

## EXERCISE NO. 1

### ACQUAINTANCE WITH MICROSCOPE AND OTHER LAB EQUIPMENTS

**1. MICROSCOPE :** it is an optical instrument in which a single convex lens or set of lenses are used to magnify an object (microorganisms) invisible to naked eyes.

**Principles :** The lenses in this instrument are so adjusted that minute objects invisible to naked eye are magnified and made visible.

**Types of microscope :**

**A. Optical microscope**

a) **Simple microscope:** Only one lens is used in between the eye and object e.g. Hand lenses and dissecting microscope.

b) **Compound microscope:** Two or more than two lenses (or a set of lenses) are used in between the eye and object and this helps in obtaining more magnification e.g. Student's microscope, Research microscope.

**B. Electron microscope :** In this type of microscope instead of optical light as a source of illumination, a electron beam and magnetic lenses are used to magnify the image of sub-microscopic object. Specimens can be observed by either transmission or scanning.

The magnification power of these microscopes range from 100X to 400000X. Measurements of microbes is recorded by using a common unit micron ( $\mu$ ). A micron is  $1/1000\text{mm}$  or  $10^{-3}\text{ mm}$  or  $0.001\text{mm}$ . The objects smaller than bacteria are measured in terms of millimicron ( $m\mu$ ). One millimicron is equal to  $1/1000\mu$  or  $10^{-3}\mu$  or  $0.001\mu$ . The technique used for microscopic measurement is known as micrometry . Stage and ocular micrometers are used for microscopic measurements.

**Types of microscopy :**

**1. Bright field microscopy :** In this type, microscopic field is brightly lighted and the microbes appear dark.

**2. Dark field microscopy :** In this, a dark background is used against which objects are brilliantly illuminated.

**3. Fluorescence microscopy :** Many chemical substances absorb light. After absorbing light of a particular wavelength and energy, some substances emit light of a longer wavelength and lesser energy content. Such substances are called as fluorescent and the phenomenon is termed as fluorescence. For this microbes are stained with a fluorescent dye and illuminated with blue light; the blue light is absorbed and green light is emitted by the dye.

**4. Phase contrast microscopy :** It is extremely valuable for studying living unstained cells and is widely used in applied and theoretical biological studies. This technique is based on the fact that light passing through one material and into

another material of a slightly different refractive index and/or thickness will undergo change in phase. This difference in phase, or wave-front irregularities are translated into variation in brightness of the structures and hence are detected by the eye.

#### **Parts of student's microscope :**

- 1) **Eye piece (Ocular)** : A single set of lenses placed on the upper end of a body tube with usual magnification of 5X, 10X, 15X etc. The function of eyepiece is to magnify the object to a certain extent.
- 2) **Body tube (Draw tube)** : Tube holding the eye piece and the objective lenses at upper and lower ends, respectively. It is fitted to the arm by rack and pinion arrangement for upward and downward movement. The length of the tube is fixed and is generally 160mm.
- 3) **Objective lens**: One or more sets of lenses are fitted to the lower end of body tube with the help of revolving nose piece. There are two objectives in student's microscope.
  - i) Low power objective: Magnification of 10X or 20X.
  - ii) High power objective: Magnification 40X, 44X, 45X or 62X.
- 4) **Arm** : It is a curved, solid, steel frame holding the drawtube at upper end and stage at lower end. It is fitted to the pillar by an inclination joint.
- 5) **Stage and Clips** : Stage is useful for placing the object slide, whereas clips are used for holding the object slide.
- 6) **Inclination joint** : It is used for changing inclination of the microscope to a certain extent for easy observation.
- 7) **Coarse or rough adjustment screw/knob** : It is meant for bringing the microscopic objects after focusing by coarse adjustment of the microscopic object under focus.
- 8) **Fine adjustment screw/knob** : It is for sharp focusing of the microscopic objects after focusing by coarse adjustment screw.
- 9) **Pillar and horse shoe shaped base** : It is the basal part holding the arm and other parts in balance.
- 10) **Mirror** : A mirror with two surfaces (concave and plain) is fitted to the pillar for reflecting light to the object.
- 11) **Diaphragm** : It is a metal disc with adjustable central holes for controlling the quantity of light.

#### **Additional parts of student's Microscope.**

- 1) **Substage or Abby's condenser** : It is meant for adjusting the intensity of light rays and it is fitted with the diaphragm.
- 2) **Mechanical or movable stage**: For movement of the object slide in any direction (to and fro or sideways) by mechanical device.
- 3) **Oil immersion objective** : This is an additional objective with higher magnification i.e. 90X, 95X, 100X,. It is so called because when in use, the air space between the lense and object is filled up with a drop of cedar wood oil

for avoiding refraction of light rays and this objective is immersed in oil. The immersion oil is provided for optically homogeneous path for light rays to travel between slide and the objective lens.

Because of the refractive index of air (1.0) is less than that of glass, light rays are refracted or bent as they pass from the microscope slide into the air. Thus many of the light rays reflected at so great an angle that they completely miss the objective. By interposing immersion oil (1.51) which has essentially the same refractive index as glass (1.51), between the slide and the objective lens, we greatly decrease refraction and a far greater per cent of the light rays from the specimen pass directly into the objective, resulting in to greater resolution and a clear image.

**Magnification:** It is defined as enlargement in original size of an object brought about by lense or lenses. Total magnification of an object is determined by the multiplication of magnifying power of the eye piece by magnifying power of objective e.g. Eye piece (10x) objective (44x) =440.

#### **Instruction for use of Microscope:**

1. It is a costly and delicate instrument. Handle with maximum care.
2. Clean all optical parts, mirror and condenser before use. Use separate cloth to clean body of microscope.
3. Use the microscope in vertical position, keep it to face the light source but avoid direct sun rays.
4. Focus light under low power objective with mirror.
5. Do not mount hot or flooded slide under the microscope.
6. Select suitable microscopic field under low power and then observe it under high power or oil immersion objective.
7. Never lower down the objective while viewing through eye piece, protect the slide from breakage. First lower down the objective just to touch the cover slip and lift it up words to focus while viewing through eye piece.
8. Clean the oil immersion objective after use with xylene and wipe out with muslin cloth.
9. Turn to low power objective while replacing the slide or closing the work.
10. All the lenses must be cleaned by using xylene with the help of expert periodically.

