AmbujaNeotia

THE NEOTIA UNIVERSITY

ज्ञानम् आत्म प्रदीपाय

PHARMACEUTICAL MICROBIOLOGY

III SEMESTER (2nd YEAR B.PHARM)

PRACTICAL LAB MANUAL

Microbiology Laboratory Manual

(III rd. Semester)

Expt. No.1

Introduction and study of different equipment and processing

BOD INCUBATOR

The full from of BOD Incubator is Biological Oxygen Demand incubator and it is widely used in microbiology laboratories for the applications that include cell culture and fungal growth, BOD test, fermentation, crop and physiology and various pharmaceutical tests etc. It is also known as low temperature incubator or refrigerated incubator because it is made with temperature range between 5°C to 60°C or with cooling and heating functions under one unit.

DIFFERENCE BETWEEN INCUBATOR AND BOD INCUBATOR
The basic difference between an incubator and BOD incubator is temperature. A general purpose incubator has only heating option and is usually operated at 37°C; while a BOD incubator also known as cooled incubator has both cooling and heating options and usually operated at low temperatures such as 10°C and 21°C.

WORKING PRINCIPLE

Electricity is supplied through mains MCB. Temperature is set through digital PID temperature controller, usually at 20°C. Machine is kept running for 5 days. Refrigeration system starts just after setting temperature. Axial fan circulates air inside chamber. Temperature sensor senses current temperature and give data to PID controller, which furthermore keeps the set temperature constant till desired time.

Laminar flow cabinet

A laminar flow cabinet or tissue culture hood is a carefully enclosed bench designed to prevent contamination of semiconductor wafers, biological samples, or any particle sensitive materials. Air is drawn through a HEPA (High Efficiency Particulate Air) filter and blown in a very smooth, laminar flow towards the user. Due to the direction of air flow, the sample is protected from the user but the user is not protected from the sample. The cabinet is usually made of stainless steel with no gaps or joints where spores might collect.

Such hoods exist in both horizontal and vertical configurations, and there are many different types of cabinets with a variety of airflow patterns and acceptable uses.

Laminar flow cabinets may have a UV-C germicidal lamp to sterilize the interior and contents before usage to prevent contamination of the experiment. Germicidal lamps are usually kept on for fifteen minutes to sterilize the interior before the cabinet is used. The light must be switched off when the cabinet is being used, to limit exposure to skin and eyes as stray ultraviolet light emissions can cause cancer and cataracts.

Aseptic Hood

Aseptic hoods are ventilation devices used within the lab to provide an aseptic work area that helps protect both the laboratory personnel and the materials they are working with. Hoods are classified as class I, II, or III depending on the level of protection they offer. Fume hoods, most often a Class I hood are designed to limit exposure of the experimenter to hazardous or toxic fumes, vapors or dusts, and are essential in any analytical chemistry or microbiology laboratory. Cell culture hoods, commonly Class II are used to create a sterile environment for cell culture experiments. These hoods protect the samples from contamination using air that is circulated within the enclosed bench and drawn through a HEPA filter. Cell culture hoods are critical in molecular biology labs, in which most work depends on sterile technique. Class III hoods offer the most protections for both the experimenter and the experiment and are commonly biosafety cabinets and/or glove boxes. These hoods are gas tight and are used for work involving known human pathogens, as well as other highly sensitive material.

Autoclave

An autoclave is a machine used to carry out industrial and scientific processes requiring elevated temperature and pressure in relation to pressure/temperature. Autoclaves are used in medical applications to perform sterilization.

It is very important to ensure that all of the trapped air is removed from the autoclave before activation, as trapped air is a very poor medium for achieving sterility. Steam at 134 °C can achieve a desired level of sterility in three minutes, in contrast to hot air at 160 °C, which can take two hours to achieve the same sterility.
Downward displacement (or gravity-type): As steam enters the chamber, it fills the upper areas first as it is less dense than air. This process compresses the air to the bottom, forcing it out through a drain which often contains a temperature sensor. Only when air evacuation is complete does the discharge stop. Flow is usually controlled by a steam trap or a solenoid valve, but bleed holes are

sometimes used. As the steam and air mix, it is also possible to force out the mixture from locations in the chamber other than the bottom.

Hot air oven

Hot air ovens are electrical devices which are used for dry heat sterilization. Generally, they use a thermostat to control the temperature. Their double walled insulation keeps the heat in and conserves energy, the inner layer being a poor conductor and outer layer being metallic. There is also an air filled space in between to aid insulation. An air circulating fan helps in uniform distribution of the heat. These are fitted with the adjustable wire mesh plated trays or aluminium trays and may have an on/off rocker switch, as well as indicators and controls for temperature and holding time. The capacities of these ovens vary.

