

Stains and Staining.

- Bacteria are semi-transparent and consist of a clear protoplasmic matter that differs slightly in refractive index from the medium in which they are growing. It is difficult to observe the bacteria in unstained state, except when special methods of illumination are used, to see them in the unstained state.

- Stains are useful for the following reasons.
- It makes the microscopic semi-transparent objects visible
- To study the shape and size
- To reveal the presence of various internal and external structures
- To produce specific chemical and physical reaction
- The term stain and dye are not the same. A colouring agent that is used for general purposes is called a dye. The one that is used for biological purposes is called a stain. Based on their chemical behaviour, the dyes are classified as acidic, basic and neutral.

- An acid (or anionic) dye has a negative charge. Eg., Eosin, Rose Bengal and Acid fuchsin.
- The negatively charged groups are carboxyls (COOH) and Phenolic hydroxyls (-OH).
- Since they are negatively charged, bind to positively charged cell structures.
- pH plays an important role in the effectiveness of staining, because the nature and the degree of the charge on cell components change with pH.

- The anionic dyes stain better under acidic conditions, where the proteins and many other molecules carry a positive charge.
- A basic dye (or cationic) carries a positive charge eg., Methylene Blue, basic fuchsin, crystal violet, malachite green, safranin.
- Basic dyes bind to negatively charged molecules like nucleic acid and many proteins.
- Since the bacterial cell surfaces are negatively charged, basic dyes are most often used in Bacteriology. Basic dyes are normally available as chloride salts.

- A neutral dye is a complex salt of a dye acid with a dye base.
- The dyes used in bacteriology have two features in common.
 1. They have chromophore groups, groups with double bonds that give the dye its colour
 2. They can bind with cells by ionic, covalent or hydrophobic bonding.

- In positive staining [procedure](#), a stain that has a positively charged chromophore (coloured portion of the stain molecule) is attracted to the negatively charged outer surface of the microbial cell.
- A stain such as Methylene blue has a positively charged blue portion of the molecule that stains the microorganisms.

- In negative staining procedures, a negatively charged chromophore is repelled by the negatively charged microorganisms, resulting in negative or indirect staining of the microbial cell.
- Nigrosin and Indian ink are frequently used for negative staining of microbial cells, and this type of staining is particularly useful for viewing some structures such as capsules that surround some bacterial cells.

Staining Reactions

- When the pH of the surroundings of the microbial cells is either neutral or alkaline, all microbial cells have a negative charge on their surface, called the surface charge.
- Many bacterial cultures produce acids, thereby adding hydrogen ions to a culture medium and decreasing its pH.
- These hydrogen ions interact with the surface of the negative charges on the surface.

- When this happens, the cell surface no longer strongly attracts positively charged dye ions (basic dyes).
- Thus the microbes from acidic environments stain poorly with basic dyes.
- For this reason, the basic dyes are made up as alkaline solutions. For example, potassium hydroxide (OH) is added to solutions of methylene blue to form the stain called Loeffler's Methylene blue.

- Some bacteria excrete alkaline materials during growth and this decreases the number of available hydrogen ions in the culture medium. Under such conditions, the cell surface has a greater negative charge, which is more attractive to basic dyes and therefore, allows greater binding, penetration and internal staining of the microbe.
- Basic dyes stain microorganisms better under neutral or alkaline conditions.
- If the dye base molecule has a negative charge, it is repelled by the cell's negatively charged surface.

- Thus negatively charged dyes neither bind to the cell's surface nor are they able to penetrate into the cell. These are called acid dyes.
- The negatively charged cells are not stained by the negatively charged dye, and they appear as clear area surrounded by a coloured back ground. Negatively charged dyes used in this way are called negative stains.
- Negative stains are of limited usefulness for those using light microscopes, but they can be used to avoid some of the disadvantages of staining with basic dyes.

Simple staining

- A simple staining solution, contains only one stain, which is dissolved in a solvent. It is applied to the microorganism in one application. The microorganisms give the colour characteristic of the staining solution. The purpose of simple staining is to reveal the size and shape of the microorganism. The simple stains that are commonly used for routine purposes are dilute solution of carbol fuchsin, crystal violet and methylene blue.
- Methylene blue is more frequently used than any other stain in bacteriology. It is because of its strong nature and it stains nuclei and nucleic acid granules very intensively. Methylene blue is used for the diagnosis of Diphtheria.

Differential staining

- In this [procedure](#), more than one dye is employed. Differential staining [procedure](#) helps to divide the bacteria into separate groups based on staining characteristics.
- The two most important differential stains used bacteriologists are Gram stain and Acid-fast stain.

Gram Staining

- The [simple staining procedure](#) makes to visualize bacteria clearly, but it does not distinguish between organisms of similar morphology.
- In 1884, a Danish Physician named, Christian Gram discovered a new technique to differentiate the bacteria of similar morphology.
- He used two dyes in sequence, each of a different colour.
- The organisms that retain the colour of the first dye are called Gram positive and those that cannot retain the first dye when washed with a decolourizing solution, but then take on the colour of the second dye are called Gram negative.

Principle of Gram Staining

- The Gram – positive bacteria will retain the crystal violet and appear deep violet in colour.
- The Gram-negative bacteria lose the crystal violet on decolorization and are counter stained by the safranin and appear red in colour.
- Iodine solution is used as a mordant that fixes the primary stain in or on a substrate by combining with the dye to form an insoluble compound-mordant, for the first stain.

- The reactions are associated with the structure and composition of the cell wall.
- The cell walls of Gram-negative bacteria are thinner than that of Gram-positive bacteria and contain a higher percentage of lipid content. During the staining of Gram-negative bacteria, the alcohol treatment extracts the lipid. This results in increased porosity or permeability of the cell wall.
- The crystal violet-iodine complex, thus can be extracted and the Gram-negative bacteria is decolorized. The cells subsequently take up the colour of the counter stain safranin.

- The cell walls of Gram-positive bacteria with lower lipid content become dehydrated during alcohol treatment. The pore size decreased, permeability is reduced and the CV-I complex can - not be extracted. Therefore, the Gram-positive cells remain purple.

Endospore Staining

- Endospore formation is a distinguishing feature of the family *Bacillaceae*, which includes members of the aerobic genus, *Bacillus* and the anaerobic genus, *Clostridium*. Endospore resists adverse environmental conditions such as dryness, heat and poor nutrient supply.
- The endospore is a highly retractile body formed within the vegetative bacterial cell at a certain stage of growth.

- The size, shape, and position of the spore are relatively constant characteristics of a given species and are thereof, of some value in distinguishing the kind of bacillus from another.
- The position of the spore in the cell may be central, sub terminal or terminal. It may be the same diameter as the cell, smaller, or larger causing a swelling of the cell.

- Endospores strongly resist application of simple dyes, but once stained are quite resistant to decolorization. This character suggests one way to make the structure visible.
- If simple stains are used, the body of the bacillus is deeply colored, whereas the spore is unstained and appears as a clear area in the organism. By vigorous staining procedures the dye can be introduced into the substance of the spore.

- When thus stained, the spore tends to retain the dye after treatment with decolorizing agents.
- To make the distinction clear between the spore and the vegetative portion of the cell, a contrasting counter stain is usually applied in the ordinary fashion and the resulting picture shows the initial stain taken up by the spore and the second stain appear in the cytoplasm.
- Thus, it makes for a very simple [method](#) of distinguishing the endospore from the vegetative cell.