#### **2. HOT AIR OVEN :**

It is a vertical steel cabinate double or triple walled partitioned with wire mesh trays inside. It is provided with heating element between the walls either at the bottom (Bottom heated) or in the three side walls of the cabinet (Universal heated). Some ovens are provided with system for uniform circulation of hot air inside the chamber to maintain uniform temperatures inside. A thermostat control is provided to maintain temperature inside with a sensitivity of  $\pm 3^{\circ}\text{C}$  or less. The range of

temperature varies from 50 - 300°C or more. Front side of the oven is provided with a digital temperature controller-Cum-indicator and power switch for ON-OFF. This device is commonly used for dry heat sterilization of many objects such as glasswares. It can be also used to dry the collected specimen for preservation. The temperature required for sterilization may vary with the time of exposure to the dry heat as mentioned below

Sr. No.	Temperature (°C)	Time (Hr.)
1	120	8
2	140	3
3	160	1
4	180	20 min

### **3. BOD INCUBATOR :**

Biological oxygen demand (BOD) incubator maintains a range of temperatures below and above the ambient temperature required for growth and multiplication of microorganisms. It is a vertical steel chamber shaped as a cupboard made up of double or triple walled body. Temperature inside may be maintained from 5°C to 50°C with an accuracy of  $\pm 1^{\circ}\text{C}$ . It is provided with both heating and cooling system. It may be bottom heated or universal heated with a thermostatic control while cooling is maintained by compressor. Also provided with fans for uniform circulation of temperature inside and fluorescent light for illumination. An inlet nozzle may also be installed for monitoring CO<sub>2</sub>/air mixture concentration inside and humidistat for control of humidity (55-95%) by natural mist. Outside on front surface it is provided with switch for manual/automatic temperature controller, heat energy regulator, digital temperature indicator, cooling/heating indicator etc.

### **4. LAMINAR AIR FLOW CABINET/BENCH :**

The cabinet is fabricated out of thick board of sunmica or is of stainless steel. Interior surface of working platform/table is of stainless steel with the sunmica clad at the top. Sides of the panel are of thick transparent flexi/acrylic glass duly framed. The unit is fitted with both pre filters and high efficiency particulate air (HEPA) filters. The air is forced through pre-filter and passed through HEPA filter having efficiency rating as high as 99.99, thus retaining all the particles of size 0.3 micron or larger. A blower and motor assembly of 1.5hp is provided for air blowing. The cabinet from inside is provided with fluorescent light for illumination and UV light for sterilization of working chamber. Laminar flow cabinet provides an aseptic or microbes free environment for performing various activities such as pouring of media in plates, isolation, transfer of cultures etc.

### **5. AUTOCLAVE / STEAM STERILIZER :**

Autoclave works on the principle that increase in pressure is directly proportional to increase in temperature. The principle behind sterilization in an

autoclave is steam under pressure due to which coagulation of cell proteins in microbes is occurred and they get killed. They may be available in various models as vertical or horizontal with different loading capacities. The equipment is provided with standard accessories such as water level indicator, pressure gauge, steam release valve, spring loaded safety valve and heating element at the bottom. Autoclave is used for sterilization of media, water, soil and other material which is sensitive to dry heat. Generally media are sterilized at 15 lbs psi for 15-20 min in an autoclave.

#### **Pressure and temperature relationship in autoclave**

Pressure per square inch (psi)	Temperature (°C)
5	107
7	110
10	115
15	121
20	126

Autoclave is not used for sterilization of heat sensitive materials such as vitamins, hormones, fatty acids, oils etc.

#### **Other equipments/tools used in microbiology laboratory**

Sr. No.	Equipment / tools	Use
1	Centrifuge	Separation of different density gradient particles.
2	Weighing balance	To weigh required ingredients / lab. Chemicals.
3	Stage and ocular micrometer	Measurement of microscopic objects.
4	Camera lucida (Prism and mirror type)	Drawings of microscopic objects with help of microscope.
5	Water bath	Melting of media, heating of liquids etc.
6	Inoculation needle (Bacterial and Fungal)	Handling of cultures of microorganisms.
7	Cork borer	Cutting circular disc of cultures.
8	Spatula	Taking out solid ingredients from bottles.
9	Microtome	Micro sectioning of plant or animal tissues.
10	pH meter	Measuring the pH of media.
11	Colony counter	Counting the number of colonies in a plate.
12	Plant press	Pressing the diseased plant specimen for dry preservation.
13	Refrigerator / Deep Freeze	Preservation of cultures and heat sensitive chemicals.
14	Lyophilizer / Freeze dryer	Lyophilization of cells.
15	ELISA reader, washer and PCR	Detection of viruses.
16	Spectrophotometer	Colorimetric observations.
17	Distillation unit	Production of distilled water.

## EXERCISE NO. 2

### METHODS OF STERILIZATION AND DISINFECTION

**Sterilization :** It is a process by means of which material is made sterile or free from living micro-organisms either by killing or separating them.

**Disinfection :** It is the process of localized removal of surface microflora or propagules of microbes.

It is essential to sterilize culture media, glasswares and other laboratory tools before their use to avoid contamination. Various physical and chemical methods used for the purpose of sterilization and disinfection are...

**Physical Agents:**

**1) Heat**

- i) Dry: (a) Direct e.g. flame, (b) Indirect i.e. Hot Air Oven.
- ii) Moist: (a) Steam without pressure – Arnold Steam Sterilizer,  
 (b) Steam with pressure – autoclave.

**2) Filters**

**For liquids:** Porcelin or glass filters like

- (a) Chamber land, (b) Berkefeld, (c) Scitz.

**Gas sterilization:** Cotton fibers etc.

**Light:** I) Ultraviolet rays, ii) Germicidal lamps.

**Chemicals:** a) Inorganic KMNO<sub>4</sub>, Idoine etc b) Organic – Phenol, formaldehyde, ethyl alcohol etc.

**Direct dry heating/flame sterilization:** It is useful for sterilization material made up of metal like inoculating needles, scalpels, forceps etc. Temperature reaches above 200° c and material is exposed for few seconds. This method is technically called as incineration. Ex. Spirit lamp, Bunsen burner.

**Indirect dry heating by hot air oven :** Materials like dry glasswares such as test tubes, flasks, Petri dishes, pipettes, funnels, etc. which get damaged by direct flame heating and which do not contain any moisture of matter likely to be burnt or charred can be sterilized by this method.

**Moist heating by steam without pressure (Arnold steam sterilizer) :** Materials like media which contain moisture or material likely to be affected or spoiled by dry heating and which requires moist heating are sterilized by this method. Further, certain media containing carbohydrates, potato cylinders, gelatin, milk media etc. are sterilized by ordinary flowing steam, as it cannot be subjected to steam pressure which affects the carbohydrate contents.

**Principles:** The material is exposed to ordinary steam i.e. 100 to 110° C for 20 to 30 minutes and the process is repeated for successive 2 more days with an interval of 24 hrs. This repeated treatment kills resistant spore formers which germinate during the intermittent period. The process is also known as 'fractional or intermittent sterilization or Tyndallization'.

The apparatus is called as 'Arnold steam sterilizer' which consists of double walled copper chamber with a water holding trays at the bottom. The material to be sterilized is placed on perforated sieves. Steam rising from bottom, heats the material, gets condensed at the top and returns to be boiling trays through the space in between two walls. Heating can done either by electric heaters or gas burners.

#### **Moist heating by steam under pressure (Autoclave)**

Culture media like P.D.A., N.B., N.A. and soil, water, sand, manure etc. which are not damaged by high temperature of the steam under pressure are sterilized by this method. Steam under pressure has high temperature and more penetrating power and hence it is the quickest method. Materials usually sterilized by direct heating or hot air can also be sterilized by this method.

