APPLIED MICROBIOLOGY

4TH SEMESTER

Rules and Regulations:

There are certain rules and regulations which should be followed in Microbiology. This ensures safety of all the workers in the laboratory.

The rules are as follows:

A. General Instructions:
   • The working area should be disinfected with suitable disinfectants.
   • Infectious materials should not be blown out of pipettes.
   • It is important to label the cultures or the samples.
   • Before centrifuging the tubes should be inspected for cracks.
   • Wetting of cotton plug should be avoided while shaking the broth culture.
   • Mixtures of infectious materials should not be prepared by blowing air through the liquid by pipette.
   • If a culture is poured or spilled, disinfectants should be poured over the area and clean the area thoroughly.
   • Before discarding the bacterial culture it should be sterilised. Used cotton papers, plastics should be disinfected by using swab.
B. Personal Care:
   • General body health should be maintained.
   • It is necessary to wear protective clothing like overcoats, aprons.
   • Open cut or wounds should be bandaged.
   • Nails should be cut. Hands should be washed preferably with soap or dettol.

1.Sterilization by Moist Heat – Autoclave

Aim: To study the physical process of sterilization using moist heat and to sterilize culture media and glasswares.

Theory and Principle:

Sterilization is freeing of an article from living organisms. Physical method using moist heat is one of the methods used for sterilization. Autoclave: When water is boiled in a closed vessel at increased pressure, the temperature at which it boils and that of the steam it forms, will rise above 100ºC. This

principle is employed in the autoclave. Moist heat denatures proteins and cause destruction of membranes and DNA. Presence of moisture significantly speeds up heat penetration. Pressure cookers are used generally to sterilize small quantities of media. When large quantities of media and other materials are to be sterilised, the use of autoclave is recommended.

Procedure:

1. The glassware are washed dried and covered with paper.
2. Media are prepared in test tube, flasks and covered with cotton plugs and paper.
3. Pipettes are wrapped in paper and placed in canisters.
4. Water is poured into the autoclave until the thermostat level is reached.
5. Things are placed in the stainless steel drum of autoclave.
6. The lid of the autoclave is closed tightly.
7. Current is switched on.
8. The steam valve is closed after steam starts leaving the steam outlet.
9. The sterilization is carried out for 15–20 minutes after the pressure guage reading reaches 15 psi.
10. Switch off the current.
11. Cool down and slowly release the lid knobs.
12. Remove the things from the autoclave for further use.

Result:

Physical method using moist heat of sterilization were studied and glasswares and media were sterilised using autoclave.

2. **Sterilization using Dry Heat – Hot Air Oven**

Aim:

To study the physical process of sterilization using dry heat and to sterilize oils, powders and glass wares.

Theory and Principle:

Sterilisation is freeing of an article from living organisms. Physical method using dry heat is one of the methods used for sterilization. Hot air oven employs dry heat at a temperature of 160ºC for one hour. Glass wares, syringes, metal instruments and paper wrapped goods which are not sensitive to high temperature are sterilised in hot air oven. This technique requires longer exposure time and higher temperatures than moist heat sterilization. Sterilizing by dry heat is accomplished by conduction. Dry heat does most of the damage by oxidizing molecules. Moisture impermeable materials like anhydrous fat, oils and powders are sterilised by hot air oven.

Procedure:

1. Switch on the hot air oven.
2. Set the required temperature and other conditions.
3. When the temperature is reached place the glass wares and other things for sterilization.
4. Switch off the oven after an hour.

5. Cool and remove the glass wares.

Result:

Physical methods of sterilization were studied and glass wares, oils and powders were sterilized using hot air oven.

3.Media Preparation – Nutrient Agar

Aim:

To prepare nutrient agar medium for cultivation of microorganisms.

Theory and Principle:

A culture medium is an environment which supplies necessary nutrients for the growth of microorganisms. Nutrient agar is a basal medium which supplies the basic requirements of carbon, nitrogen and mineral source for growth of microorganisms. It is a general purpose medium which allows the growth of wide range of nonfastidious microorganisms.

