

# **A practical manual on Agriculture Microbiology**

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## **Experiment – 1 Introduction to microbiology laboratory and its equipments.**

Following are the instrument:

### **1. Hot Air Oven for Sterilization:**

It is used for sterilization of glassware's, such as test tubes, pipettes and petri dishes. Such dry sterilization is done only for glassware's. Liquid substances, such as prepared media and saline solutions cannot be sterilized in oven, as they lose water due to evaporation. The glassware's are sterilized at 180°C for 3 hours. An oven has a thermostat-control, using which the required constant temperature can be obtained by trial and error. The thermostat dial reading is approximate and the exact temperature is read by introducing a thermometer into the oven or on a built-in L-shaped thermometer. In a modern oven there is a digital temperature display and automatic temperature controller to set the desired temperature easily. Time is set by a digital timer. After loading the glassware's, the door is closed and oven switched on.

The required temperature is set. After the oven attains the set temperature, the required time of sterilization is set on the timer. The oven switches off automatically after the set time. The oven is opened, only after its temperature comes down near to room temperature. Otherwise, if the door is opened, while the inside of the oven is still very hot, cold air may rush in and crack the glassware's.

### **2. Drying Oven:**

For preparation of certain reagents, the glassware's, after proper cleaning and rinsing with distilled water, are required to be dried. They are dried inside the drying oven at 100°C till the glassware's dry up completely.

### **3. Autoclave:**

Autoclave is the nucleus of a microbiology laboratory. It is used not only to sterilize liquid substances such as prepared media and saline (diluent) solutions, but also to sterilize glassware's, when required.

It has the same working principle as a domestic pressure cooker. The maximum temperature that can be obtained by boiling water in an open container is 100°C (boiling point of water).

This temperature is sufficient to kill only the non-spore formers, but it is difficult to kill the spore-forming bacteria at this temperature, as they escape by forming heat resistant spores. It takes very long time to kill the spores at this temperature.

On the other hand, when water is boiled in a closed container, due to increased pressure inside it, the boiling point elevates and steam temperature much beyond 100°C can be obtained. This high temperature is required to kill all the bacteria including the heat resistant spore-formers. Steam temperature increases with increase in steam pressure (Table 3.1).

In operating a standard vertical autoclave, sufficient water is poured into it. If water is too less, the bottom of the autoclave gets dried during heating and further heating damages it.

If it has in-built water heating element, water level should be maintained above the element. On the other hand, if there is too much water, it takes long time to reach the required temperature.

The materials to be sterilized are covered with craft paper and arranged on an aluminium or wooden frame kept on the bottom of the autoclave, otherwise if the materials remain half-submerged or floating, they tumble during boiling and water may enter. The autoclave is closed perfectly airtight only keeping the steam release valve open.

Then, it is heated over flame or by the in-built heating element. Air inside the autoclave should be allowed to escape completely through this valve. When water vapour is seen to escape through the valve, it is closed.

Temperature and pressure inside goes on increasing. The pressure increase is observed on the pressure dial. Usually sterilization is done at 121 °C (a pressure of 15 pounds per square inch i.e. 15 psi) for 15 minutes. The required time is considered from the point, when the required temperature-pressure is attained.

Once required temperature-pressure is attained, it is maintained by controlling the heating source. After the specified time (15 minutes), heating is discontinued and steam release valve slightly opened. If fully opened immediately, due to sudden fall in pressure, liquids may spill out from the containers.

Gradually, the steam release is opened more and more, so as to allow all steam to escape. The autoclave is opened only after the pressure drops back to normal atmospheric pressure (0 psi). The autoclave should never be opened, when there is still pressure inside. The hot sterilized materials are removed by holding them with a piece of clean cloth or asbestos- coated hand gloves.

In case of a steam-jacketed horizontal autoclave, a boiler produces the steam. It is released at a designated pressure, into the outer chamber (jacket). Air is allowed to escape and then its steam release valve is closed.

The hot jacket heats the inner chamber, thereby heating the materials to be sterilised. This prevents condensation of steam on the materials. Now, steam under pressure is released from the jacket into the inner chamber and air is allowed to escape from it.

Then, its steam release valve is closed. The steam under pressure in the inner chamber reaches temperatures in excess of 100°C, which can sterilise the materials kept inside it. The autoclave also has automatic shutting system i.e. unless temperature and pressure comes down near to room conditions, the door cannot be opened.

Besides the pressure dial, it also has separate temperature dial to indicate the temperature inside the inner chamber. Moreover, the autoclave maintains the temperature and pressure automatically and switches off after the set time of sterilization.

#### 4. Microbiological Incubator:

Profuse growth of microbes is obtained in the laboratory by growing them at suitable temperatures. This is done by inoculating the desired microbe into a suitable culture medium and then incubating it at the temperature optimum for its growth.

Incubation is done in an incubator (Figure 3.7), which maintains a constant temperature specifically suitable for the growth of a specific microbe. As most of the microbes pathogenic to man grow profusely at body temperature of normal human being (i.e. 37°C), the usual temperature of incubation is 37°C.

The incubator has a thermostat, which maintains a constant temperature, set according to requirement. The temperature reading on the thermostat is approximate. Accurate temperature can be seen on the thermometer fixed on the incubator. Exact temperature, as per requirement, is set by rotating the thermostat knob by trial and error and noting the temperature on the thermometer.

Most of the modern incubators (Figure 3.8) are programmable, which do not need trial and error temperature setting. Here, the operator sets the desired temperature and the required period of time.

The incubator automatically maintains it accordingly. Moisture is supplied by placing a beaker of water in the incubator during the growth period. A moist environment retards the dehydration of the media and thereby, avoids spurious experimental results.

#### 5. BOD Incubator (Low Temperature Incubator):

Some microbes are to be grown at lower temperatures for specific purposes. The BOD low temperature incubator (Figure 3.9), which can maintain temperatures from 50°C to as low as 2-3°C is used for incubation in such cases.

The constant desired temperature is set by rotating the knob of the thermostat. Rotation of the thermostat knob moves a needle on a dial showing approximate temperature. Exact required temperature is obtained, by rotating the knob finely by trial and error and noting the temperature on the thermometer fixed on the incubator.

Most of the modern BOD incubators (Figure 3.10) are programmable, which do not need trial and error temperature setting. Here, the operator sets the desired temperature and the required period of time. The incubator automatically maintains it accordingly.

**6. Fridge (Refrigerator):**

It serves as a repository for thermo labile chemicals, solutions, antibiotics, serums and biochemical reagents at cooler temperatures and even at sub-zero temperatures (at less than 0°C). Stock cultures of bacteria are also stored in it between sub-culturing periods. It is also used for the storage of sterilized media, so as to prevent their dehydration.

**7. Deep-fridge:**

It is used to store chemicals and preserve samples at very low sub-zero temperatures.

**8. Electronic Top-pan Balance:**

It is used for weighing large quantities of media and other chemicals, where precise weighing is not of much importance.

**9. Electronic Analytical Balance:**

It is used to weigh small quantities of chemicals and samples precisely and quickly.

**10. Double-pan Analytical Balance:**

It is used to weigh chemicals and samples precisely. Weighing takes more time, for which it is used in emergency only.

