INTRODUCTION:

- **Microbial Staining – giving colour to microbes.**
- **Because microbes are colourless and highly transparent structures.**
- **Staining – process in which microbes are stained.**

INTRODUCTION - STAINS

- **Stains/dyes - organic compounds which carries either positive charges or negative charges or both.**

- **Based on the charges:**
- **Basic stain/dyes – stain with +ve charge.**
- **Acidic stain/dyes – stain with –ve charge.**
- **Neutral stain/dyes – stain with both charges.**

- **Based on function of stain:**

1. **Simple staining – only one dye is used- differentiation among bacteria is impossible- Eg. Simple Staining.**

2. **Differential staining- more than one dye is used- Differentiation among bacteria is possible- Eg. Gram's staining, Acid-fast staining.**

3. **Special staining – more than one dye used - Special structures are seen.**
   Eg. Capsule staining, Spore staining.

Principle of staining:

- Each staining methods have own principles but the following steps may be common:

- Basic stain(+ve charge) –
- To stain -ve charged molecules of bacteria
- Mostly used because cell surface is –ve charge.

- Acidic Stain(-ve charge)
  To stain +ve charged molecules of bacteria.
  Used to stain the bacterial capsules.

- As cell surface is –ve charged- Basic dyes mostly used.

Basic requirements for staining:

- Clean grease-free slide.
- Bacteria tobe stained.
- Inoculating loops- to transfer bacterial suspension to slide.
- Bunsen burner – to sterilise inoculating loops before and after smear preparation.
- Pencil marker – to mark (particularly central portion of slide) where bacterial smear is applied

Basic initial steps before staining:

- ***Smear preparation:***
- **Putting of bacterial suspension (bacteria in liquid) tobe stained on the central portion of slide in a circular fashion, air-dried, heat-fixed, the resultant preparation called *bacterial smear*- appears dull white.**

:Smear preparation

:The goals of preparing a good smear are

Allow the cells to adhere to the surface of the slide so that they are .1 .not washed off during staining

Allow the cells to adhere properly so that they do not shrink during .2 .staining(preserve their morphology)

.To attenuate pathogenicity of bacteria and work with it safely .3

A thin smear allows only one layer of cells to adhere otherwise you .4 will get layers of cells on top of each other and you will not be able to !examine the individual cells

SIMPLE STAINING:

- Simple to perform- only one basic stain used.

Eg. Crystal violet, Methylene blue, Basic fuschin, Malachite green etc.,

Principle:

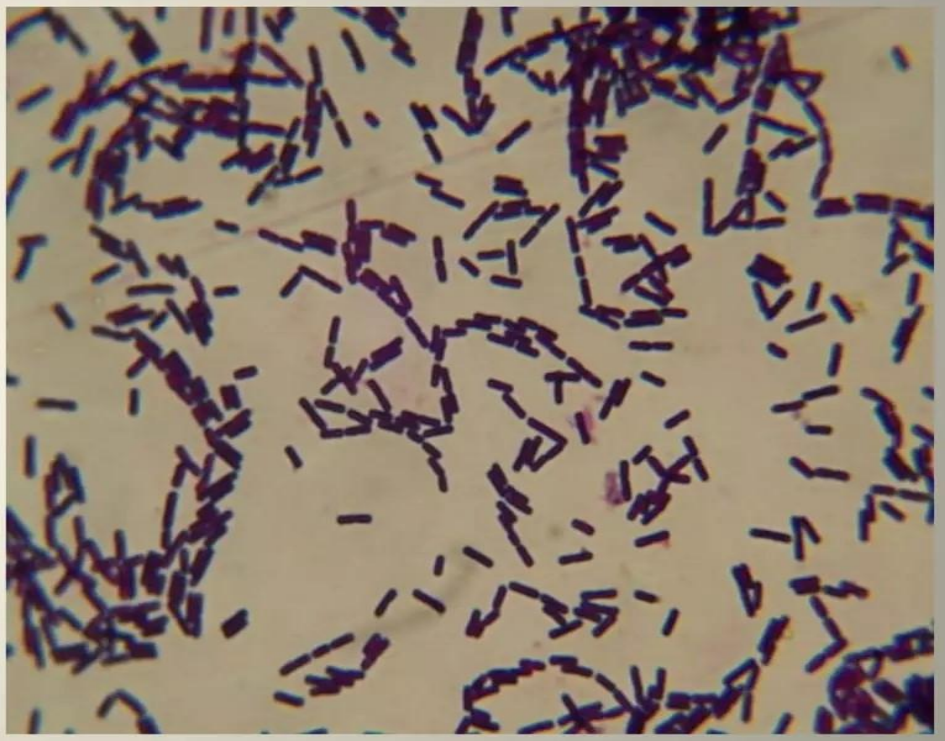
- All bacteria in smear takes stain and appears in colour of stain.
- Basic stain more affinity towards bacterial surface & stains the bacteria.

