model of predator-prey interaction.

The model assumes that a stable equilibrium of predator and prey is not necessary for satisfactory biological control. Pest populations may fluctuate wildly without pest densities exceeding the economic threshold. According to Krebs (1994), the non-equilibrium model is a meta-population model and, as such, emphasizes that population in different patches may fluctuate independently.

Biological Control by Predators and Parasitoids:

Although predators are considered poor candidates for biological control, they have been used in a number of cases. For example, a small predaceous ladybird beetle, Rodolia cardinalis, commonly called vedalia, has been used to control the cottony- cushion scale insect (Icerya purchasi), a pest of citrus trees. Adult Parasitoids (Hymenoptera) lay their eggs in or near other insects. The larval parasitoid then develops inside its host and kills it before or during the pupal stage.

Biological Control by Parasites:

Some calcid wasps control a number of major pests. The oriental fruit fly, Dacus dorsalis, a pest of ripe fruits in Hawaii has been controlled by three species of parasitic wasps of the genus Opius (O. vandenboschi, O. longicaudatus and O oophilus). This example also illustrates that several parasites of the same pest can be released without having any adverse effect on the overall control. Although the three control agents competed for the same host, the one with superior qualities displaced the others and became dominant.

In this case O. vadenboschi derived the advantage from attacking first instar larvae and thereby inhibiting the development of the eggs and larvae of O. longicaudatus, which favoured older host larvae for oviposition. Likewise, O. oophilus, which oviposits in the eggs of the host, are already present as larvae by the time hosts are suitable for attack by O. vadenboschi.

The geometrid moth Operophetera brumata or winter moth, a pest (defoliator) of hardwood forest and ornamental trees in Canada and Europe, has been controlled by a tachinid fly, Cyzenis albicans, and a wasp Agrypon flaveolatum. However, in this case there was no displacement. Instead, the two species that are compatible and complimentary to each other were able to bring about control. C. albicans was very effective at high host densities, whereas the superior searching ability of A. flaveolatum made it effective at low host densities.

(a) Bacteria:

The use of spore-forming bacteria as a means of controlling the larvae of the Japanese beetle (Popillia japonica), a serious pest of fruits and vegetables, provided the first encouragement for the application of bacteria in insect control. Bacillus popilliae and Bacillus lentimorbus that cause types A and B milky disease of Japanese beetle can both be mass- produced and are sold as a spore dust for injection into the soil. Infected larvae that die in the soil become 1 source of contamination for other larvae feeding in the vicinity. Larval population can be substantially reduced in this way and the Bacillus spores persist in the soil to infect larvae from generation to generation.

Another spore- forming bacteria Bacillus thuringiensis is a facultative pathogen that infects a variety of insects, including the larvae of lepidopterans, flies, and beetles. The bacteria can be cultured on artificial media and is therefore quite economical to produce. Commercial preparations of Bacillus thuringiensis (Biotsol, Dipel, Thuricide) containing both spores and crystals are used is a biological insecticide on a variety of crops. The rather specific nature of Bacillus thuringiensis to kill a few groups of foliage feeders and not to harm beneficial species is of great value in management programmes.

(b) Fungi:

Most entomogenous fungi are internal pathogens. They belong to all the four major taxonomic groups of true fungi, but only a few are frequently associated with insect disease outbreaks. The most commonly used in insect control are Beauvaria bassiane (white muscardine disease) and Metarrhizium anisopliae (green muscardine disease), both of which are fungi imperfect. The infective unit of an entomogenous fungi is usually a spore which germinates on the surface of the host's integument. Once the host tissue is invaded, the fungus can complete its life cycle, but the survival and germination of spores is critical to the development of an epidemic.

Facultative fungi such as Beauvaria and Metarrhizium can be cultured on artificial media, thereby facilitating the production of spore preparations which may be used in biological control. As with most biological control agents, fungi can be used for either persistent or short-term control. A fungus can be introduced into an area where it becomes established and kills the host year after year. Alternatively, fungal spore preparations can be used as microbial insecticide similar 10 the way Bacillus thuringiensis is used.

However, few attempts have been made to colonize entomogenous fungi. Most projects have involved the redistribution of indigenous fungi or those associated with introduced pests, rather than the importation of foreign species. The best example of attempts to establish new fungal pathogens in disease free areas involves the introduction of Coelomomyces against mosquito larvae, but so far the success has been limited.

The successful use of repeated application of fungal spores as microbial insecticides has been reported for achieving short-term reductions of pest populations. The major limiting factor in initiation of fungal disease in insect populations is the effect of the microclimate on spore survival and germination.