**11. Distilled Water Plant:**

Water is used in the preparation of media and reagents. If the media are prepared using tap water, the chemical impurities present in it may interfere with the growth of the microorganisms in the media. Moreover, the higher is the bacteria content of the media, the longer is the time required for their sterilization and greater is the chance of survival of some bacteria.

Distilled water, though not bacteria-free, contains less number of bacteria. That is why; it is preferred in the preparation of microbiological media. It is also used in the preparation of reagents, because the chemical impurities present in tap water may interfere with the proper functioning of the reagent chemicals.

As manufacture of distilled water by Liebig condenser is a time-taking process, in most laboratories, it is prepared by 'distilled water plants'. Usually a distilled water plant is made of steel or brass. It is also called distilled water still.

It has one inlet to be connected to the water tap and two outlets, one for distilled water to drop into a container and the other for the flow out of hot cooling water into the sink. The still is installed on the wall. It is heated by in-built electric heating elements (immersion heater).

The still works efficiently, when the water in-flow is so adjusted that, the temperature of the cooling water flowing out from the still into the sink is neither too high nor too low i.e., warm water should flow out. The distilled water may contain traces of metals corroded from the steel or brass container.

To get metal-free distilled water, glass distillation apparatus is used and still better is quartz distillation apparatus. However, for a microbiology laboratory, a steel or glass distillation apparatus is sufficient. For precision analyses, double- or triple- distilled water is used.

#### **12. Ultrapure Water Purification System:**

For precision analytical works, now-a-days, instead of using double- or triple-distilled water, micro-filtered water is used. In case of distilled water, there is chance that, few volatile substances present in the water get volatilized during heating of the water and subsequently get condensed into the distilled water collected.

Thus, there may be traces of such substances in the distilled water. To overcome this, ultrapure water is used. Here, water is allowed to pass through very fine microscopic pores, which retain the microscopic suspended particle including the microbes.

Then, the water passes through two columns of ion exchange resins. The anion exchange resin adsorbs the cations present in the water, while the cation exchange resin adsorbs the anions. The water that comes out is extremely pure.

#### **13. Homogeniser:**

For microbiological analysis, liquid samples are directly used, whereas solid samples have to be mixed thoroughly with a diluents (usually physiological saline), so as to get a homogenous suspension of bacteria. This suspension is assumed to contain bacteria homogeneously.

The mixing of solid samples and diluents is done by a homogenizer, in which a motor rotates an impeller with sharp blades at high speed inside the closed homogenizer cup containing the sample and the diluents. It has a speed regulator for controlling the speed of rotation of the impeller.

In some laboratories mixing is done manually by sterilized pestle and mortar. In modern laboratories, a disposable bag is used, inside which the solid sample and liquid diluents are put aseptically and mixed mechanically by peristaltic action of a machine on the bag. This machine is called stomacher.

#### **14. pH Meter:**

A pH meter is an instrument for determining the pH of liquid media, liquid samples and buffers. It has a glass pH electrode. When not in use, it should be kept half immersed in water contained in a small beaker and preferably be covered by a bell jar to avoid dust accumulation in the water and loss of water through evaporation.

Before use, the meter is calibrated using two standard buffers of known pH. Usually buffers of pH 4.0, 7.0 and 9.2 are available commercially. The instrument is switched on and left for 30 minutes to warm up. The temperature calibration knob is rotated to the temperature of the solutions whose pH is to be measured.

Then, the electrode is dipped into the buffer (pH 7.0). If the reading is not 7.00, the pH calibration knob is rotated till the reading is 7.00. Then, the electrode is dipped in another buffer (pH 4.0 or 9.2).

If the reading is same as the pH of the buffer used, the instrument is working properly. Otherwise, the electrode is activated by dipping in 0.1 N HCl for 24 hours. After calibration, the pH of samples is determined by dipping the electrode into them and noting the reading.

Every time, before dipping into any solution, the electrode should be rinsed with distilled water. The samples should not contain any suspended sticky materials, which may form a coating on the tip of the electrode and reduce its sensitivity.

The old model pH meters have double electrodes (one of them acting as reference electrode), while new models have single combined electrode. Moreover, to overcome the problem of temperature correction, now pH meters with automatic temperature correction are available.

Here, another 'temperature electrode' is also put into the solution along with the pH electrode, which measures the temperature of the solution and automatically corrects the influence of temperature variations.

Sophisticated pH meters have single gel electrode. Such electrodes have very little chance of breakage, as they are almost completely enclosed in a hard plastic casing except at the tip. The tip has both pH and temperature sensors.

Moreover, they are easy to maintain, as they do not require constant dipping in distilled water, because the electrode tip is closed with a plastic cap containing saturated solution of potassium chloride, when not in use. However, in preparation of microbiological media, pH is determined by narrow-range pH papers and is adjusted to the required pH by adding acids or alkalis as required.

#### **15. Hot Plate:**

Hot plate is used to heat chemicals and reagents. The hot plate is made of an iron plate, which gets heated by an electric heating element from below. The required degree of heating is obtained by a regulator.

#### **16. Shaking Water Bath:**

Sometimes, heating at very precise temperatures is required. Such precise temperatures cannot be obtained in an incubator or oven, in which temperature fluctuates, though slightly. However, precise temperatures can be maintained in a water bath, which provides a stable temperature.

A water bath consists of a container containing water, which is heated by electric heating elements. The required water temperature is obtained by increasing or decreasing the rate of heating by rotating the thermostat by trial and error.

In a shaking water bath, the substance is heated at the required temperature and at the same time, it is shaken constantly. Shaking is done by a motor, which rotates and moves the containers to and fro in each

rotation. The rate of shaking is again controlled by a regulator. Shaking agitates the substance and enhances the rate of the process.

Most modern water baths are programmable and do not need trial and error temperature setting. A desired water temperature can be maintained over a desired period of time by programming accordingly. It is used for cultivation of bacteria in broth medium at a specific temperature.

#### **17. Quebec Colony Counter:**

In enumeration of bacteria in samples, it is assumed that a single bacterium gives rise to a single visible colony, when grown on a plate of solidified nutrient medium. Thus, by counting the number of colonies, the number of bacteria in a sample can be estimated.

Sometimes, colonies are very small and too much crowded making it difficult to count. Counting becomes easy, when a mechanical hand counter, called Quebec colony counter (Figure 3.11), is used. It divides the plate into several square divisions and the colonies are magnified 1.5 times by a magnifying glass, which makes counting easy.

#### **18. Electronic Colony Counter:**

Electronic colony counter is of two types:

- (1) Hand-held electronic colony counter and
- (2) Table-top electronic colony counter.

The hand-held electronic colony counter is a pen-style colony counter with an inking felt-tip marker. For counting of colonies of bacteria grown in a petri dish, it is kept in an inverted position, so that the colonies are visible through the bottom surface of the petri dish.

The colonies are marked by touching the glass surface of the petri dish with the felt-tip of the colony counter. Thus, each colony is marked by a dot made by the ink of the felt-tip on the bottom surface of the petri dish. In a single motion, the electronic colony counter marks, counts and confirms with a beep sound.

The cumulative count of colonies is displayed on a four-digit LED display. In case of table-top electronic colony counter, the petri dish containing the colonies of bacteria is placed on an illuminated stage and the count bar is depressed. The precise number of colonies is instantly displayed on a digital read out.