Culture media, water etc. are sterilized at 15 lbs. Pressure for 15 minutes, while bulky materials like soil, sand, manure etc. required 30 lbs psi for 30 minutes.

The apparatus is called as 'Autoclave'. It consists of a thick walled vessel made of alloy (metal), with a close fitting, which can be tightened with fly-nuts. The lid is provided with air or steam cock, safety valve and pressure gauge. Water is to be filled at the bottom and a separator stand or frame is provided at the bottom. It is operated by electric current or in some cases gas burners can also be used.

Normally it takes 11-12 minutes at 121°C (moist heat) to kill oospores of thermophilic bacteria. Culture media are usually sterilized at 15 lbs. pressure maintained for 15 to 20 minutes and soil at 25 to 30 lbs. pressure for 30 to 60 minutes depending upon the volume of materials in autoclave. Oils, fats and gasses cannot be sterilized in autoclave.

#### **Filtration**

- 1) **Liquids:** Heat sensitive materials like sugars, blood serum, enzymes, hormones, vitamins, antibiotics, vaccines, etc. require sterilization by filtration. Bacterial filters are made up of porcelain (diatomaceous earth) sintered glass etc. e.g. chamber land, Berkefeld, seitz filter. The pores of the filters are so small that the bacteria cannot pass through and are, therefore, hold back. Actually this is not the sterilization but micro-organisms are separated from the media. Filtration occurs only when Vacuum is created in receiving flask. The filters are of various designs and grades (Pore size).
- 2) **Gaseous sterilization ( Cold sterilization ):** Ethylene oxide vapours under pressure in special equipment is becoming common method of cold sterilization. Ethylene oxide is highly toxic to viruses, bacteria, fungi as well as the heat

resistant bacterial endospore It is easy to handle, inexpensive, noncorrosive and nondeleterious to materials being sterilized. Ethylene gas is inflammable but in mixute with 90% carbon dioxide it becomes noninflammable and more effective, sterilizing agent.

**3) Irradiation:** High energy ionizing radiations with gamma rays from a cobalt 60 or caseium 139 source and cathod rays from electron generators and accelerators are used in sterilization of certain pharmaceuticals. Irradiation with ultraviolet light is used in operation theaters and isolation chambers. e.g. Laminar air flow cabinet, and Germicidal lamp.

**Chemicals:** Chemicals used for sterilization are called disinfection.

**I) Organic chemicals :**

1. Phenol (Carbolic acid) : 2 to 4% used for dis-infection of wounds. It is a standard disinfectant against which other disinfectants are compared.
2. Alcohol : 50 to 70% used for plant material and skin.
3. Lysol (Cresol + linseed oil) : 1 to 5% in water used for pots etc.
4. Formaldehyde : 5% used for preserving plant materials and soil sterilization.

**II) Inorganic chemicals :**

1. Mercuric chloride : 0.1% used for plant materials, working surfaces etc.
2. Chlorine : 1 to 5% for skin, plants, drinking water etc.
3. Silver nitrate : 1 to 5% for skin.
4. Iodine : 2 to 5% alcoholic solution (tincture) used for skin.
5. Potassium permanganate : Used for skin at 1% and in drinking water.
6. Soaps and detergents : Used for skin sterilization.

### EXERCISE NO. 3 NUTRITIONAL MEDIA AND THEIR PREPARATIONS

**Culture:** It is the growth of microorganisms on a medium. If a culture contains the growth of only one type of microorganisms it is called as **pure culture**, on the other hand when it contains growth of more than one type of microorganisms it is called as '**Mixed**' or '**Contaminated culture**'. Obligate parasites such as rusts, downy mildew, powdery mildew causing fungi and viruses can't be grown artificially on culture media.

The main elements required for bacterial growth are carbon and nitrogen with certain amount of minerals and vitamins with water. Carbon is usually supplied as carbohydrates, water supplies the hydrogen, and nitrogen can be supplied in organic or inorganic form. Plant or animal tissue extract supplying organic nitrogen with carbon are usually more favorable. In the laboratory Peptone is commonly employed in the form of organic nitrogen, it also supplies carbon. Since different organisms requires various nutrients in different proportions, general media supplying all the essential nutrients are commonly used e.g. Nutrient broth for bacteria and P.D.A. (Potato Dextrose Agar) for Fungi.

**Culture medium:** A nutrient or combination of nutrients prepared for the growth of microorganisms in laboratory is called as a culture medium.

The medium is prepared by mixing or dissolving nutrients in water that makes a liquid medium (broth). Solidifying agents (Agar or gelatin) is added to prepare a liquefiable solid medium.

#### Requirement of a good culture medium.

- 1) It should supply required nutrients in proper proportion.
- 2) It should have a proper pH.
- 3) It should be free from all living organisms and unwanted materials.
- 4) It should have proper moisture content.
- 5) It should have considerable physical properties e.g. Solid, liquid.

#### Commonly used solidifying agents :

- 1) **Gelatin** – It is an incomplete protein compound prepared from animal bones, horns, hoofs, etc. It is used by certain bacteria producing gelatines called gelatin liquefying organisms. It melts at 37° C and solidifies at 20° C. The medium-containing gelatin is transparent. It is used @ 10 to 20 % in the medium for solidification.
- 2) **Agar-Agar** – It is a complex carbohydrates prepared from sea weed or marine algae (*Gelidium* spp.). It is used as solidifying agent in culture media @ 1.5 to 2% concentration. It melts at 98° C and solidifies at 37° C. The medium is opaque when solid. It does not supply any nutrient to the organisms.

### Classification of media

#### 1) According to composition.

- a) Natural media: Media prepared from natural basic materials like host tissue extracts, vegetable extracts, fruit extracts, oat meal, yeast extract etc. are known as natural media. e.g. oat meal agar, V8 juice agar etc.
- b) Synthetic media: Exact chemical composition of ingredients is known e.g. Richard's medium, Ashby's medium, Conn's medium.
- c) Non/semi-synthetic media: Exact chemical composition of ingredients is not known e.g. P.D. A, N. A. etc.

#### 2) According to use:

- a) General or common medium - e.g. P.D.A, N. A.
- b) Selective or special medium: Used for specific organisms. Ashby's medium for *Azotobacter* and Yeast extract mannitol agar medium for *Rhizobium*.

#### 3) Differential Media:

These are used to detect the production of certain characteristic growth of the various species of bacteria e.g. Eosine methylene blue agar for testing coliform bacteria.

Solid media are used for studying cultural characters, for maintenance and obtaining pure cultures by streaking, plating etc. while liquid media are commonly used for large scale multiplication of the organisms.

### Preparation of common culture media :

#### 1) Nutrient Agar medium :

1. Beef extract	-	3 g.
2. Peptone	-	5 g.
3. Agar-agar	-	20 g.
4. Distilled water	-	1000 ml.

Weigh all the ingredients properly. Dissolve or dispense the taken ingredients in 1000ml of distilled water. Plug the flask with non-absorbent cotton plus and wrap it with paper to prevent entry of steam inside the flask. Sterilize in an autoclave at 15 lbs psi for 20 minutes.