The optimal temperature range for the growth of entomogenous fungi is fairly narrow, and relatively high humidity is needed by most fungi to germinate and successfully penetrate their host before they can produce the new spores required to spread the disease. Sunlight also kills the spores. Consequently, the application of a spore preparation must coincide with both the presence of susceptible hosts and suitable environmental conditions. Best success can usually be obtained by applying the spores in the absence of sunlight such as on a warm evening after either rain or irrigation which provides the needed humidity.

(c) Viruses:

The insect pathogenic viruses are called inclusion viruses, as opposed to non-inclusion viruses in which the virus particles or viruses are free within the cells of the host. The virus particles first multiply in the nuclei, but later continue to replicate in the cytoplasm. The disease eventually kills the insect, leaving it hanging as a fragile sac of virus like the one which results from nuclear polyhedroses infection.

A few non-inclusion viruses also attack insects. But with the exception of Tipula Iridescent Virus (TIV) and Mosquito Iridescent Virus (MIV) that might prove useful in mosquito control, most attention has been given to the inclusion viruses. The very fact that the virus particles enclosed in a protein matrix maintain their infectivity for many years means that the inclusion viruses can be stored as concentrated preparations for later application with conventional pesticide spray equipment.

It has been shown that a nuclear polyhedroses virus is highly effective against a variety of forest sawflies and, as it persists in the environment, it provides continuous regulation of the

pest in some areas. Several nuclear polyhedroses virus are being mass produced for possible use against a variety of pests, including cotton bollworm, tobacco budworm, com earworm, cabbage looper, forest tent caterpillar, and alfalfa butterfly. However, one of the problems with viruses is that there are periods when they have little effect on the pest populations. A virus may remain latent in a pest population for several generations and then develop epizootics when the pest population comes under stress.

Generally, short-term control can be achieved by frequent applications of virus preparations so that there is an active innoculum in the pest environment for a long period.

Genetic Control:

Genetic control is a type of biological control that uses two strategies to reduce pest problems. First, crop plants can be manipulated to increase their resistance to pests. Second, we can attempt to alter the genome of the pest species so that they become sterile or less harmful.

Resistant varieties of many crop plants have been developed by selective breeding (Maxwell and Jennings, 1980). However, resistant plants do not necessarily have chemical defenses. Strains of cotton plant produced with low gossypol (a chemical that occurs in green parts and seeds of cotton plant and is toxic to chickens and pigs) content are quite low in resistance to insect pests. Resistant crop plants have also been developed by genetic engineering.

Genes that produce resistance in one species can be transferred into a crop plant to make the crop genetically resistant to specific pests. Bacteria may also be used as vehicles to carry bio-pesticide genes. For example, in 1987 the first success was reported of inserting a gene (the toxin gene of Bacillus thurengiensis) into tobacco plants, conferring resistance against Lepidoptera. Bacillus thurengiensis (Bt) is the main focus at present for developing insect – resistant crops (Lambert and Peferoen 1992).

This bacteria normally lives in the soil and carries a gene for a toxic protein that kills the larvae of moths and butterflies. By splicing this gene into bacteria that normally live on crop plants, genetic engineers have produced insect- resistant crops. Insect pests would inject the bacteria while feeding on the plant and thereby is poisoned.

Alternatively, the Bt genes that produce the toxins can be transferred directly into the plant's genome, so that the plant would protect itself As of 1992 tobacco, potato, cotton and tomato

plants have been genetically engineered with Bt genes (Lambert and Peferoen, 1992). The development and use of such transgenic plants has immense potential. However, one major problem is that pest insects will become resistant to the bio-pesticide, just as they become resistant to chemical pesticides (Pimentel 1991).

The simplest genetic manipulation that can be carried out on a pest species is sterilization. A large number of pests are sterilized by radiation or by chemicals and released into the wild where they can mate with normal individuals. This technique leads to a decrease in birth rate of the pest and control can be achieved. The most notable success of this technique is the near extinction of the screw-worm fly, Cochliomyia hominivorax, which lays its eggs on fresh wounds of livestock and wild animals.

Another example of successful use of sterile-insect method was the suppression of mosquito Culex pipiens quinquefasciatus on a small island off Florida (Patterson, et.al. 1970). However, the sterile insect method cannot be used for all pest populations because it requires the rearing and sterilizing of a large number of individuals and isolation of target area so that natural males from outside the area may not be able to reach there to undrmine the programme.