#### **19. Magnetic Stirrer:**

In the preparation of solutions, certain chemicals require stirring for long time, to be dissolved in certain solvents. Magnetic stirrer is used to dissolve such substances easily and quickly. A small teflon-coated magnet, called 'stirring bar', is put into a container containing the solvent and the solute.

Then, the container is placed on the platform of the magnetic stirrer, below which a magnet rotates at high speed by a motor. Attracted by the rotating magnet, the teflon-coated magnet rotates inside the container and stirs the contents. Now, the solute dissolves quickly.

The teflon coating prevents the magnet from reacting with the solution, which comes in contact with it. After complete dissolution, the teflon-coated magnet is removed from the solution by mean of a long retriever, called 'stirring bar retriever'.

#### **20. Sonicator:**

It is used to rupture cells using high frequency waves.

#### **21. Vortex Mixer:**

It is an instrument used for thorough mixing of liquids in test tubes. It has a rotor, whose speed can be controlled. On the tip of the rotor is a foam-rubber top. When the bottom of a test tube is pressed upon this foam-rubber top, the rotor starts rotating, thereby rotating the bottom of the test tube at high speed.

Due to centripetal force, the solution gets mixed thoroughly. It is particularly helpful during serial dilution in enumeration of bacteria, which needs homogenous suspension of bacteria cells.

#### **22. Laminar Flow Chamber:**

It is a chamber (Figure 3.12) used for aseptic transfer of sterilized materials, as well as for inoculation of microbes. Dust particles floating in the air harbour microbes. These microbe-laden dust particles may enter into the sterilized media and contaminate them, when they are opened for short periods of time during inoculation of microbe or transfer from one container to another.

To overcome this, when inoculation is done in open air, the air of the small inoculating area is sterilized by the flame of a bunsen burner. The heated air becomes light and moves upwards, thereby preventing the dust particles from falling on the media during the short opening process.

To further reduce the chance of contamination by the microbe-laden air, a laminar flow chamber is used. It is a glass-fitted cuboidal chamber. An air blower blows air from the surrounding and passes it through a HEPA filter (High Efficiency Particulate Air filter), so as to make it dust free (microbe-free).

This microbe-free air passes through the chamber in a laminar manner and comes out from the chamber through the open front door. This laminar flow of microbe-free air from the chamber to outside through the open door prevents the outside air from entering into the chamber.

Thus, the chamber does not get contaminated with the microbes present in the outside air, though the door is kept opened during inoculation or transfer of media. An UV lamp fitted inside the chamber sterilizes the chamber before operation.

It has a stainless steel platform with provision for gas pipe connection for a bunsen burner. Before use, the platform is cleaned and disinfected with lysol, the bunsen burner is connected and then the glass door is closed.

The UV light is switched on for 10 minutes to sterilise the environment inside the chamber and then switched off. The glass door should never be opened when the UV light is on, because UV light has detrimental effect on skin and vision. The blower is switched on and then the glass door is opened.

Now, the bunsen burner is lighted and media transfer or inoculation is carried out in the chamber aseptically. If extremely hazardous microbes are to be handled, a laminar flow chamber with gloves projecting into the chamber from the front glass door is used, as inoculation has to be done keeping the front door closed.

### **23. Electronic Cell Counter:**

It is used to directly count the number of bacteria in a given liquid sample. An example of electronic cell counter is the 'Coulter counter'. In this equipment, a suspension of bacteria cells is allowed to pass through a minute orifice, across which an electric current flows.

The resistance at the orifice is electronically recorded. When a cell passes through the orifice, being non-conductor, it increases resistance momentarily. The number of times resistance increases momentarily is recorded electronically, which indicates the number of bacteria present in the liquid sample.

### **24. Membrane Filtration Apparatus:**

Certain substances like urea disintegrate and lose their original properties, if sterilized by heat. Such substances are sterilized by membrane filtration apparatus. In this apparatus, the solution of the substance to be sterilized is filtered through a membrane filter, which does not allow bacteria cells to pass down. Filtration is done under suction pressure to increase the rate of filtration (Figure 2.19, page 30).

### **25. Microscopes:**

Different types of microscopes are used for visual observation of morphology, motility, staining and fluorescent reactions of bacteria.

### **26. Computers:**

Computers are generally used for analysis of results. They are also used for identification of bacteria easily within few hours. Otherwise, identification of bacteria is a tedious process and takes days together to identify one bacteria species.

The computers used for identification of bacteria are Apple II, IBM PC and TRS-80 and their modern variants. Each research personnel of the laboratory should be provided with a computer, along with internet facility.

### **27. Spectrophotometer:**

It is an instrument for measuring the differences in color intensities of solutions. A beam of light of a particular wavelength is passed through the test solution and the amount of light absorbed (or transmitted) is measured electronically.

A simple visible spectrophotometer can pass light with wavelengths within visible range, whereas a UV-cum-visible spectrophotometer can pass light with wavelengths in ultraviolet as well as in visible range. In microbiology lab, it is used for direct counting of bacteria in suspension as well as for other purposes.

### **28. Electrical Devices:**

A fluctuation of electric voltage in the laboratory is one of the most important reasons, which reduces the longevity of the equipments and sometimes damage them. Therefore, all the voltage-sensitive equipments should be provided with voltage protection devices like stabilizers, servo stabilizers or constant voltage transformers (CVT) as per the recommendations of the manufacturers of the equipments.

Computers, balances and some sophisticated equipments should be connected through uninterrupted power supply (UPS), as any breakdown in the electric power supply during their operation may severely damage some of their sensitive components.

The laboratory should have a high capacity generator to supply electric current to the whole laboratory in case of power failure. This is because, power failure not only brings the activities of the laboratory to a standstill, it also brings about undesirable irreversible changes in the samples stored in the deep-fridges and refrigerators.