#### 2) Potato Dextrose Agar (P. D. A.) medium :

1. Potato (peeled)	-	200 g.
2. Dextrose	-	20 g
3. Agar-agar	-	20 g
4. Distilled water	-	1000 ml.

Weigh all the ingredients properly. Chop the peeled potatoes into small pieces and boil/cook them in 500ml of distilled water till desired extract is obtained. Strain the extract through clean muslin (Sol<sup>n</sup> A). Dissolve or dispense remaining ingredients in remaining 500ml of water and heat for some time to dissolve agar-agar (Sol<sup>n</sup> B). Mix both in third container and make the volume to 1000ml by adding more water. Plug

the flask with non-absorbent cotton plus and wrap it with paper to prevent entry of steam inside the flask. Sterilize in an autoclave at 15 lbs psi for 20 minutes.

#### **Adjustment of pH of Culture Medium**

pH is the measurement of acidity or alkalinity of a solution or it is the hydrogen-ion concentration of medium. Different organisms grow well in the culture medium having specific pH, Bacteria require slightly alkaline medium (pH 7 to 7.5), while fungi require slightly acidic media (pH 6 to 7).

#### **Methods of pH Adjustment**

1. Colorimetric method
2. Electrolytic method

#### **Requirement of a good plug.**

1. It should be tight enough to bear the weight of the container.
2. It should be 1" to 1 ½" inside the mouth
3. It should be easily removable or replaceable.

**EXERCISE NO. 4**

**ENUMERATION OF MICROBIAL POPULATION IN SOIL – BACTERIA, FUNGI  
AND ACTINOMYCETES**

**Serial dilution and plate technique**

Serial dilution and plate method is one of the good and dependable methods available for determining the quantitative and qualitative distribution of microorganisms in soil. The total number of bacteria, fungi and actinomycetes in the soil samples are determined by this method.

**Media required :**

**A. For bacteria :**

**Nutrient agar medium :**

Beef extract	3.0 g
Peptone	5.0 g
Agar	20.0 g
Water	1000 ml

**B. For fungi :**

**Martin's rose bengal agar medium:**

Glucose	10.0 g
Peptone	5.0 g
KH <sub>2</sub> PO <sub>4</sub>	1.0 g
MgSO <sub>4</sub>	0.05g
Streptomycin	30.0 mg*
Agar	15.0 g
Rose Bengal	0.035 g
Distilled water	1000 ml

**C. For Actinomycetes :**

**Kenknights agar medium:**

Dextrose	1.0 g
KH <sub>2</sub> PO <sub>4</sub>	0.1 g
NaNO <sub>3</sub>	0.1 g
KCL	0.1 g
MgSO <sub>4</sub>	0.1 g
Agar	15.0 g
Distilled water	1000 ml

**Material required :**

1. Sterile water blanks
2. Sterile Petri dishes, pipettes, etc.
3. Soil sample

### **Procedure**

Firstly prepare a series of water blank by pouring 9 ml of sterile water in each sterile test tube (6 tubes). Weigh 1 g of soil sample and suspend in first water blank, shake for 1 minute and allow the soil particles to settle to obtain  $10^{-1}$  dilution. Using 10<sup>-2</sup> dilution Similarly, samples are diluted up to 10<sup>-4</sup> to 10<sup>-6</sup> dilution levels.

One ml quantity of the 10<sup>-3</sup> 10<sup>-4</sup> and 10<sup>-6</sup> dilution is transferred aseptically to the individual sterilized Petri plates (at least three replications for each dilution are needed) for the enumeration of fungi, actinomycetes and bacteria respectively.

Respective agar media are melted and cooled down to 40-42°C and approximately 15-20 ml of the medium is poured aseptically to each Petri dish. Immediately after adding the medium, the Petri dish is gently rotated in clockwise and anti-clockwise direction for uniform distribution of the population. The plates are incubated at room temperature.

In the case of fungal enumeration, in the Martin's rose Bengal agar medium just before pouring the medium, the antibiotic solution (30 mg streptomycin sulphate)

The moisture content in the soil is determined by keeping the known quantity of soil in hot air oven at 80°C for 24 h. The weight loss is the soil sample actually denotes the moisture content in the soil sample.

### **Observations:**

Bacteria are counted after 24-48 hrs, fungi after 72-120 hrs and actinomycetes after 168 hrs. The number of colonies in the Petri plates are counted using a colony counter. The mean of the colony numbers is worked out for each group of organisms and the data are further calculated to one gram of soil sample on moisture free basis.

$$\text{Number of colonies/gm} = (\text{Mean number of colonies} \times \text{dilution factor}) / \text{Oven dry weight of } 1 \text{ g soil sample}$$

### EXERCISE NO. 5

## METHODS OF ISOLATION AND PURIFICATION OF MICROBIAL CULTURES

### **Isolation:**

It is a technique of separating the organisms from the diseased host tissue and soil and growing them on artificial media for the purpose of further study or investigation.

#### **A. Isolation of fungal pathogen :**

Isolation of fungal plant pathogens from diseased host tissues when causal organism is located inside the host tissues.

#### **Tissue isolation :**

1. Wash the diseased part with tap water to remove dirt and soil and cut the affected part in to small convenient pieces of 2 to 3 mm size in such a way that half the portion of healthy tissue must be present along with half diseased portion..
2. Surface sterilize the bits by immersing in 0.1%  $HgCl_2$  (Mercuric chlorides) solution for 1 to 2 minutes or sodium hypochlorite ( $NaOH$ ) 1% for 2-3 minutes.
3. Rinse the surface sterilized bits in distilled sterilized water for three or four times to remove the traces of  $HgCl_2/NaOH$ .
4. Dry these pieces on the sterilized blotter paper.
5. Place the pieces/bits on solid medium (potato dextrose agar medium) already poured in sterilized Petri plates. Place 2 or 3 bits in a plate.
6. Incubate the inoculated plates in inverted position at room temperature ( $25-30^{\circ}C$ ) for 3-5 days and observe regularly for growth of fungus if any.
7. Transfer the fungal growth obtained in slants immediately after its appearance. If mixed or contaminated culture is obtained, purify the culture either by hyphal tip isolation or single spore isolation method.

**NOTE :** If the infection is due to fungus, few drops of 2% lactic acid are mixed in sterilized water, in last rinse with water or add the antibiotics like Penicillin or Streptocyclin @ 100 ppm of medium in order to avoid the bacterial contamination.

#### **B. Isolation of bacterial pathogen :**

1. Collect the samples suspected to be infected with bacterial pathogen.
2. Wash the sample under running tap water to remove soil or dust particles. Cut small bits of convenient size and dip them in sodium hypochlorite solution (4%) for surface sterilization for 1-2 minutes and rinse the pieces in distilled sterilized water for 3-4 times to remove the traces of disinfectant.

