Exercise No. 13

Object : *To demonstrate the technique for pouring a petri-dish.*

Requirements : Culture tube with medium and plug, burner, petri-dish.

Method:

1. Remove cotton plug from the tube containing medium and pass the mouth of this tube through the flame of burner.
2. Raise one edge of the cover of petri-dish and pour medium into the lower plate as shown in Fig. 133.

Result: Pouring process of petri-dish is complete.

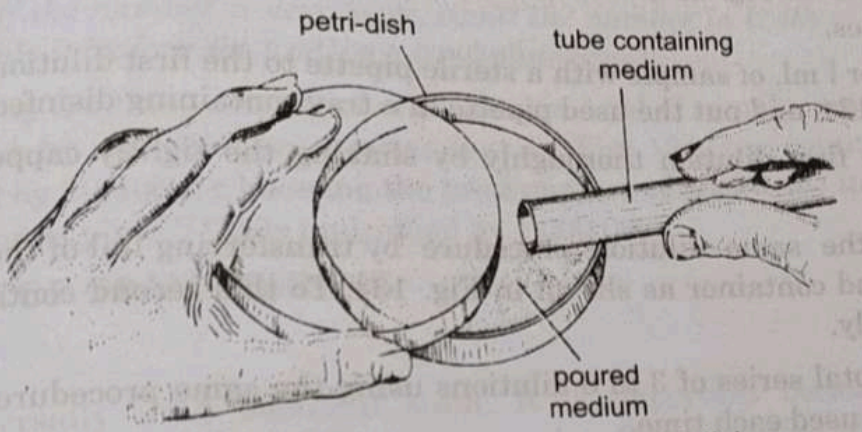

Fig. 133. Technique for pouring a petri-dish.

TECHNIQUE OF DILUTION AND COUNTING THE NUMBER OF MICROORGANISMS

Exercise No. 14

counting the number of micro

Gram-negative).

1.1 Gram-staining of Bacteria

Gram-staining technique of bacteria is differential stain technique developed by **Hans Christian Gram** i **1884**. It is extremely popular technique to differentiate bacteria species into **Gram-positive** an **Gram-negative**. The basis of Gram's staining technique is the ability of a bacterial cell stained with crysta violet to retain the colour when treated with a differentiating agent (usually alcohol). Bacteria that retain th violet/purple are called **Gram-positive**, because crystal violet is more extensively on trapped in thic peptidoglycan layer, while those loose colour when reacting with alcohol (decolourizing agent) are calle **Gram-negative**. In Gram-negative, peptidoglycan layer is very thin.

1.2 Requirements

1. 24 hours old culture of *E. coli* and *Staphylococcus aureus.*
2. Gram staining reagents :
   Crystal violet (Primary stain)
   Gram's iodine solution (Mordant)
   95% Alcohol (decolourizing agent)
   Saffranin (counter stain)
3. Staining tray
4. Inoculation loop
5. Glass slides
6. Microscope
7. Bunsen burner or spirit lamp
8. Blotting paper/absorbing paper
9. Dropper
10. Wash bottle of distilled water

1.3 Bacterial Smear Preparation by Heat Fixation

Unless fixed on the glass slide, the bacterial smear will wash away during the staining procedure. This is avoided by heat fixation, during which the bacterial proteins are coagulated and fixed to the glass surface. Heat fixation is performed by the rapid passage of the air dried smear 2-3 times over the flame of the **Bunsen burner.**