## **Experiment 2 Microscope- parts, principles of microscopy, resolving power and numerical aperture.**

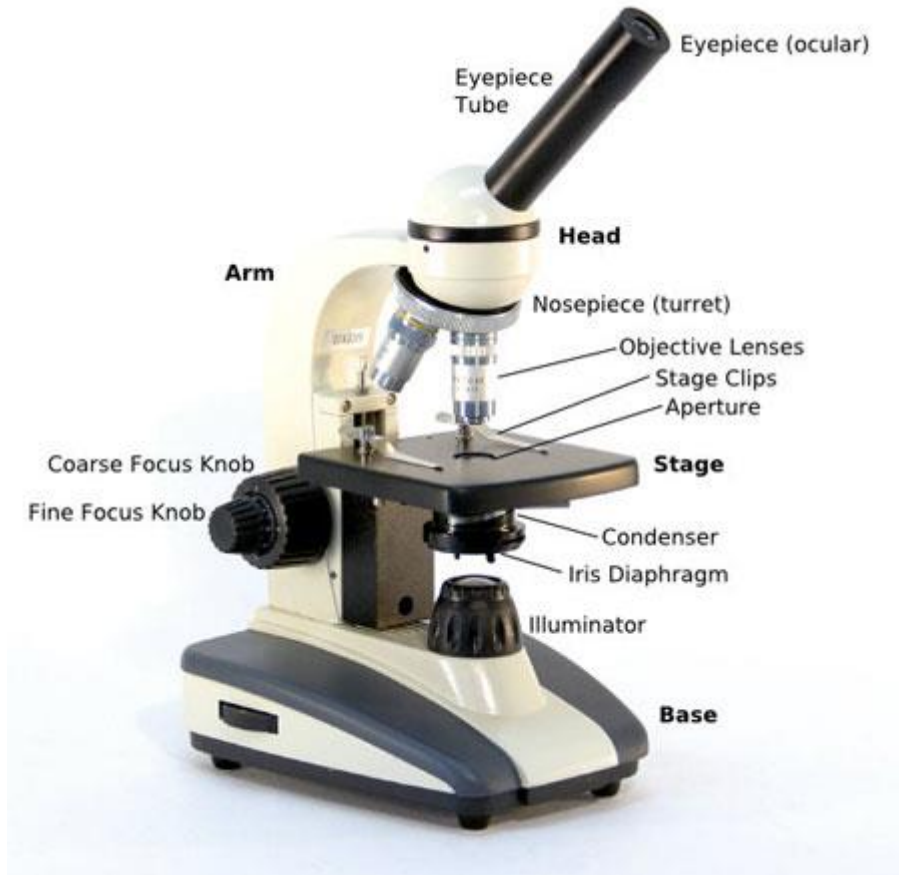
A high power or compound microscope achieves higher levels of magnification than a stereo or low power microscope. It is used to view smaller specimens such as cell structures which cannot be seen at lower levels of magnification. Essentially, a compound microscope consists of structural and optical components. However, within these two basic systems, there are some essential components that every microscopist should know and understand. These key microscope parts are illustrated and explained below.

### **STRUCTURAL COMPONENTS**

The three basic, structural components of a compound microscope are the head, base and arm.

- Head/Body houses the optical parts in the upper part of the microscope
- Base of the microscope supports the microscope and houses the illuminator
- Arm connects to the base and supports the microscope head. It is also used to carry the microscope.

When carrying a compound microscope always take care to lift it by both the arm and base, simultaneously.



## OPTICAL COMPONENTS

There are two optical systems in a compound microscope: Eyepiece Lenses and Objective Lenses:

Eyepiece or Ocular is what you look through at the top of the microscope. Typically, standard eyepieces have a magnifying power of 10x. Optional eyepieces of varying powers are available, typically from 5x-30x.

Eyepiece Tube holds the eyepieces in place above the objective lens. Binocular microscope heads typically incorporate a diopter adjustment ring that allows for the possible inconsistencies of our eyesight in one or both eyes. The monocular (single eye usage) microscope does not need a diopter. Binocular microscopes also swivel (Interpupillary Adjustment) to allow for different distances between the eyes of different individuals.

Objective Lenses are the primary optical lenses on a microscope. They range from 4x-100x and typically, include, three, four or five on lens on most microscopes. Objectives can be forward or rear-facing.

Nosepiece houses the objectives. The objectives are exposed and are mounted on a rotating turret so that different objectives can be conveniently selected. Standard objectives include 4x, 10x, 40x and 100x although different power objectives are available.

Coarse and Fine Focus knobs are used to focus the microscope. Increasingly, they are coaxial knobs - that is to say they are built on the same axis with the fine focus knob on the outside. Coaxial focus knobs are more convenient since the viewer does not have to grope for a different knob.

Stage is where the specimen to be viewed is placed. A mechanical stage is used when working at higher magnifications where delicate movements of the specimen slide are required.

Stage Clips are used when there is no mechanical stage. The viewer is required to move the slide manually to view different sections of the specimen.

Aperture is the hole in the stage through which the base (transmitted) light reaches the stage.

Illuminator is the light source for a microscope, typically located in the base of the microscope. Most light microscopes use low voltage, halogen bulbs with continuous variable lighting control located within the base.

Condenser is used to collect and focus the light from the illuminator on to the specimen. It is located under the stage often in conjunction with an iris diaphragm.

Iris Diaphragm controls the amount of light reaching the specimen. It is located above the condenser and below the stage. Most high quality microscopes include an Abbe condenser with an iris diaphragm. Combined, they control both the focus and quantity of light applied to the specimen.

Condenser Focus Knob moves the condenser up or down to control the lighting focus on the specimen.

### **Experiment 3 Methods of sterilization**

**Sterilization:**-It is defined as the process where all the living microorganisms, including bacterial spores are killed. Sterilization can be achieved by physical, chemical and physiochemical means. Chemicals used as sterilizing agents are called chemosterilants.

**Physical Methods of Sterilization:**

**Sunlight:** The microbicidal activity of sunlight is mainly due to the presence of ultra violet rays in it. It is responsible for spontaneous sterilization in natural conditions. In tropical countries, the sunlight is more effective in killing germs due to combination of ultraviolet rays and heat. By killing bacteria suspended in water, sunlight provides natural method of disinfection of water bodies such as tanks and lakes. Sunlight is not sporicidal, hence it does not sterilize.

**Heat:** Heat is considered to be most reliable method of sterilization of articles that can withstand heat. Heat acts by oxidative effects as well as denaturation and coagulation of proteins. Those articles that cannot withstand high temperatures can still be sterilized at lower temperature by prolonging the duration of exposure.

**Red heat:** Articles such as bacteriological loops, straight wires, tips of forceps and searing spatulas are sterilized by holding them in Bunsen flame till they become red hot. This is a simple method for effective sterilization of such articles, but is limited to those articles that can be heated to redness in flame

**Flaming:** This is a method of passing the article over a Bunsen flame, but not heating it to redness. Articles such as scalpels, mouth of test tubes, flasks, glass slides and cover slips are passed through the flame a few times. Even though most vegetative cells are killed, there is no guarantee that spores too would die on such short exposure. This method too is limited to those articles that can be exposed to flame. Cracking of the glassware may occur.

**Hot air oven:** This method was introduced by Louis Pasteur. Articles to be sterilized are exposed to high temperature (160°C) for duration of one hour in an electrically heated oven. Since air is poor conductor of heat, even distribution of heat throughout the chamber is achieved by a fan. The heat is transferred to the article by radiation, conduction and convection. The oven should be fitted with a thermostat control, temperature indicator, meshed shelves and must have adequate insulation.

**Articles sterilized:** Metallic instruments (like forceps, scalpels, scissors), glassware (such as Petri-dishes, pipettes, flasks, all-glass syringes), swabs, oils, grease, petroleum jelly and some pharmaceutical products.

Autoclave: Sterilization can be effectively achieved at a temperature above 100°C using an autoclave. Water boils at 100°C at atmospheric pressure, but if pressure is raised, the temperature at which the water boils also increases. In an autoclave the water is boiled in a closed chamber. As the pressure rises, the boiling point of water also raises. At a pressure of 15 lbs inside the autoclave, the temperature is said to be 121°C. Exposure of articles to this temperature for 15 minutes sterilizes them. To destroy the infective agents associated with spongiform encephalopathy (prions), higher temperatures or longer times are used; 135°C or 121°C for at least one hour are recommended.