3. Place the bits of diseased host tissues on glass slide in a drop of sterilized water and again cut into small pieces with sterilized scalpel or blade. The bacteria will ooze out in about 5 minutes.

**Method I :**

Take a loopful of bacterial suspension and streak it on Petri plate containing the nutrient agar medium and incubate it at a temp. of  $27 \pm 2^{\circ}\text{C}$  for 72 to 96 hrs. After 72 hours if colonies appear along the streak, collect such a single colony and transfer it on nutrient agar slant.

**Method II :**

1. Dilute the bacterial suspension with the help of sterilized distilled water. Add the suspension to the liquefied medium when the temp. is approximately  $40^{\circ}\text{C}$  and mix thoroughly by shaking the medium and pour in sterilized Petri plates.
2. Incubate these Petri dishes in inverted position in order to avoid contamination in an incubator at  $27 \pm 2^{\circ}\text{C}$  for 72 to 96 hours.
3. After 72 hours if colonies appear on the medium, collect a single colony and transfer it on the nutrient agar slant.

### Purification of fungal culture

Following two methods are used for the purification of the fungal cultures.

**A. Hyphal tip isolation method :**

1. In this method initially growing hyphal tip is selected and transferred in the slant to obtain the pure culture.
2. Grow the colony of the fungus on the PDA medium.
3. Select single growing hyphal tip of the mycelium with the help of microscope and mark it with glass marking pencil.
4. Cut the marked portion with the help of cork borer and transfer it on the PDA slant and incubate at the temp. of  $27 \pm 2^{\circ}\text{C}$  and observe for pure growth of culture.

**B. Single spore isolation method :**

1. Prepare the spore suspension from the sporulating fungus in order to have  $10^6$  spores/ml.
2. Add above 10 ml of spore suspension in 250 ml of liquified potato dextrose agar medium when the temp. of medium is around  $40^{\circ}\text{C}$ .
3. Add the medium in sterilized Petri dish and allow to it solidify.

4. After solidification of the medium locate single spore with the help of microscope and mark with a glass marking pencil or immediately after appearance of fungal growth, locate single well isolated colony and mark it.
5. Cut marked portion with the help of cork borer and transfer it on PDA slant.
6. Incubate the slants at the temp. of  $27 \pm 2^{\circ}\text{C}$  and observe for pure growth of culture.

### **Purification of bacterial cultures**

#### **Streak method :**

The bacterial cultures are generally purified by mean of streak method. In this method the loopful contaminated bacterial culture is streaked consecutively on three to four preoured Petri plates containing nutrient agar medium. Then the plates are incubated at  $27 \pm 2^{\circ}\text{C}$  temp. for 72 to 96 hrs. The isolated colonies from the last streaked plate are generally picked up to obtain pure culture. Select the isolated colony and streak it on the slant containing nutrient agar medium.

**EXERCISE NO. 6**  
**ISOLATION OF RHIZOBIUM FROM LEGUME ROOT NODULE**

*Rhizobium* is a bacterium which fixes atmospheric  $N_2$  symbiotically in legume root nodules. It was first discovered in 1888 by Beijerinck. Symbiosis is a phenomenon of living together with mutual benefits. The legume crop is benefited by the supply of  $NH_3$  fixed by bacteroids (forms of *Rhizobium*) in nodules, while *Rhizobium* is benefited by shelter in root nodules and receipt of carbohydrates from legume plant. The capacity of rhizobial isolate is to invade roots of a restricted number of plant species in addition to the legume from which it is isolated. This indicates host specificity of *Rhizobium* and taken as a basis for different cross-inoculation groups of *Rhizobium*. For isolation generally, bigger nodules with pink colour are effective. From such nodules it is possible to isolate pure culture of *Rhizobium*. Pink coloration of nodules is due to a pigment called leg-haemoglobin.

**Material required.**

Any leguminous crop with vigorous nodules (Groundnut/pigeonpea/red gram), 1:1000  $HgCl_2$  solution, 75% alcohol, sterile petri dishes, test tubes, glass rod, scalpel, forceps, razorblade, sterile water and Yeast extract mannitol agar containing Congo red.

**Composition of Congo Red Yeast Extract mannitol Agar Medium**

Mannitol	-	10.0g
$K_2HPO_4$	-	0.5g
$MgSO_4, 7H_2O$	-	0.2 g
NaCl	-	0.1 g
$CaCO_3$	-	3.0 g
Yeast Extract	-	1.0 g
Agar	-	15.0 g
Distilled water	-	1000 ml
Congo-red	-	15 ml of $\frac{1}{400}$ aqueous solution

**Procedure**

1. Uproot carefully vigorously growing legume crop preferably at flowering and bring the plants with intact nodules to the laboratory.
2. Wash the root system under running tap water to remove adhering soil.
3. Detach carefully the nodules from the root system with the help of sterile sharp razor blade leaving a small portion of root attached to nodules.
4. Place the nodules in a Petri dish containing 1:1000  $HgCl_2$  solution and agitate the nodules using sterile forceps for 1 to 2 minutes.
5. Take care that sufficient  $HgCl_2$  solution is taken in Petri dish so as to achieve complete dipping of nodules.

6. Transfer the nodules with sterile forceps to a sterile Petri dish containing 75% alcohol and agitate for half minutes
7. After surface sterilization, wash the nodules in sterile distilled water 3-4 times to make them free from the excessive chemicals.
8. Collect one or two surface sterilized nodules in a sterile test tube containing 1 ml of sterile water. Crush the nodule with sterile blunt ended glass rod. Mix the nodule exudates and water. From this transfer one or two loopful of exudates to other sterile test tube containing 1 ml of sterile water to dilute the nodule exudates.
9. Add 1 ml of diluted nodule exudates to a sterile Petri dish.
10. Pour Petri dish with 15-20ml of solidifiable Congo red yeast extract mannitol agar medium ( $45^{\circ}\text{C}$ ). Mix the nodule exudates and medium by rotating the plates gently.
11. Allow the medium to solidify and incubate the plates at  $28^{\circ}\text{C}$  or at room temperature for 4 to 5 days.
12. Transfer growth from rhizobial colony to the slants of yeast mannitol agar medium.

#### **Observation:**

*Rhizobium* forms white, translucent, glistening, elevated and comparatively small colonies on the medium. Moreover, *Rhizobium* colonies do not take up the colour of Congo red dye added in the medium. Those colonies, which readily take up the Congo red stain, are not rhizobia but presumably *Agrobacterium*, a soil bacterium closely related to *Rhizobium*.

After careful selection, a loopful of *Rhizobium* colony is purified by streak plate method on fresh YEM agar plates for single colony. These purified cultures of rhizobia are maintained on agar slants of the same YEMA medium.