The main principle of sterilization is based on the principle of thermal inactivation by oxidation

A complete cycle involves heating the oven to the required temperature, maintaining that temperature for the proper time interval for that temperature, turning the machine off and cooling the articles in the closed oven till they reach room temperature. The standard settings for a hot air oven are, 1.5 to 2 hours at 160 °C or 6 to 12 minutes at 190 °C and the time required to preheat the chamber before beginning the sterilization cycle. If the door is opened before time, heat escapes and the process becomes incomplete. Thus the cycle must be properly repeated all over.

These are widely used to sterilize articles that can withstand high temperatures and not get burnt, like glassware and powders. Linen gets burnt and surgical sharps lose their sharpness.

Preparation and Sterilization of different Medium

Expt. No.2

Preparation and Sterilization of Agar Medium

Nutrient Agar is a general purpose, nutrient medium used for the cultivation of microbes supporting the 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

It typically contains, in mass/volume

- **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 6.8± 0.2 at 25 °C.

Procedure-

These ingredients are combined and boiled for approximately one minute to ensure they are mixed and then sterilized by autoclaving, typically at 121 °C for 15 minutes. Then they are cooled to around 50 °C and poured into Petri dishes which are covered immediately. Once the dishes hold solidified agar, they are stored upside down and are often refrigerated until used. Inoculation takes place on warm dishes rather than cool ones: if refrigerated for storage, the dishes must be rewarmed to room temperature prior to inoculation.

Expt. No.3

Preparation and Sterilization of Potato Dextrose Agar Medium

Potato Dextrose Agar (PDA) is used for the cultivation of fungi. Potato Dextrose Agar (PDA) is a general purpose medium for yeasts and molds that can be supplemented with acid or antibiotics to inhibit bacterial growth.

Composition of Potato Dextrose Agar (PDA)

Potato infusion	200 gm
Dextrose	20 gm
Agar	20 gm
Distilled water	1 litre

Note: 200 gm of potato infusion is equivalent to 4.0 gm of potato extract.

Procedure of Potato Dextrose Agar (PDA)

- To prepare potato infusion, boil 200 g sliced, unpeeled potatoes in 1 liter distilled water for 30 min.
- Filter through cheesecloth, saving effluent, which is potato infusion (or use commercial dehydrated form).
- Mix with Dextrose, Agar and Water and boil to dissolve.
- Autoclave 15 min at 121°C.
- Dispense 20-25 ml portions into sterile 15 × 100 mm petri dishes.
- Final pH, 5.6 ± 0.2.

Expt. No.4

Preparation and Sterilization of Nutrient Broth Medium

Nutrient Broth is a general purpose medium used for cultivating a broad variety of fastidious and non-fastidious microorganisms with non-exacting nutritional requirements

Composition of Nutrient Broth

It typically contains, in mass/volume

- **0.5% Peptone**
- **0.3% beef extract/yeast extract**
- **0.5% NaCl**
- **Distilled water**
- pH is adjusted to neutral 6.8± 0.2 at 25 °C.

Procedure-

These ingredients are combined and boiled for approximately one minute to ensure they are mixed and dispense into appropriate containers and sterilize in autoclave at 121°C for 15 minutes.

Expt. No.5

Cultivation/Culturing of Bacteria

A microbiological culture is a method of multiplying microbial organisms by letting them reproduce in predetermined culture medium under controlled laboratory conditions.

It is often essential to isolate a pure culture of microorganisms. A pure (or axenic) culture is a population of cells or multicellular organisms growing in the absence of other species or types. A pure culture may originate from a single cell or single organism, in which case the cells are genetic clones of one another. For the purpose of gelling the microbial culture, the medium of agarose gel (agar) is used. Agar is a gelatinous substance derived from seaweed.

REQUIREMENTS

Materials: Microorganism source like stock culture, agar plate, pure agar plate.

Equipment: Inoculating loop, Bunsen burner, Paper towels

Procedure

Microbiological cultures can be grown in petri dishes of differing sizes that have a thin layer of agar-based growth medium. Once the growth medium in the petri dish is inoculated with the desired bacteria, the plates are incubated at the best temperature for the growing of the selected bacteria (for example, usually at 37 degrees Celsius for cultures from humans or animals or lower for environmental cultures).

Another method of bacterial culture is liquid culture, in which the desired bacteria are suspended in liquid broth, a nutrient medium. Inoculate liquid broth with bacteria and let it grow overnight (they may use a shaker for uniform growth). Then take aliquots of the sample to test for the antimicrobial activity of a specific drug or protein (antimicrobial peptides).