### **FILTRATION:**

Filtration does not kill microbes, it separates them out. Membrane filters with pore sizes between 0.2-0.45 µm are commonly used to remove particles from solutions that can't be autoclaved. It is used to remove microbes from heat labile liquids such as serum, antibiotic solutions, sugar solutions, urea solution. Various applications of filtration include removing bacteria from ingredients of culture media, preparing suspensions of viruses and phages free of bacteria, measuring sizes of viruses, separating toxins from culture filtrates, counting bacteria, clarifying fluids and purifying hydrated fluid. Filtration is aided by using either positive or negative pressure using vacuum pumps. The older filters made of earthenware or asbestos are called depth filters.

## **Experiment 4 Nutrient Agar media and their preparation**

### **Nutrient Agar: Composition, Preparation and Uses**

Nutrient Agar is a general purpose, nutrient medium used for the cultivation of microbes supporting growth of a wide range of non-fastidious organisms. Nutrient agar is popular because it can grow a variety of types of bacteria and fungi, and contains many nutrients needed for the bacterial growth.

#### Composition of Nutrient Agar

- 0.5% Peptone

It is an enzymatic digest of animal protein. Peptone is the principal source of organic nitrogen for the growing bacteria.

- 0.3% beef extract/yeast extract

It is the water-soluble substances which aid in bacterial growth, such as vitamins, carbohydrates, organic nitrogen compounds and salts.

- 1.5% agar

It is the solidifying agent.

- 0.5% NaCl

The presence of sodium chloride in nutrient agar maintains a salt concentration in the medium that is similar to the cytoplasm of the microorganisms.

- Distilled water

Water is essential for the growth of and reproduction of micro-organisms and also provides the medium through which various nutrients can be transported.

- pH is adjusted to neutral (7.4) at 25 °C.

#### Preparation of Nutrient Agar

1. Suspend 28 g of nutrient agar powder in 1 litre of distilled water.
2. Heat this mixture while stirring to fully dissolve all components.
3. Autoclave the dissolved mixture at 121 degrees Celsius for 15 minutes.
4. Once the nutrient agar has been autoclaved, allow it to cool but not solidify.
5. Pour nutrient agar into each plate and leave plates on the sterile surface until the agar has solidified.
6. Replace the lid of each Petri dish and store the plates in a refrigerator.

## Uses of Nutrients Agar

1. It is frequently used for isolation and purification of cultures.

2. It can also be used as a means for producing the bacterial lawns needed for antibiotic sensitivity tests.

In actuality, antibiotic sensitivity testing is typically performed on media specially formulated for that purpose.

### **Experiment 5: Enumeration of microbial population in soil.**

Soil microorganisms live in thin films of water that surround soil particles. These tiny organisms include microflora (bacteria, fungi and actinomycetes) and microfauna (protozoa and nematodes). In terms of numbers and biological activity the microflora are dominant. Bacteria are small (about 1 - 10  $\mu\text{m}$ ) and occur in three general shapes rod (bacillus), spherical (coccus) and spiral (spirilla). Bacilli and cocci are more common in soil. Fungi are filamentous and much larger. The branched hyphae exhibit cell divisions and fungal mycelia (hyphal mass) are often macroscopic. Actinomycetes are also filamentous and branched but smaller.

### **Agar Plate Method for Microbial Count**

In this method, soil is dispersed in an agar medium so that individual microbial cells, spores or mycelial fragments develop into macroscopic colonies. The procedure involves successive dilutions of soil. Depending upon extent of dilution, plates may be filled with a huge number of colonies or very few. Enumeration of colony-forming units initially present in the soil is from plates in between these extremes. This method requires sterile technique to avoid introduction of extraneous microbes.

### **Procedure**

A homogenized, field-moist sample of topsoil and bottles containing 90 mL of sterilized water was taken to perform the experiment.

1. Add a 10 g sub-sample of topsoil to the bottle of sterilized water. Tightly cap and shake vigorously for 10 minutes to disperse the soil. This is the  $10^{-1}$  dilution.
2. Transfer 10 mL of the  $10^{-1}$  dilution to another bottle of sterilized water. Use a sterile pipette. Take sample from the middle. Tightly cap and shake to uniformly mix. This is the  $10^{-2}$  dilution.

3. Repeat step 2 using the  $10^{-2}$  dilution to make a  $10^{-3}$  dilution and proceed similarly, making  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$  and  $10^{-7}$  dilutions.
4. From the  $10^{-7}$  dilution, transfer 1 mL to each of 2 sterile petri dishes using a sterile 1 mL pipette. Make similar transfers from the  $10^{-6}$ ,  $10^{-5}$  and  $10^{-4}$  dilutions.
5. Into each seeded petri dish, pour enough sterile, melted agar to fill dish about 2/3 full. Immediately swirl it around to ensure good mixing of soil inoculant and agar.
6. After the agar has solidified, invert plates and incubate at  $35^{\circ}\text{C}$  for 1 week.
7. Next week, count the number of colonies on plates from the dilution that contains from 30 to 300 colonies. Don't count from those plates that contain colonies larger than 2 cm diameter. Multiply by dilution, take the average and correct to oven-dry moisture content of the soil. This gives the number of colonyforming units (CFUs) per gram of soil.

## **Experiment 6: Methods of isolation and purification of microbial cultures**

There are special techniques employed to obtain pure cultures of microorganisms. In few cases it is possible to secure pure culture by direct isolation or direct transfer. This can be done only in those situations in which pure culture occurs naturally. Kinds of specimens taken for culturing will depend on the nature and habitat of microbes.

Different pathogens can be isolated from body tissues and fluids such as blood, urine, sputum, pus, faces, spinal fluid, bile, pleural fluids, stomach fluids etc. In the blood stream of a patient suffering with typhoid fever, the bacteria *Salmonella typhosa* may be present.

A pure culture of this bacterium may be obtained by drawing blood sample using a sterilized hypodermic syringe and treating the blood with anticoagulant such as heparin and potassium oxalate. The presence of the anticoagulant prevents the pathogenic microbe from entrapping in fibrin clot. The sample of the blood may be inoculated into a suitable medium.

1. Streaking
2. Plating
3. Dilution
4. Enriched procedure, and
5. Single cell technique.

### **1. Streaking:**

This is most widely used method of isolation. The technique consists of pouring a suitable sterile medium into sterile petriplate and allowing the medium to solidify. By means of a sterile loope or straight needle or a sterile bent glass-rod a small amount of growth preferably from a broth culture or bacterial suspension is streaked back and forth across the surface of agar until about one third of the diameter of the plate has been covered.

The needle is then flamed and streaking is done at right angles to and across the first streak. This serves to drag bacteria out in a long line from the initial streak. When this streaking is completed the needle is again flamed and streaking is done at right angles to the second streak and parallel to the first.

**2. Plating:** It includes diluting of a mixture of microorganisms until only a few hundred bacteria are left in each millilitre of the suspension. A very small amount of the dilution is then placed in a sterile petriplate by means of a sterile loop or pipette. The melted agar medium is cooled to about 45°C and is poured into plate. The microorganism and agar are well mixed. When the agar is solidified the individual

bacterium will be held in place and will grow to a visible colony.

**3. Dilution:** This method is used for the microorganisms which cannot be easily isolated by streaking or plating method. Sometimes when several organisms are present in a mixture, with one organism predominating, the predominating form may be isolated by this method. For example, when raw milk is allowed to sour at room temperature it will, at the time of curding, have a mixture of microorganisms with high percentage of *Streptococcus lactis*.