**EXERCISE - 7**  
**ISOLATION OF AZOTOBACTER FROM SOIL**

The *Azotobacter* is an aerobic, Gram -ve, short rod, heterotrophic, free living, non-symbiotic nitrogen fixing bacterium and prefers to live in high organic matter content and in neutral pH soils. Some species of *Azotobacter* are *A. chroococcum*, *A. vinelandii*, *A. beijerinckii*, *A. agilis*, *A. paspali*, *A. macrocytogenes* and *A. insignis*. It can be isolated from rhizosphere soil of any cereal crop by two methods viz., (1) Soil dilution and plate method and (2) Enrichment culture technique. A quantitative study of these bacteria in soil would reflect the natural fertility of soil. The principle involved is that the growth medium used for enumeration of *Azotobacter* is devoid of any combined nitrogen and if organisms appear on N-free medium, it is presumed that they are nitrogen fixers.

**Materials required:**

**1. Ashby's Mannitol Agar Medium (N-free Mannitol Agar Medium)**

Mannitol	:	20.0 g
Ca CO <sub>3</sub>	:	5.0 g
K <sub>2</sub> HPO <sub>4</sub>	:	0.2 g
Mg SO <sub>4</sub> .7H <sub>2</sub> O	:	:0.2 g
NaCl	:	0.2 g
Ferric chloride	:	Trace
MnSO <sub>4</sub> , 4H <sub>2</sub> O	:	Trace
N-free washed Agar	:	20.0 g
pH	:	7.0
Distilled Water	:	1000 ml

**2. Jensens Medium :**

Sucrose	:	20.0 g
Ca CO <sub>3</sub>	:	2.0 g
K <sub>2</sub> HPO <sub>4</sub>	:	1.0 g
Mg SO <sub>4</sub> .7H <sub>2</sub> O	:	0.5 g
NaCl	:	0.5 g
FeSO <sub>4</sub>	:	0.1 g
Na <sub>2</sub> MO <sub>4</sub>	:	0.005 g
N-free washed Agar	:	20.0 g
pH	:	7.0
Distilled Water	:	1000 ml

Soil samples, Sterile water blanks, Sterile pipettes and Petri dishes

**Procedure:**

**Method 1 : Serial dilution and plate technique**

Firstly prepare a series of water blank by pouring 9 ml of sterile water in each

sterile test tube (6 tubes). Weigh known quantity of soil sample (1g) and suspend in first water blank, shake for 1 minute and allow the soil particles to settle to obtain  $10^{-1}$  dilution. Using sterile pipette transfer 1 ml of solution from  $10^{-1}$  dilution to next water blank to have  $10^{-2}$  dilution. Similarly, samples are diluted upto  $10^{-4}$ . Because the populations of these bacteria are not very high in soils, dilutions are restricted to  $10^{-2}$  or  $10^{-3}$ . Using sterile pipettes one ml of the each dilution is transferred aseptically to the sterile Petri plates. Three plates are used for each dilution. To each Petri plate 15-20 ml of selective media is added and incubated at room temperature. Colonies of these bacteria would appear after 3-5 days. If necessary, the plates are to be incubated for 7 days as these bacteria are slow growers.

Azotobacter grow as raised, slimy colonies on agar surface and aged cultures shows yellowish brown/black colouration due to pigment production.

**Method 2 : Enrichment culture technique**

In enrichment culture technique, the nutrient medium used is enriched with defined chemical components which are necessary for a particular organism so as to enhance its predominance by its ability to grow more rapidly than others. Azotobacter being N<sub>2</sub> fixer, it can use atmospheric N<sub>2</sub> and so far its isolation, the nutrient medium that is free of combined nitrogen but contains other minerals and carbon source is used. Jensen's medium is most suitable for the isolation of Azotobacter by enrichment culture technique.

1. Collect rhizosphere soil of any cereal crop growing profusely.
2. Add 0.5 g of rhizosphere soil separately to 3 to 4 conical flasks containing 100 ml of sterile Jensen's broth and shake well to mix the soil with broth.
3. Incubate the flasks at 28°C ( $\pm 2^\circ\text{C}$ ) for a week.
4. After incubation, transfer 1 to 2 ml of growth suspension from each of these flasks separately to another flasks containing Jensen's liquid medium. Incubate newly transferred flasks at 28°C ( $\pm 2^\circ\text{C}$ ) for a week.
5. Similarly make 2 to 3 more transfers and observe for the growth of Azotobacter obtained from the finally transferred flasks.
6. Transfer loopful of growth on the slants of Jensen's agar.
7. Examine the growth of Azotobacter from flasks and slants.

**EXERCISE - 8**  
**ISOLATION OF AZOSPIRILLUM FROM ROOTS**

The diazotrophic bacteria, *Azospirillum* are common inhabitants in the rhizosphere of wide variety of crop plants of diverse geographical regions and are frequently isolated from tropical, semiarid and temperate zones.

The bacterium known as *Spirillum lipoferum* was first described by Beijerinck (1922), followed by Schroder (1932) and Becking (1963). The importance of these organism was realized when Dobereiner and Day (1976) isolated these organisms from the roots of *Digitaria decumbens* cv *transvala* which exhibited nitrogenase activity.

*Azospirilla* are Gram negative, vibrio or spiral or curved shaped and  $1.15\ \mu$  in diameter, possessing peritrichous flagella for swarming and a polar flagellum for swimming. It contains poly-B-hydroxy-butyrate granules. The genus *Azospirillum* consists of five species viz. *A. brasiliense*, *A. lipoferum*, *A. halopraeferens*, *A. amazonense* and *A. irakense*.

**Materials required:**

1. Freshly collected root samples of cereals, sterile water blanks, alcohol (80%), mercuric chloride (0.1%), nitrogen free malic acid semi solid medium, sterile Petri plates, test tubes and forceps.

**Composition of the Nitrogen Free semisolid malate medium**

Malic acid	5.0 g
Potassium hydroxide	4.0 g
Dipotassium hydrogen orthophosphate	0.5 g
Magnesium Sulphate	0.2 g
Sodium chloride	0.1 g
Calcium chloride	0.2 g
Fe-EDTA (1.64 per cent W/V aqueous)	4.0 ml
Trace element solution	2.0 ml
Vitamin solution	1.0 ml
Bromothymol blue (0.5% alcoholic solution)	2.0 ml
Agar	1.75 g
Distilled water	1000 ml
pH	6.8

**Procedure:**

The root samples are washed free of soil particles and cut into small bits of 0.5-1.0 cm size. The root bits are first surface sterilized using mercuric chloride (0.1%) for one minute, followed by 80 per cent alcohol for one minute. Then root bits are washed in several changes in sterile distilled water, to remove the excess of

chemicals. The bits are then aseptically transferred to test tubes (1-2 root bits/test tube) containing 5 ml of nitrogen free semi solid malate medium and incubated for 3-5 days at 30°C. The tubes are observed for growth of *Azospirillum* after incubation. For comparison uninoculated tubes are maintained as controls. The positive tubes are observed for the development of small white globular subsurface pellicles in the medium. Moreover, the change of colour of the medium from yellowish green to blue is an indication of the growth of *Azospirillum*.

### Purification

A loopful of the subsurface pellicle is transferred and streaked over the same medium in Petri dishes (with 1.5 per cent agar). From the characteristic single colonies, the organism is picked up and maintained over yeast extract agar slants.