Expt. No.6
Sub culturing of Bacteria

Sub culturing is the method of transferring microorganisms from one growth container to another, thereby offering them with fresh supply of nutrients either in a solid or a liquid medium. So the basic objective of this test is to prepare subculture of bacteria and fungus.

REQUIREMENTS
Materials required: Bacterial Culture in Nutrient agar plate, Fungal culture in Potato Dextrose agar plate, sterile medium, Nutrient agar plate, Potato dextrose agar

Requirements: Inoculating loop, Bunsen burner

PROCEDURE
Sub culturing method of bacteria
Inside the laminar airflow, take the plate cultures containing the isolated colonies of bacteria grown on them. Pick up an isolated colony or more colonies in the inoculating loop. And then close this plate. Now transfer this single colony or few colonies of bacteria into the new plate by streaking in a zig-zag method. To avoid contamination, it is advisable to lift the lid of the plate to minimum. Then close the lid, invert it and incubate the plate for 24-48 hours. Identify the microbes.

Staining methods- Simple, Grams staining

Expt. No.7

Simple Staining

Simple staining is one of the conventional methods of staining techniques. As from the name, it is quite clear that it is very simple and direct staining method which makes the use of a single stain only. The microorganism is invisible to the naked eye, therefore to make it visible, the staining is performed, which gives divergence to a microscopic image. Simple staining makes the use of basic dyes like methylene blue, safranin, crystal violet, malachite green etc. which refers as "Simple or Direct stains".

Procedure of Simple Staining

The method of simple staining involves three steps like:

1. Smear preparation
2. Heat fixing
3. Staining

1. Smear Preparation

Bacterial smear consists of a thin film of bacterial culture or inoculum. For the preparation of smear, we need to perform the following steps like:

- Take a clean, grease-free glass slide.
- Add a drop of distilled water at the centre of the glass slide.
- Then add inoculum from the bacterial culture with the help of sterilized inoculating loop on the glass slide.
- After that, mix the inoculum with a drop of distilled water to make a thin film by uniformly rotating the inoculating loop.

2. Heat Fixing

There are many reasons to perform heat fixing, and it cannot be skipped because:

•Heat fixing helps in the fixation of a specimen to the glass slide.
•Heat fixing helps the stain to penetrate into the smear.

After smear preparation, heat fixes the smear by passing the slides through the flame of Bunsen burner for at least three times. Then, allow the slide to air dry.

3. Staining

It is the last and the most crucial step which colours the bacterial cells and makes it visible, through which one can identify the morphological characteristics of the bacteria. This stage involves the following steps as follows:

- Add stain to the heat fixed smear.
- Allow the stain to stand for at least 1minute so that it can penetrate between the cells.
- Wash off the glass slide carefully.
- Blot dry the slide with absorbent paper but do not wipe the slide.
- Examine the glass slide under the microscope from low to high power objective to get a magnified view of the specimen. One can also add a drop of oil immersion over the stained area of the glass slide and observe it under suitable magnification.

Expt. No.8

Gram Staining

Gram staining is used widely technique and the most popular method in the laboratories. It is the type of "Differential staining" which makes the use of more than one stain to differentiate the bacteria. The gram staining method was first given in 1884 by the Danish scientist and Physician Han's Christian Gram.

The Gram stain is the differential stain that stains the bacterial cells differently according to the type of cell wall. Gram stain is used to differentiate the bacterial cells by staining the Cell wall and distinguish two major groups of bacteria that are gram-positive and gram-negative. Gram-positive bacteria appear violet in colour and gram-negative bacteria appear pink in colour as a result of Gram staining.

Chemicals required: Crystal violet solution, Gram's iodine solution, Ethyl alcohol (95%), Safranin

Process of Gram staining

1. Take a clean dry slide and place one drop of distilled water at the center.
2. Prepare bacterial smear by taking small inoculum from the bacterial culture by the help of the inoculating loop.
3. Then mix the inoculum with the water drop to prepare thin bacterial smear by rotating the inoculating loop in a clockwise and anticlockwise direction.
4. After that, heat fixes the bacterial smear under the spirit lamp.
5. Then flood the bacterial smear with crystal violet and allow to stand for 1minute.
6. Then flood the smear with Gram's iodine.
7. After that, add the decolourizing agent i.e. 95% Ethanol to the smear.
8. At last, add counter stain i.e. Safranin to the smear and allow to stand for 1minute.
9. Then wash the slide with water and air dry the slide.
10. Then observe the glass slide under the microscope by adding oil immersion to the stained cells to differentiate gram-positive and negative cells.