Uses:

To study morphology and arrangement of bacteria.

Procedure:

- A bacterial smear is prepared, air-dried and heat-fixed.
- A Heat-fixed smear is flooded with either one of the basic stain and allowed to react for 1-2 minutes and then washed under running tap water.
- Air dried and focussed with 10x,45x & 100x.



Results:

- Morphology – spherical / rod.
- Arrangement – cocci – clusters/chains.

GRAM STAINING :

- **DANISH BACTERIOLOGIST HANS CHRISTIAN GRAM (1880)**
- **Based on this reaction, bacteria classified into Gram positive and Gram negative bacteria.**
- **The cell wall compostion differences makes difference.**

REQUIREMENTS – STAINING REAGENTS:

1. Crystal violet – Primary stain
2. Gram's iodine- mordant/fixative
3. Acetone (95%)- decoloriser
4. Safranine/dilute carbol fuchsin – counterstain

PRINCIPLE:

1. Crystal violet - all bacteria take crystal violet- so all appears violet.
2. Iodine – Crystal Violet-iodine(CV-I) complex is formed.
3. Acetone- bacteria with high lipid content loose CV-I complex(appear colouless) but bacteria with less lipid content retains CV-I complex (appear violet).
4. Safranine/ dilute carbol fuchsin – only colouless bacteria takes – appear pink.

Principle of Gram Stain

1) In aqueous solutions crystal violet dissociates into CV+ and Cl – ions that penetrate through the wall and membrane of both gram-positive and gram-negative cells. The CV+ interacts with negatively charged components of bacterial cells, staining the cells purple.

When added, iodine interacts with CV+ to form large Crystal violet iodine (CV-I) (2 complexes within the cytoplasm and outer layers of the cell

The decolorizing agent, (95% ethanol or an ethanol and acetone solution), interacts (3 with the lipids of the membranes of both gram-positive and gram-negative bacteria.The outer membrane of the gram-negative cell (lipopolysaccharide layer) is lost from the cell, leaving the peptidoglycan layer exposed. Gram-negative cells have thin layers of peptidoglycan. With ethanol treatment, gram-negative cell walls become .leaky and allow the large CV-I complexes to be washed from the cell

4) The highly cross-linked and multi-layered peptidoglycan of the gram-positive cell is dehydrated by the addition of ethanol. The multi-layered nature of the peptidoglycan along with the dehydration from the ethanol treatment traps the large CV-I complexes within the cell.

5) After decolorization, the gram-positive cell remains purple in color, whereas the gram-negative cell loses the purple color and is only revealed when the counterstain, the positively charged dye safranin, is added.

PROCEDURE:

- Crystal violet – 1 min - wash.
- Iodine – 1 min – wash.
- Acetone add drop by drop and watch out colour comes out – wash immediately.
- Safarnine/dilute carbol fuchsin – 1 min- wash.
- Allow to dry – examine under microscope.

Note: Results should be confirmed only with 100x.