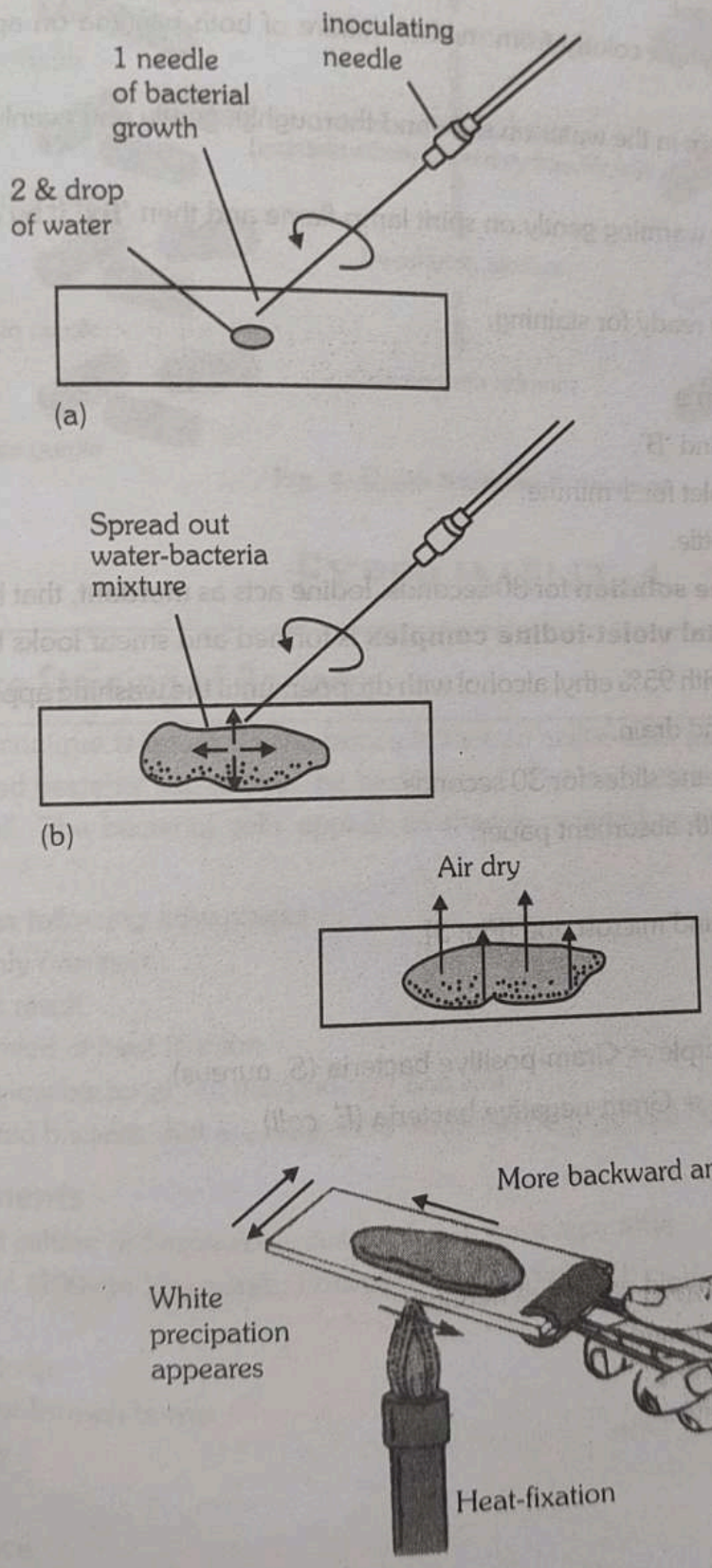

Fig. 3: Bacterial Smear Preparation

1.4 Making Smear

1. Take two clean sterilized glass slides and level with marker 'A' and 'B' transfer.
2. With the help of flame sterilized loop, two loop full of distilled water on slide.
3. Flame the loop and allow to cool.
4. Aseptically transfer a port of single colony from mother culture of both bacteria on agar plate into the distilled water.
5. Make a suspension of the culture in the water on slide and thoroughly, gently and evenly spread over the slide in a 2 cm area.
6. Now dry the suspension on by warming gently on spirit lamp flame and then **'fix'** it by quickly passing it through the flame a few times.
7. This **heat fixed smear** is now ready for staining.

1.5 Gram-Staining Procedure

1. Take both heat fixed slide 'A' and 'B'.
2. Stain separately with crystal violet for 1 minute.
3. Rinse with water using wash bottle.
4. Now treat the smear with **iodine solution** for 30 seconds. Iodine acts as mordant, that helps the stain to adhere to the specimen, a **crystal violet-iodine complex** is formed and smear looks black.
5. Dicolourize the iodine solution with 95% ethyl alcohol with dropper, until the washing appears pale violet.
6. Rinse immediately with water and drain.
7. Counter stain with safranin both the slides for 30 seconds.
8. Wash with water and blot dry with absorbent paper.
9. Let the slides dry.
10. Examine the slide under compound microscope (Fig. 3).