## 2. Enumeration of *Azospirillum* from the rhizosphere soil

- Rhizosphere soil is collected from cereal plants
- One gram of representative sample is placed in 9ml water blank and mixed thoroughly by shaking which will provide a  $10^{-1}$  dilution of the sample.
- Serial dilutions are prepared up to  $10^{-6}$  dilutions by transferring one ml of sample starting from  $10^{-1}$
- The diluents  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  are selected for enumeration. One ml each of the suspension from  $10^{-4}$  dilution is transferred aseptically to each of the five tubes containing 5 ml (approximate) of malate semisolid medium. Similarly another five tubes each of media are inoculated with  $10^{-5}$  dilution and  $10^{-6}$  dilution suspension respectively.
- The inoculated 15 tubes with appropriate uninoculated control are incubated at room temperature for one week period and undisturbed.
- After incubation the tubes are observed for the growth of *Azospirillum*. The *Azospirillum* growth is evidenced by the change of colour of the medium from greenish yellow to blue and the presence of white dense sub surface pellicles.

### EXERCISE - 9

#### ISOLATION OF BLUE GREEN ALGAE (BGA)

The occurrence of algae is reported in most of the soils. Algae are never numerous as bacteria, actinomycetes or fungi. The algae are abundant in habitats where moisture is adequate and light is accessible. The algae are abundant in habitats unicellular or they may occur in short filaments.

Soil algae are divided on the basis of pigmentation into: (1) Cyanophyta (blue-green algae), (2) Bacillariophyta (diatoms), (3) Xanthophyta (yellow-green algae), (4) Euglenophyta (euglenoid flagellates) and (5) Chlorophyta (green algae). The red algae (Rhodophyta) and brown algae (Phaeophyta) are totally lacking in the soils.

Some species in the genera of blue-green algae (such as *Anabaena*, *Calothrix*, *Nostoc*, *Plectonema* etc.) fix atmospheric nitrogen and may contribute significantly to the fertility of the soils. BGA are also known as 'cyanobacteria'. Blue pigment in BGA is 'phycocyanin' and site of nitrogen fixation in BGA is 'heterocysts'. BGA are used as biofertilizer in puddle paddy crop. It is photoautotrophic non-symbiotic N<sub>2</sub> fixer.

The algae living at the surface of the soil convert carbon dioxide to carbonaceous materials through their photoautotrophic nutrition. The photosynthetic process in algae liberates molecular oxygen (which will be of great use in improving soil structure and erosion control because of the binding together of soil particles).

In this experiment the different groups of algae population in three different soil samples are enumerated and the isolation of algae into pure culture is carried out.

#### **Material required:**

**Soil samples:** Wet land soil, sterile water blanks 100 ml and 90 ml, sterile pipettes.

#### **Medium No. 1**

NaNO <sub>3</sub>	-	1.5 g
K <sub>2</sub> HPO <sub>4</sub>	-	0.039 g
MgSO <sub>4</sub> 7H <sub>2</sub> O	-	0.075 g
Na <sub>2</sub> CO <sub>3</sub>	-	0.020 g
CaCl <sub>2</sub> 2H <sub>2</sub> O	-	0.027 g
Na <sub>2</sub> SiO <sub>5</sub> 9H <sub>2</sub> O	-	0.058 g
Ethylenediamine tetra acetic acid	-	0.001 g
Citric acid	-	0.006 g
Ferric citrate	-	0.006 g
Distilled water	-	1000 ml
Micronutrient solution	-	1.0 ml

### MICRONUTRIENT MIXTURE

$H_3BO_4$	-	
$MnCl_2 \cdot 4H_2O$	-	2.86 g
$ZnSO_4 \cdot 7H_2O$	-	0.181 g
$Na_2MoO_4 \cdot 2H_2O$	-	0.222 g
$CuSO_4 \cdot 5H_2O$	-	0.391 g
$Co(NO_3)_2 \cdot 6H_2O$	-	0.079 g
Distilled water	-	0.049 g

Medium No.: 2  
1000 ml.

Above medium without addition of  $NaNO_3$ . (medium 2 is recommended for the cultivation of blue-green algae and particularly useful to detect only those species capable of fixing atmospheric nitrogen).

### Procedure

1. Ten gram of wet land soil is weighed and transferred the same into  $\frac{100}{10}$  ml sterile water blank to get  $1/10$  ( $10^{-1}$ ) dilution. The mixture is shaken well in a rotary shaker for 5-10 minutes. Ten ml of this suspension is transferred to 90 ml water blank to obtain  $1/100$  ( $10^{-2}$ ) dilution and this procedure is repeated to get the dilution series up to  $1/100,000$  ( $10^{-5}$ ).
2. Ten tubes containing medium 1 are inoculated with 1.0 ml portions of the soil suspension for each of three dilutions. (A reasonable series of dilutions to use is  $1/1000$ ,  $1/10000$ ,  $1/100000$  unless it is known that algae are unusually abundant or scarce in the soil tested).
3. Similarly, 10 tubes containing medium 2 are inoculated with 1.0 ml portions of each solution from  $1/100$  through  $1/10000$  so as to obtain the blue-green algae capable of fixing atmospheric nitrogen.
4. After inoculation, all the tubes are exposed to diffuse light in a green house or window and maintained at a temperature of  $25^{\circ}C$ . The tubes are examined for the growth of algae once in 7 days interval and final observations are made in about 30 days. (In these media, it is reported that algal growth becomes visible after incubation for 10-14 days).
5. The above steps (1 to 4) are repeated for the other soils samples also.

**EXERCISE - 10 & 11**  
**STAINING OF BACTERIA**

Bacteria being colourless (hyaline), it is rather difficult to observe their morphological features (size, shape, cell grouping, presence of flagella etc.) without staining directly under microscope. Therefore, bacterial cells needs to be stained with suitable dyes. The method of bacterial staining was introduced by Weiger (1875).

**Methods of staining :**

1. Simple staining
2. Differential staining – e. g. Gram staining

**1. Simple staining of bacteria :**

**Material :** Bacterial culture, methylene blue or carbol fuchsin, slides, spirit lamp, glass wash bottle, muslin cloth, microscope, slide holder etc.

**Procedure:**

- 1) Clean the slide with detergent powder to wash off greasy surface and air dry.
- 2) Sterilize the slide over the flame of spirit lamp from both the slides.
- 3) **Preparation of smear :** A little drop of bacterial suspension is mixed in distilled water and placed on the slide, spread it uniformly and thinly with a glass rod or needle to form a very thin film of smear.
- 4) **Drying:** Allow the smear to air dry. Do not dry on flame as it forms clusters on flame.
- 5) **Fixing:** Warm the slide slightly by passing over flame of spirit lamp for two to three times to fix the bacteria on the slide.
- 6) **Staining:** Place two or three drops of any simple stain over the smear and allow to react for specific time (Methylene blue 1 to 1  $\frac{1}{2}$  minutes, carbol fuchsin –5 to 10 seconds).
- 7) **Washing :** Wash the slide under gentle flow of water, wipe out the lower surface and dry the upper surface in air.
- 8) **Mounting :** Observe the slide under low power objective lens of microscope, and then under high power and oil immersion objective.

