## LAB WORK 6.

**Subject: Differential Staining.Gram Staining.**
**Session Purpose:** To study the organization of the bacterial cell wall, determining the relationship of microorganisms to Gram staining.
**Objectives**:
1. Determine the ratio of Gram staining an unknown culture, to take control cultures of *Staphylococcus aureus* and *Escherichia coli*.
2. Master the technique ofmaking the short test with 3% KOH.
3. Performexercises 1-3.

**Differential Vs Simple Staining:**

- Simple staining use single dyes while differential staining procedures take use of more than a single dye for visualization.
- As there are more than one dyes are used for differential staining purposes the cell appears multicoloured and cellular organization can be visualized easily and more confidently.
- Simple staining technique can only give informations about cell shape, cell size clustering or arrangement of the cells while differential procedures can give additional informations in terms of presence or absence of any specific cellular part, or substance in cells, making characterization and identification of microorganisms easy.

Differential staining techniques are for making cellular differences visible, giving opportunity to distinctly compare or characterize microorganisms. There are two major powerful applications of differential staining, viz. characterization and identification.

**Characterization of microorganisms:**every microbial cell is not alike and the classification and characterization of these microbial cells requires revelation of these differing characters. If these differences are based on structural levels, it can be easily traced using differential staining techniques. Gram's staining technique is one of the most important and versatile technique of differential staining which is necessarily applied for classification of bacteria at initial levels of study. This differential staining is the most significant technique in whole microbial staining approaches. Acid-fast staining, Endospore staining etc are some other differential staining procedures used for identification and characterization of microorganisms.

**Locating structural variations:** Every microorganism is cellular and contains similar features but they also have some distinct variations. These variations may be in form of chemical composition of any specific cellular part or complete presence or absence of any cellular component. Differential staining can also locate these structural variations such as endospores, flagella, capsules, pilli etc.

In 1884 Hans Christian Gram, a Danish physician, developed the Gram stain. Gram-stain is a method for the differential staining of bacteria. Gram-positive microorganisms stain purple. Gram-negative microorganisms stain pink. *Staphy-lococcusaureus,* a common bacterium that causes food poisoning, is gram-positive. *Escherichia coli* is gram-negative.

**Lab Exercise 1.Gram Stain Procedure (variant A).**

**Methodical instructions:** In one glass of skim make strokes of different microorganisms in the center - a smear of cells studied culture, left and right - the control of microorganisms. The cells of one of the test organisms (eg, *Staphylococcus aureus*) Gram stain, and the cells of another (for example, *Escherichia coli*) - not colored. Smears must be

thin so that the cells are uniformly distributed over the surface of the glass and did not form clusters, since the thickness of the stroke depends on the results of staining. Smears air-dried and fixed over the burner flame.

**Procedure:**

1.Prepare the specimen using the heat fixation process.
2. Place a drop of crystal violet stain on the specimen for 1-2 min.Poured into the dye, not washing the smear with water.
3. Apply iodine on the specimen using an eyedropper for 1-2 minutes. The iodine helps the crystal violet stain adhere to the specimen. Iodine is a mordant, which is a chemical that fixes the stain to the specimen.
4.Wash the specimen with an ethanol during 0.5-1 min.
5.Wash the specimen with water to remove the dye.
6.Apply the fuchsin stain to the specimen using an eyedropper.
7.Wash the specimen.
8. Use a paper towel and blot the specimen until the specimen is dry.

The specimen is ready to be viewed under the microscope. Gram-positive bacteria appear blue-violet and gram-negative bacteria appear pink.

When stained by Gram, the **following errors**:

a) All cells are blue due to lack of bleaching;

b) All cells are pale pink, gentian violet staining due to insufficient or excessive treatment with alcohol.

For comparison, you can use an accelerated test for determining membership gram-positive bacteria or gram-negative species.

**Lab Exercise 2. Gram Stain Procedure (variant B).**

**Methodical instructions:** In one glass of skim make strokes of different microorganisms in the center - a smear of cells studied culture, left and right - the control of microorganisms. The cells of one of the test organisms (eg, *Staphylococcus aureus*) Gram stain, and the cells of another (for example, *Escherichia coli*) - not colored. Smears must be thin so that the cells are uniformly distributed over the surface of the glass and did not form clusters, since the thickness of the stroke depends on the results of staining. Smears air-dried and fixed over the burner flame.

**Procedure:**

1. Prepare the specimen using the heat fixation process.
2.Place a drop of crystal violet stain on the specimen for 1-2 min,and then the dye is washed off**.**
3. Apply iodine on the specimen using an eyedropper for 1-2 minutes and washed off. The iodine helps the crystal violet stain adhere to the specimen. Iodine is a mordant, which is a chemical that fixes the stain to the specimen.
4. Wash the specimen with an alcohol-acetone decolorizing solution during 0.5-1 min.
5. Wash the specimen with water to remove the dye (now the gram-positive cells are purple and the gram-negative cells are colorless).
6. Apply thesafranin stain to the specimen using an eyedropper.
7. Wash the specimen.
8. Use a paper towel and blot the specimen until the specimen is dry.
9. And examined microscopically.

Gram-positive bacteria retain the purple dye, even through alcohol wash. Gram-negative bacteria appear pink because they pick up the safranin counterstain.

**Lab Exercise 3.Rapid method for determining membership gram-positive bacteria or gram-negative species.**

**Procedure:**

1. The culture of the bacteria studied by means of a loop is transferred to the dense medium on a glass slide in a drop of 3% KOH solution and mix thoroughly.
2. After 10 sec loop drops sharply raised above.
3. Under these conditions, Gram-negative bacteria is characterized by mucus, which stretches for a loop at 0.5-1.0 cm mucus occurs as a result of the destruction of the cell walls of gram-negative bacteria and leaving the nucleic acids. If the mucus is not produced, the bacterium belongs to the gram-positive.

**Equipment:**

- Microscope
- Slide
- Dropper bottle of water
- Disinfectant tray
- Culture of Bacteria in slant tubes
- Inoculation loop
- Burnerflame
- Staining material - Methylene blue and Fuchsine stains
- 3% KOH solution
- Immersion oil