Composition of Nutrient Agar

1. Peptone: 1gm
2. 2. Beef Extract: 0.5gm
3. 3. Sodium Chloride: 0.3gm
4. 4. Agar: 2.8gms
5. 5. Distilled Water: 100ml
6. 6. pH: 7.4 (neutral)

Requirements:

1. Peptone 2. Beef Extract 3. Sodium Chloride 4. Agar 5. Distilled Water 6. Measuring Cylinder 7. Petri dishes 8. Non Absorbent Cotton 9. Rubber bands and Newspaper.

Procedure:

1. Weigh the ingredients and transfer it to 250ml conical flask.
2. With the help of measuring cylinder measure 100ml distilled water.
3. Dissolve the weighed ingredients in 50ml of distilled water and make up the volume to 100ml. Molten Nutrient Agar Nutrient Agar Plate Non absorbant cotton plug 22
4. Adjust the pH to 7.4 using sodium hydroxide with the help of pH meter or paper.
5. Cover the flask with cotton plug and paper and label it.
6. Sterilise the medium in an autoclave.
7. Cool the medium to 45ºC and pour the medium into petriplates, or tubes as per the requirement.

Observation: Nutrient agar medium is in the molten state after autoclaving. It solidifies after cooling to form a solid medium to be further used for isolation and growth of microorganisms.

Result:

Nutrient agar medium was prepared and sterilised. Further Nutrient agar plates and slants were made for isolation of microorganisms.

4. Isolation and enumeration of microorganism.

AIM:

To prepare serial dilutions of sample and perform pour plate, spread plate & streak plate method for isolation and enumeration of microorganism.

Requirements:

Nutrient agar, petriplates, saline solution (0.85%), cotton plugs, micropipette, Laminar flow, etc.

Theory:

The microbial population in our environment is diverse and contains species of bacteria, fungi, yeast and molds. The study of specific micro-organism for different purpose is very important. This requires the dilution of the sample initially for accurate enumeration in distilled water or saline solution. Then the micro-organism can be isolated using different isolation methods-

1. Streak plate technique.
2. Pour plate technique
3. Spread plate technique.

Procedure-

Preparation of serial dilution:

1. Prepared saline solution (0.85%) and poured 9 ml of solution in 5 tests tube each.
2. Cotton plugged the tubes and autoclaved.
3. Prepared the stock solution with sample.
4. Under the aseptic conditions in laminar air flow, prepared the dilution with the sample upto 10-5 .
5. Different plating techniques were performed·

Streak plate technique:

By means of a transfer loop, a portion of the mixed culture is placed on the surface of an agar medium and streaked across the surface. The manipulation thins out the bacteria are separated from each other. When streaking is done properly the colonies grow sufficiently far apart showing no merge of colonies. The assumption is made that colony is derived from a single cell &therefore the colony is a clone.

Pour plate technique:

In this mixed culture is diluted directly in tubes of liquid agar medium. The medium is made liquid state at a temperature of 450C to allow thorough distribution of the inoculums. The inoculated medium is dispensed into petri-dishes, allowed to solidify and then incubated.

Spread-plate method:

The mixed culture is not diluted in the culture medium instead it is diluted in a series of tubes containing a sterile liquid. A sample is removed from each tube, placed onto the surface of an agar plate by means of bent glass rod. One plate of the series bacteria will be in numbers sufficiently low as to allow the development of well separated colonies.

5.Gram's staining

AIM :

To perform gram staining of given sample.

MATERIAL REQUIRED :

Glass slides, bunsan burner , cotton , sample , microscope.

REAGENTS REQUIRED: Crystal violet dye, iodine, alcohol (95% ethyl alcohol), saffranin dye.

PRINCIPLE: Gram staining is most widely staining technique used in m/o examination. It was discovered by Danish scientist and physician Hans Christain Joachin Gram in 1884. This technique differentiates bacteria in 2 groups i.e. Gram positive and Gram negative bacteria. The procedure is based on the ability of m/o to retain colour of the stain during Gram reaction. Gram negative bacteria are decolourised by alcohol losing the colour of primary stain , purple. Gram positive bacteria are not decolourised by alcohol and will remain as purple. After decolourisation stop , a counter stain is used to impart pink colour to the gram negative m/o. Gram positive bacteria have a thick mesh like cell wall which is made up of peptidoglycan (50-90%) of cell wall, which stain purple. Gram negative bacteria have a thinner layer of peptidoglycan (10% of cell wall) and lose the crystal violet iodine complex during decolourisation with alcohol rinse but retain the counter stain safarin thus appearing reddish or purple.