Expt. No.9
Isolation of Bacteria

Isolation of Pure Cultures by Using Streak Plate Method

REQUIREMENTS
Materials: Microorganism source like stock culture, agar plate, pure agar plate.
Equipment: Inoculating loop, Bunsen burner, Lysol, Paper towels

PROCEDURE
First sterilize the inoculating loop by keeping the tip of loop in the flame of Bunsen burner until it is red hot. Let it cool down. Then pick a microbial colony from the stock culture and then streak the first quadrant as four parallel streaks. Show the loop to flame and then let it cool down. Then go to the edge of each line of the first quadrant and extend this to second quadrants sets. Again, sterilize the loop and in the same manner streak the third set of quadrants. Repeat the technique for the fourth set of quadrants. Show the inoculating loop to the flame and keep it. Now close the inoculated plate carefully to avoid contamination. Then incubate the plates for growth. After growth the plate should be checked for the purity of culture by seeing that pure isolated colonies are formed towards the last quadrant. These pure isolated colonies can be again subcultured and kept in slants or they can be used for studying various properties.

Isolation of Pure Cultures by Using Pour Plate Method

REQUIREMENTS
Materials: Test microbial sample, sterile water
Equipment: Sterile capped test tubes, Agar medium, Flame, Pipettes, Petri plates

PROCEDURE
First make the dilution of the test microbial sample. Accordingly, mark the plates in which dilution you have to pour. Then inoculate about 0.1 ml of the diluent in the molten agar and mix it thoroughly. Now take the sterile molten agar and remove the cap of the container or the cotton plug in left hand. Now show the neck of the flask in the flame and lift the lid of the petri plate and then pour the molten agar in the plate uniformly and cover the lid. Slowly rotate the plate so that the agar is distributed in the plate in a very evenly manner. In most of the times the agar migrates towards one side of the plate. Try to avoid this by rotating the plate. Then allows the plates to solidify. As soon as the

plate settles down incubate the plate at the required temperature and duration.

Expt. No.10 Identification of Bacteria

A) Indole Test

REQUIREMENTS
Samples: Bacterial samples
Chemicals: Tryptone broth, Kovac reagent
Apparatus: Test tubes, Incubator, Inoculation tube

PROCEDURE
Take the bacterial sample and inoculate it carefully in tryptone broth. Then incubate it at a temperature of 37^{0}C.To this adds about some drops of the Kovac reagent. Be careful while observing without shaking the tube, as it may disturb the experiment.
If a red colour or pink colour ring is formed in the top then it is taken as indole positive and if there is no colour change after adding Kovac's reagent, then the bacterium is indole negative.

B) Methyl Red Test

REQUIREMENTS
Sample: Unknown bacterial sample.
Chemicals: MR-VP medium, Methyl red reagent
Apparatus: Test tubes, Inoculating loop, Incubator

PROCEDURE
In two test tubes take freshly prepare MR-VP medium. To one of the test tube inoculate the sample microorganism, with the help of an inoculating loop. The other test tube containing the broth is used as a standard. Then keep both the tubes in incubator at 37^{o}C for about 24-48 hours for proper growth of organism. After incubation, bring both tubes and to it add about 4-5 drops of Methyl Red reagent. Now leave for some time undisturbed and observe for the colour change. In the standard test tube if there is no colour change, then there is no contamination and vice-versa. If red colour is formed then it is MR positive. If no colour change then MR negative.

C) Voges-Proskauer's Test

REQUIREMENTS
Sample: Bacterial sample.
Media: MR-VP Broth
Reagents: Baritt reagent A – 5G Alpha naphthol in 100 ml absolute ethyl alcohol
Baritt reagent B – Potassium Hydroxide 400 gm in 1000 ml Distilled water

PROCEDURE
Take the pure culture of the test organism and inoculate it in MR-VP broth. Then keep it for incubation for about 24 hours at a temperature 35^{0}C. After bringing out the inoculated broth from incubator, take some clean test tubes and to it add 1 ml of broth. Then to it add 0.6 ml of 5% alpha naphthol, then 0.2 ml of 40% KOH. Then shake the test tube well and then allow the tube to remain stagnant for about 10-15 min. If after 15 min red colour appears, it indicates the presence of diacetyl and if no colour change then negative test.

D) Citrate Utilization Test

REQUIREMENTS
Sample: Bacterial sample
Media: Simmon citrate agar, Water
Apparatus: Test tube, Inoculation tube, Incubator

PROCEDURE
Prepare Simmons citrate agar medium and autoclave it. Then after, it becomes little cool, prepare the slant, One for control and another for inoculating organism. Then in the test tube for inoculation take s small amount of organism in the inoculating loop and streak it in the slant agar. Then incubate it at 37^{o}C for about 24 hours. Then after incubation observe for the colour observation. If the colour changes to blue colour then it is citrate positive, if no colour change then it is citrate negative.