If 1 ml of the sour milk is taken into a tube containing 9 ml. of sterile milk (in which no organisms are present) then 1 ml. of this mixture is transferred with a sterile pipette into a second tube of sterile milk and the procedure is repeated i.e. from second to third tube, third to fourth tube until a series of about 10 tubes are inoculated. By this serial dilution, the chances are that a pure culture of *S. lactis* will be obtained.

#### **4. Enrichment Procedure:**

This procedure involves the use of media and conditions of cultivation which favour the growth of the desired species. For example, when a man suffers with typhoid, the intestinal discharge posses small number of *Salmonella typhosa* when compared with *E. coli* and other forms.

It is almost impossible to isolate the typhoid organisms because they represent only a fraction of a per cent of the total microorganisms present. The media are therefore derived, which allow the rapid growth of the desired organisms, at the same time inhibiting the growth of other microorganisms.

#### **5. Single Technique:**

This is one of the most ideal and difficult method of securing pure culture. In this method a suspension of the pure culture is placed on the under-side of a sterile cover-glass mounted over a moist chamber on the stage of the microscope.

While looking through the microscope, a single cell is removed with the help of sterile micropipette and transferred to a small drop of sterile medium on a sterile cover-glass and is mounted on a sterile hanging drop slide, which is then incubated at suitable temperature. If the single cell germinates in this drop, few cells are transferred into a tube containing sterile culture medium which is placed in the incubator to obtain pure culture originated from single cell.

#### **Other methods:**

The isolation of anaerobic microorganisms is very difficult. There are certain special techniques by which these organisms are isolated.

Cultivation of Microorganisms:

For cultivating microbes in laboratory, we require culture media. The various mixtures of nutritive substances used for the laboratory cultivation of microorganisms are collectively known as culture media. The culture media serve as soil in which bacteria are planted for the purpose of study.

*Culture Media:*

Culture media must contain all the essential nutrients required by the organism for its growth and reproduction. A suitable source of energy, building materials and growth factors must be supplied in adequate amounts.

**So a culture medium must contain:**

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<b>Carbohydrate (Sugar)</b>	-	<b>source of hydrogen, carbon &amp; chemical-bond energy.</b>
<b>Nitrite ions or Ammonium ions</b>	-	<b>Source of nitrogen</b>
<b>Sodium / Magnesium sulphate</b>	-	<b>Minerals</b>
<b>Micronutrients</b>		
<b>Growth factors, Vitamins &amp; certain amino acids.</b>		

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Since microorganisms show a considerable variation in their nutritional requirements, no single medium is suitable for growth of all of them.

**The different types of culture media employed fall into three groups:**

**1. Defined or synthetic media:**

These are the media prepared from chemical compounds. They are highly purified and specific, an investigator working in another laboratory can duplicate them.

**2. Complex or non-synthetic media:**

Media that are prepared from ingredients that have not been precisely defined. It contains hydrolysed proteins and vitamin extracts. This type of medium cannot be duplicated by another worker in another laboratory. Peptone is usually produced by boiling beef, by the hydrolysis of its protein. Casein peptone and milk peptone are also used in complex media as the source of amino acids and nitrogen.

All liquid media, whether complex or synthetic may be converted to solid media by adding either gelatin (a protein) or agar-agar, (a complex polysaccharide) extracted from red marine algae. The use of agar has an advantage. The most bacteria are unable to hydrolyze this molecule into more simple components. Since gelatin is a liquid at room temperature, the use of agar allows the medium to remain in a solid form while microbes are growing on its surface.

### **3. Living cells:**

These are used for the cultivation of viruses. For example, fertilized eggs incubated for 8 to 12 days are able to support the growth of many viruses.

**In another classification culture media are grouped into following four types:**

#### **1. Natural media:**

Includes substances occurring in nature, as 1) Milk 2) Eggs 3) Blood 4) Extract of plant and animal tissues.

#### **2. Derived media:**

Includes known substances but the chemical composition of each is unknown. Examples are 1. Nutrient broth (prepared by boiling beef to extract nutrients and adding an amino acid-nitrogen source.) 2. Nutrient agar 3. Nutrient gelatin.

#### **3. Chemically defined media:**

Exact chemical composition known.

#### **4. Special media:**

Include combinations of the other three types of media.

**There are four categories of media used in laboratory:**

**They are:**

1. Enrichment
2. Selective
3. Differential and
4. Propagation.

### **1. Enrichment media:**

They are prepared with ingredients that will enhance the growth of certain microbes. Enrichment media encourage the growth of the suspected pathogen so that it will become the most pre-dominant type of microbe in the culture. Two types of enrichment media are blood agar and chocolate agar.

### **2. Selective media:**

They are prepared with ingredients that inhibit the growth of unwanted microbes which might be in the specimen. The inhibitor may be an antibiotic, salt or other chemical. Mixed culture of microbes originally grown in enrichment media may be inoculated into selective media to isolate the desired microbe.

### **3. Differential media:**

They are designed to differentiate among microbes. Different bacterial species may produce dissimilar colony colours when grown on differential agar. While in differential broth cultures, the media change colour. Differential media are used to confirm the identity of a microbe that has already been isolated by culturing in enrichment and selective media.

### **4. Propagation media:**

They are used to propagate, or keep microbes growing for a long time. Samples grown on these media may be taken for analysis. The most common propagation media are nutrient broth and agar.

#### *Preparation of Media:*

#### **There are three main steps in the preparation of media:**

- (a) Preparation as solutions of chemicals and adjusting the pH.
- (b) Dispensing the media, and
- (c) Sterilization.

A broth is prepared by dissolving the appropriate amount of the components in distilled water and pH is adjusted by the addition of either dilute NaOH or HCl. The broth is dispensed into clean rimless 'Pyrex' test tubes which are plugged with non-absorbant cotton wool plugs. The test tubes are placed in wire baskets which are covered with grease proof paper.

The media are sterilized by autoclaving at a temperature of 121 °C and a pressure of 151 b/in<sup>2</sup> for 15 minutes. But medium containing heat- sensitive substances are sterilized either by filtering the solution at room temperature, using bacteria-proof filter or by a process called Tyndallization.

In this method, the liquids are steamed for one hour a day on three consecutive days and the liquids are incubated at 25-30°C. During the first steaming, all the heat sensitive vegetative cells are killed, leaving only the spores. During the first incubation period, most of the spores germinate in to vegetative cells. These vegetative cells are killed by the second steam period.

In the second incubation period, the rest of the spores germinate into vegetative cells which are killed by the third steaming period. In this way, the liquids are sterilized without temperature rising above 100°C.

#### *Maintenance of Pure Culture:*

**After obtaining the pure culture of a particular microbe, it may be grown and maintained as a pure culture in different ways:**

1. The most common practice is to grow the culture on suitable medium until it reaches the stationary phase of growth, and then store in a refrigerator. If they are to be kept alive for a long period all culture must be transferred to a fresh sterile medium. Thus by successive transfer, a culture may be kept for an indefinite period.
2. A second method involves freezing of young culture and desiccating it under vacuum. The cells of a pure culture will remain viable for a long period of time if they are mixed with sterile blood serum, sterile skimmed milk, before freezing and drying. They dried cultures are kept in the sealed, evacuated tubes and are stored in cool places.
3. This method of maintaining pure culture is most suitable for spore forming species. The microorganisms are grown in pure culture in suitable media. A suspension of microorganisms is then transferred to cotton stoppered tubes of sterilized dry soil. The spores remain viable, though dormant, for long periods of time, in dry soil. The organism can be grown after a desired period, by transferring the soil into a suitable medium and incubating it under suitable temperature.