STAIN REACTION :

1. Application of crystal violet to heat fixed smear : CV dissociates in aqueous solution into CV+ and Cl- ions. These two penetrate the cell wall and cell membrane of both gram positive and gram negative . CV+ interact with negative component of bacterial cell and stain it purple.
2. Addition of gram iodine : Iodine acts as a mordant and a trapping agent. A mordant is a substance that increase the affinity of cell wall for a stain by binding to primary stain , thus forming a insoluble complex that get trapped in cell. During the reaction CV-I compex is formed and all the cells turn purple.
3. Decolourization with ethyl alcohol : Alcohol dissolve the lipid outer membrane of gram negative bacteria, thus leaving the petidoglycan layer exposed and increase the porosity of cell wall. The CV-I complex is then washed away from the peptidoglycan layer leaving gram negative bacteria colourless.In gram positive bacteria , alcohol has dehydrating effect on cell wall causing cell wall to shrink , then CV-I complex get tightly bound into multi layered leaving the cell with purple colour.
4. Counter stain with safranin dye : The decolourised gram negative cell can be visible with a suitable counter stain which is usually positively charged safarnin, which stained it pink.

PROCEDURE :

1. Prepared very thin smear of sample on glass slide and heat fixed it.
2. Flooded the smeared slide with crystal violet dye. Avoid over flooding and kept it for 1 minute.
3. Washed the slide under running tap water.
4. Applied iodine solution gently all over the slide and kept for 1 minute.
5. Washed it under tap water.

6. Applied 95% ethyl alcohol all over the slide drop wise and kept for 10 second.
7. Immediately rinsed with water.
8. Finally, flooded the sample with saffranin dye to counter stain and kept for 45 seconds.
9. Washed the slide with running water.

Result:

Observed it under microscope.

Positive result Gram positive organisms stain deep blue/purple.
Negative result Gram negative organisms stain pink/red.
Other counterstains (such as carbol fuchsin) used may give more intense colours.

Quality control organisms

A culture containing Gram positive and Gram negative organisms may be used for quality control.

6. motility test

AIM:

Hanging Drop method to test Bacterial Motility.

Materials required:

1. Glass slides (glass slide with depression) or normal glass slide with adhesive or paraffin ring
2. Paraffin wax
3. Loop
4. Coverslip
5. Microscope
6. Bunsen burner
7. Young broth culture of motile bacteria (e.g. *Proteus mirabilis*)

Theory

A tiny drop of bacteria is hung from the middle of the cover slip into the cavity slide. It is then observed under the microscope using the oil-immersion objective. The movement in the medium and its surrounding indicates that the bacteria are motile.

On the other hand, if the bacteria remain calm in the medium, it means that the bacteria is non-motile. Hanging drop preparation is a special type of wet mount (in which a drop of medium containing the organisms is placed on a microscope slide), often is used in dark illumination to observe the motility of bacteria.In this method a drop of culture is placed on a coverslip that is encircled with petroleum jelly (or

any other sticky material). The coverslip and drop are then inverted over the well of a depression slide. The drop hangs from the coverslip, and the petroleum jelly forms a seal that prevents evaporation..

Procedure

1. Petroleum jelly is applied around the clean and dry cover slip.
2. The loop is put over the flame and allow to cool. Using the sterilized loop, a bacteria is taken from the broth culture.
3. A drop of suspension is put at the coverslip's center.
4. The slide is inverted and put on the coverslip. The two are pressed gently to seal the cavity. Make sure that not a single part touches the drop.
5. The slide is inverted so that the drop hangs into the cavity.
6. The slide is then clipped to the stage and will be examined under the low power objective of the microscope.

Result

With the hanging drop method, the bacteria will be checked for its motility, shape, arrangement, and size.