**Composition of Methylene blue (aqueous 3%)**

Methylene blue - 0.30g.

Ethanol (95%) - 30.0ml

Distilled water - 100ml.

Dissolve methylene blue in ethanol and then mix in distilled water.

## 2. Gram's staining of bacteria :

**Principle:** The bacteria are first treated with a main stain, then subjected to decolorizing agent. Later on they are counterstained with other dye. The bacteria which retain the colour of the main stain are called as 'Gram positive', whereas those loose the colour of the main stain during decolorization and take up the colour of counter stain are called as a 'Gram Negative' bacteria.

The stain was first used by 'Christien Gram' in 1984 to demonstrate the proportion of bacteria in the diseased tissue. There are different modifications of gram staining using the same principle.

**Materials :** Glass slide, 24 hrs. fresh bacterial culture, Gram A and Gram B solution, Gram's Iodine solution, 50% Acetone-Alcohol soln. Basic fuschin, spirit lamp, glass rod, wash bottle, muslin cloth, slide holder, microscope.

### Procedure: Kopeloff and Beerman's Method.

- 1) Prepare a thin smear of bacterial suspension on clean slide, dry in air and then fix by passing the slide 2-3 times over flame of spirit lamp.
- 2) Cover the smear with gram 'A' and Gram 'B' solution in 3:1 proportion (6 drops of Gram 'A' and 2 drops of Gram 'B') and allow it to react for 2 minutes. Drain off excess stain, wash under gentle flow of water and air dry.
- 3) Treat with iodine solution (Mordant) which helps in fixing the colour of main stain. It is done by dipping the slide in a jar containing Iodine solution for 1 minutes. Again wash the slide as usual and air dry.
- 4) Decolourization : Pass the slide serially through three jars containing 50% acetone-alcohol solution, wash the slide as usual and air dry.
- 5) Counter stain with carbol fuchsin or safranin for 30 seconds.
- 6) Wash the slide, air dry and examine under microscope.

### Results:

If the bacteria retain colour of main stain and appears violet or blue, they are 'Gram +ve' and if they take up the colour of counter stain showing red colour they are 'Gram -ve'.

### Definitions:

1. **Mordant** : It is a chemical agent which helps in fixing the colour of the main stain e.g. Iodine solution.
2. **Decolourising agent**: It is a chemical which decolourises (removes) the colour of the main and thus the colour is lost.
3. **Counter stain** : It is stain used for staining after the main stain is decolourised..

### *Composition of stains*

i) Main stain (A)	:	Crystal violet	- 1.0 gm.
	:	Distilled water	- 100 ml.
ii) Gram -B	:	Sodium carbonate	- 1.0g.
	:	Distilled water	- 20 ml.
III) Counter Stain			
i) Basic fuchsin/Safranin	:	1.0g.	
ii) Distilled water	:	100 ml.	
IV) Mordant			
i) Iodine crystals	:	2 g.	
ii) NaOH (N/10)	:	10ml.	
		Make volume upto 100ml.	

### **Examples of Gram +ve and Gram -ve bacteria**

#### Gram +ve

*Bacillus subtilis*  
*Bacillus anthracis*  
*Lactobacillus bulgaricus*  
*Mycobacterium tuberculosis*  
*Streptomyces scabies*  
*Corynebacterum diphtheriae*  
*Clostridium tetanae*  
*Diplococcus pneumoniae*  
*Mycobacterium leprae*  
*Streptococcus lactis*

#### Gram -ve

*Escherichia coli*  
*Azotobacter chroococcum*  
*Rhizobium leguminosarum*  
*Xanthomonas citri*  
*X. malvacearum*  
*Pseudomonas soalanacearum*  
*Erwinia amylovora*

## EXERCISE - 12

### ISOLATION OF PHOSPHATE AND SILICON SOLUBILIZING MICROBES

#### 1. Isolation of phosphate solubilizing microbes :

Fixation of added phosphorus through chemical fertilizers in soil poses the problem of availability of this essential element to crops and crops suffer from P deficiencies. Some of the microorganisms present in soil solubilize the fixed form of phosphate and make it available to crops. Phosphate solubilizing microorganisms can be isolated from soil using Pikovskaya's medium containing tricalcium phosphate. On plating aliquot of soil dilution, the 'P' solubilizing microorganisms show clear zones of 'P' solubilization around the colony. Such colonies are further sub-cultured and pure cultures of 'P' solubilizing microorganisms can be maintained on slants of Pikovskaya's medium.

#### Materials

Rhizosphere soil of legume crop, 9 ml sterile water blanks, sterile Petri plates, pipettes, inoculation needle, incubator, test tubes, Pikovskaya's medium.

#### Composition of Pikovskaya's Medium

Glucose	-	10.0 g
$(\text{NH}_4)_2 \text{SO}_4$	-	0.5 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	-	0.1 g
Yeast Extract	-	0.5 g
$\text{Ca}_3(\text{PO}_4)_2$	-	5.0 g
KCl	-	0.2 g
$\text{MnSO}_4$	-	Trace
$\text{FeSO}_4$	-	Trace
Agar	-	15 g
Distilled water	-	1000 ml

#### Procedure

1. Prepare 10-fold dilutions serially up to  $10^{-6}$
2. Transfer 1 ml each of  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$  and  $10^{-5}$  dilution aliquots in quadruplicate plates, separately.
3. Pour about 15 ml molten and cooled Pikovskaya's medium into the above plates and mix the soil suspension by gently rotating the plates and allow the medium to solidify.
4. Incubate the plates at room temperature for 3 to 4 days.
5. Observe for the growth of microbial colonies showing clear zone around them.

## *2. Isolation of silicon solubilizing microbes :*

The silica solubilizing thermophilic fungi were isolated from the soils of silica rich environments by serial dilution and plate technique using malt extract agar medium or silica peptone agar medium. One gram of soil sample was vortexed for 5 minutes with 99 ml of sterilized distilled water to make uniform suspension. Heavy particles were allowed to settle and clear supernatant was used for serial dilution. One ml of serially diluted sample was pour plated on malt extract agar medium (MEA) and silica peptone agar medium (SPA), each containing chloramphenicol (50 mg /lit) and incubated at  $50\pm 2^{\circ}\text{C}$ . The isolated colonies were transferred thrice on fresh agar plates to purify the cultures. The isolates were maintained on malt extract medium and silica peptone agar (SPA) medium by monthly transfers.

### **Composition silica peptone agar (SPA) medium**

Silica	-	10.0 g
Peptone	-	2.0 g
Methyl red	-	0.1%
Agar-agar	-	20.0 g
Distilled water	-	1000 ml

Change in colour the medium from yellow/orange to pink/light pink indicates the conversion of silica to silicic acid and hence, silica solubilizing potential of fungal cultures is confirmed.