## **Experiment 7: Isolation of Rhizobium from Legume root nodules**

Rhizobium Medium is used for cultivation and isolation of Rhizobium species.

### **Composition\*\***

#### **Ingredients Gms / Litre**

Mannitol 10.000

Dipotassium phosphate 0.500

Magnesium sulphate 0.200

Yeast extract 1.000

Sodium chloride 0.100

Agar 20.000

Final pH ( at 25°C) 6.8±0.2

\*\*Formula adjusted, standardized to suit performance parameters

### **Directions**

Suspend 31.8 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and pour into sterile Petri plates.

### **Principle and Interpretation**

Rhizobium Medium is used in the large scale production of legumes and in their isolation from root nodules.

Rhizobium Medium is recommended for isolation and cultivation of mannitol-positive Rhizobium species. It is also useful for the maintenance of Rhizobium species by adding extra 1% mannitol to the medium as specified by the American Type Culture Collection (1).

The medium is well buffered for pH changes and osmotic changes by presence of phosphate and sodium chloride salts. Yeast extract provides nitrogenous nutrients. Mannitol is the energy source while magnesium sulphate provides essential ions. The inocula are transferred from agar slants into starter flasks containing Rhizobium Medium. After 4 days of growth, the culture from starter flasks is transferred into a small seed tank fermentor. At this stage, Rhizobium Medium is used for large scale production. Rhizobium may be isolated from the root system of the leguminous plant. The healthy, pinkish nodule on the tap root is carefully cut out. The nodule is surface sterilized for 5 minutes and then washed with solvents like ethanol etc. The nodule is then crushed with a sterile glass rod in a small aliquot of sterile water. Serial dilutions are subsequently made to get sparse and distinct colonies. The dilutions are plated on Rhizobium Medium and incubated for upto 4 days at 25-30°C (2).

### **Quality Control**

#### **Appearance**

Cream to yellow homogeneous free flowing powder

**Gelling**

Firm, comparable with 2.0% Agar gel.

**Colour and Clarity of prepared medium**

Yellow coloured clear to slightly opalescent gel forms in Petri plates

**Reaction**

Reaction of 3.18% w/v aqueous solution at 25°C. pH :  $6.8 \pm 0.2$

**pH**

6.60-7.00

**Cultural Response**

M408: Cultural characteristics observed after an incubation at 25-30°C for upto 4 days.

**Storage and Shelf Life**

Store below 30°C in tightly closed container and prepared medium at 2-8°C. Use before expiry period on the label.

## **Experiment 8: Isolation of Azotobacter from soil**

### **Azotobacter Broth (Mannitol) M1722**

Azotobacter Broth (Mannitol) is recommended for cultivation of mannitol positive *Azotobacter* species from soil.

#### **Composition\*\***

##### **Ingredients Gms / Litre**

Dipotassium phosphate 1.000

Magnesium sulphate 0.200

Sodium chloride 0.200

Ferrous sulphate TRACE

Soil extract 5.000

Mannitol 20.000

Final pH (at 25°C) 8.3±0.2

\*\*Formula adjusted, standardized to suit performance parameters

#### **Directions**

Suspend 26.4 grams in 1000 ml distilled water. Heat if necessary to dissolve the medium completely. Dispense as desired. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Slight precipitate may occur after autoclaving, however it will not interfere with growth performance nor interfere with the interpretation of results.

#### **Principle and Interpretation**

*Azotobacter* is a free-living nitrogen-fixing bacterium (3), which is used as a biofertilizer in the cultivation of most crops. *Azotobacter* is found on neutral to alkaline soils, in aquatic environments, in the plant rhizosphere and phyllosphere. *Azotobacter* species are gram-negative aerobic soil-dwelling bacteria and are usually motile, oval, or spherical bacteria, form thick-walled cysts, and may produce large quantities of capsular slime. They are typically polymorphic, i.e. of different sizes and shapes. Their size of the cells ranges from 2-10 µm long and 1-2 µm wide. Plant needs nitrogen for its growth and *Azotobacter* fixes atmospheric nitrogen non-symbiotically. Therefore, all plants, trees, vegetables, get benefited. Beyond *Azotobacter* is used as a model it has biotechnological applications like use for alginate production and for nitrogen production in batch fermentations. This medium contains necessary nutrients for growth of *Azotobacter* species. For cultivation of mannitol positive *Azotobacter* species from soil, Azotobacter broth (Mannitol) can be used (1). It is used for cultivation of mannitol positive *Azotobacter* species from soil. It can also be useful for maintenance of *Azotobacter* species by adding extra 1%

Mannitol to the medium containing agar i.e solid media as specified by the American Type Culture Collection (2).

**Quality Control**

**Appearance**

White to Cream homogeneous free flowing powder

**Colour and Clarity of Prepared medium**

Colourless to off-white coloured clear to slightly opalescent solution with slight precipitate forms intubes

**Reaction**

Reaction of 2.64% w/v aqueous solution at 25°C. pH : 8.3±0.2

**pH**

8.10-8.50

**Cultural Response**

Cultural characteristics observed after an incubation at 25-30°C for 24-48 hours.

**Storage and Shelf Life**

Store below 30°C in tightly closed container and the prepared medium at 2 - 8°C. Use before expiry date on the label.

## **Experiment 9: Isolation of Azospirillum from roots.**

### **Azospirillum Medium w/ 0.17% Agar (Twin Pack) M518**

#### **Intended use**

Azospirillum Medium with 0.17% Agar is used for the cultivation of *Azospirillum* species.

#### **Composition\*\***

##### **Ingredients Gms / Litre**

Part A -

Malic acid 5.000

Dipotassium hydrogen phosphate 0.500

Ferrous sulphate 0.500

Manganese sulphate 0.010

Magnesium sulphate 0.200

Sodium chloride 0.100

Bromo thymol blue 0.002

Sodium molybdate 0.002

Calcium chloride 0.020

Agar 1.750

Part B -

Potassium hydroxide 4.000

Final pH (at 25°C) 6.8±0.2

\*\*Formula adjusted, standardized to suit performance parameters

#### **Directions**

Suspend 8.08 grams of dehydrated Part A in 950 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and aseptically add required quantity of Potassium hydroxide (Part B) dissolved in 50 ml of sterile distilled water to obtain pH of 6.8±0.2. As per standard it is recommended to use 4.000 grams of Potassium hydroxide (Part B)

#### **Principle And Interpretation**

*Azospirillum* species occur as free-living in soil or in association with the roots of cereal crops, grasses and tuber plants

(1). *Azospirillum* species are plant-associated diazotrophs of the alpha subclass of *Proteobacteria*. Azospirillum Medium with 0.17% Agar is used for cultivation of *Azospirillum* species. Malic acid is used

as the carbon source. *Azospirillum* species grow well in presence of Malic acid and are not overgrown by other nitrogen fixers. Dipotassium phosphate provides buffering effect and other inorganic salt ingredients provide necessary growth nutrients. Agar at 0.17% concentrations provides microaerophilic conditions necessary for nitrogen fixation by *Azospirillum* species (1).