1.6 Observation

1. Bacterial cells appearing violet/purple = Gram-positive bacteria (*S. aureus*)
2. Bacterial cells appearing red/pink = Gram-negative bacteria (*E. coli*)

1.7 Precautions

1. Use only fresh culture.
2. Prepare smear by gentle heating, avoid excessive heating.
3. Smear should be thin and evenly spread.
4. Avoid using excessive alcohol.

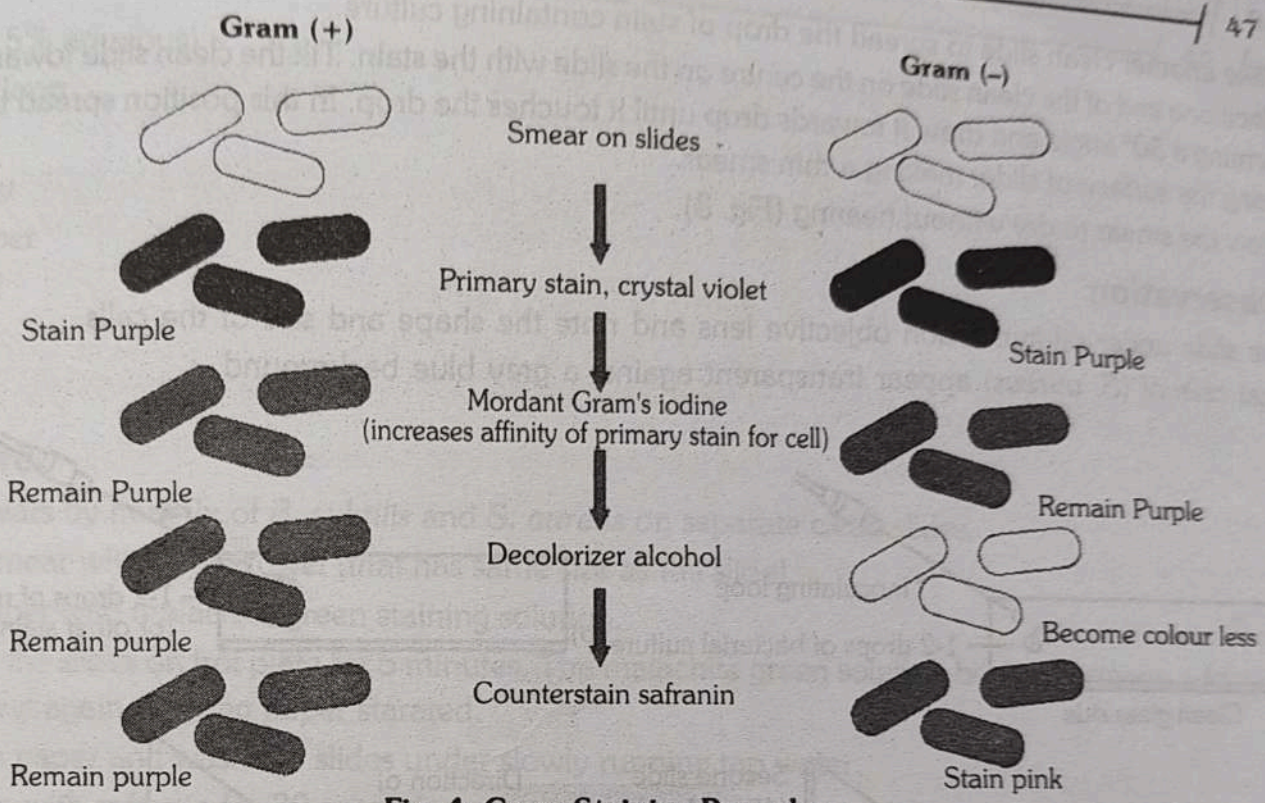

Fig. 4: Gram Staining Procedure