### **Type of specimen**

Soil samples: For soil samples, follow appropriate techniques for sample collection, processing (1) After use, contaminated materials must be sterilized by autoclaving before discarding.

### **Specimen Collection and Handling:**

#### **Warning and Precautions:**

Read the label before opening the container. Wear protective gloves/protective clothing/eye protection/face protection. Follow good microbiological lab practices while handling specimens and culture. Standard precautions as per established guidelines should be followed while handling specimens. Safety guidelines may be referred in individual safety data sheets

#### **Limitations:**

Further biochemical tests must be carried out for confirmation.

### **Performance and Evaluation**

Performance of the medium is expected when used as per the direction on the label within the recommended temperature.

### **Quality Control**

#### **Appearance**

Part A: Cream to yellow homogeneous free flowing powder Part B :White to cream pellets

#### **Gelling**

Semisolid, comparable with 0.17 % Agar gel.

#### **Colour and Clarity of prepared medium**

Light yellow to pale green coloured clear to slightly opalescent solution.

#### **Reaction**

Reaction of 0.81% w/v aqueous solution (containing KOH) at 25°C pH : 6.8±0.2

#### **pH**

6.60-7.00

#### **Cultural Response**

M518: Cultural characteristics observed after an incubation at 30°C for upto 8 days.

### **Storage and Shelf Life**

Store between 10-30°C in a tightly closed container and the prepared medium at 15-25°C. Use before expiry date on the label. On opening, product should be properly stored dry, after tightly capping the bottle in order to prevent lump formation due to the hygroscopic nature of the product. Improper storage of the product may lead to lump formation. Store in dry ventilated area protected from extremes of temperature and sources of ignition Seal the container tightly after use. Use before expiry date on the label. Product performance is best if used within stated expiry period.

### **Experiment 10: Isolation of Blue Green Algae (BGA)**

#### **Cyanophycean Agar M699**

Cyanophycean Agar is used for the isolation and cultivation of Blue green Algae.

#### **Composition\*\***

##### **Ingredients Gms / Litre**

Potassium nitrate 5.000

Dipotassium phosphate 0.200

Magnesium sulphate 0.100

Agar 15.000

\*\*Formula adjusted, standardized to suit performance parameters

#### **Directions**

Suspend 20.3 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 3 minutes. Cool to 45°C and aseptically add one drop of 1% separately autoclaved solution of ferrous ammonium citrate to 100 ml sterile medium. Mix well and pour into sterile Petri plates.

### **Principle And Interpretation**

Blue green algae are a type of photosynthetic bacteria, called *Cyanobacteria* that rely on sunlight for energy. They are present in almost all aquatic ecosystems, including creeks, rivers, lakes and wetlands. Algal blooms can cover large areas of a water supply. Like all photosynthetic organisms, blue-green algae rely on sunlight for energy, with their growth rate determined by the level of nutrients available in the water.

Cyanophycean Agar is used for the isolation and cultivation of blue green algae. Potassium is required for maintenance of maximum growth rate of blue green algae (1). Nitrate serves as nitrogen source. Dipotassium phosphate buffers the media. Magnesium sulphate is a source of divalent cations.

### **Quality Control**

#### **Appearance**

White to cream homogeneous free flowing powder

#### **Gelling**

Firm, comparable with 1.5% Agar gel

#### **Colour and Clarity of prepared medium**

Colourless clear to slightly opalescent gel forms in Petri plates

#### **Cultural Response**

M699: Cultural characteristics observed after an incubation at 35-37°C for 18-48 hours.

#### **Storage and Shelf Life**

Store below 30°C in tightly closed container and the prepared medium at 2-8°C. Use before expiry date on label.

## **Experiment 11: Gram Staining and microscopic examination of microbes.**

### **Materials Required:**

1. Clean glass slides
2. Inoculating loop
3. Bunsen burner
4. Bibulous paper

5. Microscope
6. Lens paper and lens cleaner
7. Immersion oil
8. Distilled water
9. 18 to 24 hour cultures of organisms

**Reagents:**

1. Primary Stain - Crystal Violet
2. Mordant - Grams Iodine
3. Decolourizer - Ethyl Alcohol
4. Secondary Stain - Safranin

**Procedure:**

**Part 1: Preparation of the glass microscopic slide**

Grease or oil free slides are essential for the preparation of microbial smears. Grease or oil from the fingers on the slides is removed by washing the slides with soap and water. Wipe the slides with spirit or alcohol. After cleaning, dry the slides and place them on laboratory towels until ready for use.

**Part 2: Labeling of the slides**

Drawing a circle on the underside of the slide using a glassware-marking pen may be helpful to clearly designate the area in which you will prepare the smear. You may also label the slide with the initials of the name of the organism on the edge of the slide. Care should be taken that the label should not be in contact with the staining reagents.

**Part 3: Preparation of the smear**

- **Bacterial suspensions in broth:** With a sterile cooled loop, place a loopful of the broth culture on the slide. Spread by means of circular motion of the inoculating loop to about one centimeter in diameter. Excessive spreading may result in disruption of cellular arrangement. A satisfactory smear will allow examination of the typical cellular arrangement and isolated cells.

- **Bacterial plate cultures:** With a sterile cooled loop, place a drop of sterile water or saline solution on the slide. Sterilize and cool the loop again and pick up a very small sample of a bacterial colony and gently stir into the drop of water/saline on the slide to create an emulsion.
- **Swab Samples:** Roll the swab over the cleaned surface of a glass slide.

**Please note:** It is very important to prevent preparing thick, dense smears which contain an excess of the bacterial sample. A very thick smear diminishes the amount of light that can pass through, thus making it difficult to visualize the morphology of single cells. Smears typically require only a small amount of bacterial culture. An effective smear appears as a thin whitish layer or film after heat-fixing.

#### **Part 4: Heat Fixing**

Heat fixing kills the bacteria in the smear, firmly adheres the smear to the slide, and allows the sample to more readily take up stains.

- Allow the smear to air dry.
- After the smear has air-dried, hold the slide at one end and pass the entire slide through the flame of a Bunsen burner two to three times with the smear-side up.

Now the smear is ready to be stained.

Please Note: Take care to prevent overheating the slide because proteins in the specimen can coagulate causing cellular morphology to appear distorted.

#### **Part 5: Gram Stain Procedure**

1. Place slide with heat fixed smear on staining tray.
2. Gently flood smear with crystal violet and let stand for 1 minute.
3. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
4. Gently flood the smear with Gram's iodine and let stand for 1 minute.

5. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. The smear will appear as a purple circle on the slide.
6. Decolorize using 95% ethyl alcohol or acetone. Tilt the slide slightly and apply the alcohol drop by drop for 5 to 10 seconds until the alcohol runs almost clear. Be careful not to over-decolorize.
7. Immediately rinse with water.
8. Gently flood with safranin to counter-stain and let stand for 45 seconds.
9. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
10. Blot dry the slide with bibulous paper.
11. View the smear using a light-microscope under oil-immersion.