RESULT:

Colour:

Purple colored bacteria – Gram positive

Pink colored bacteria – Gram negative

Shape:

Spherical – cocci

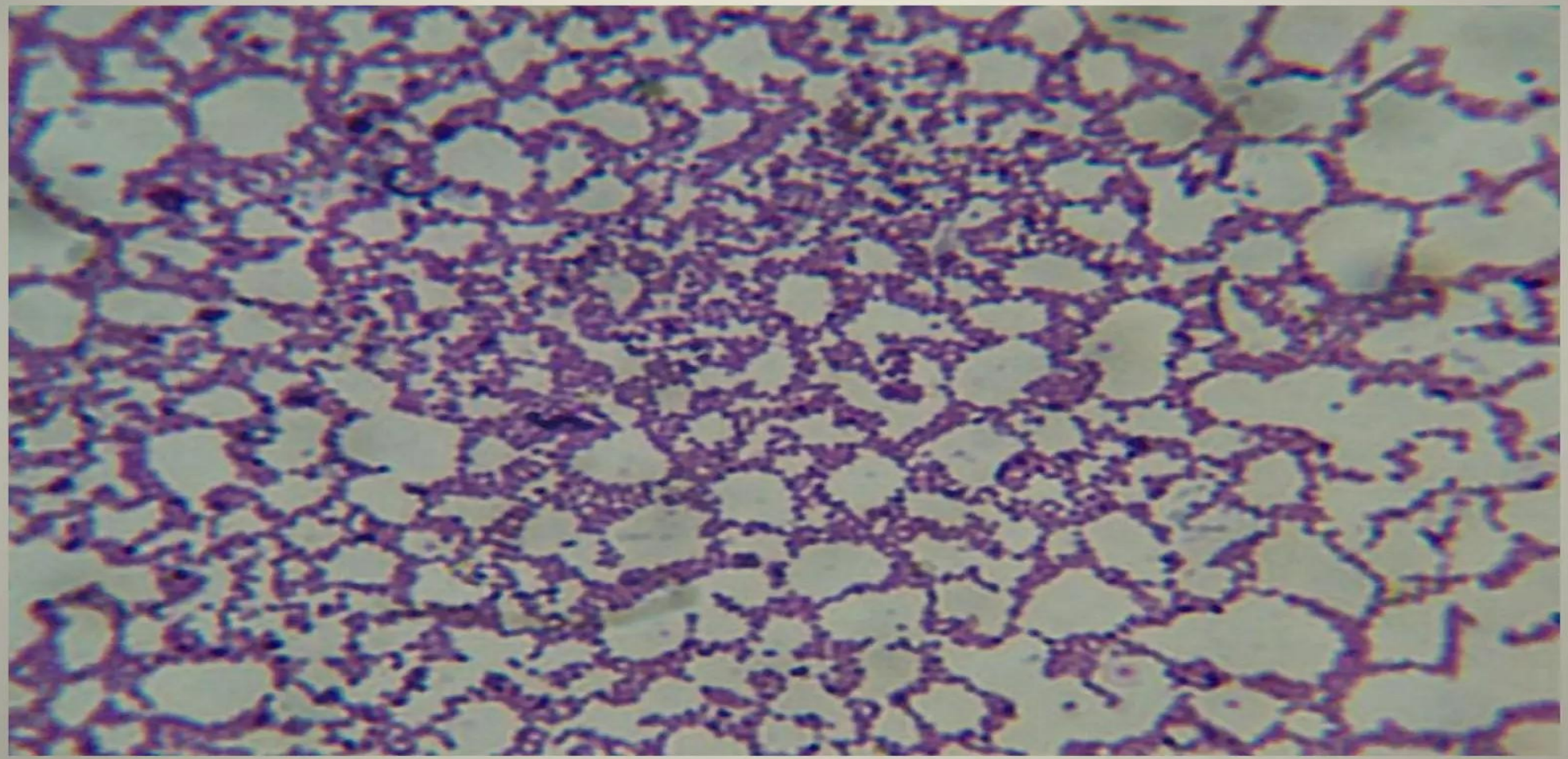
Rod – bacilli

Arrangement

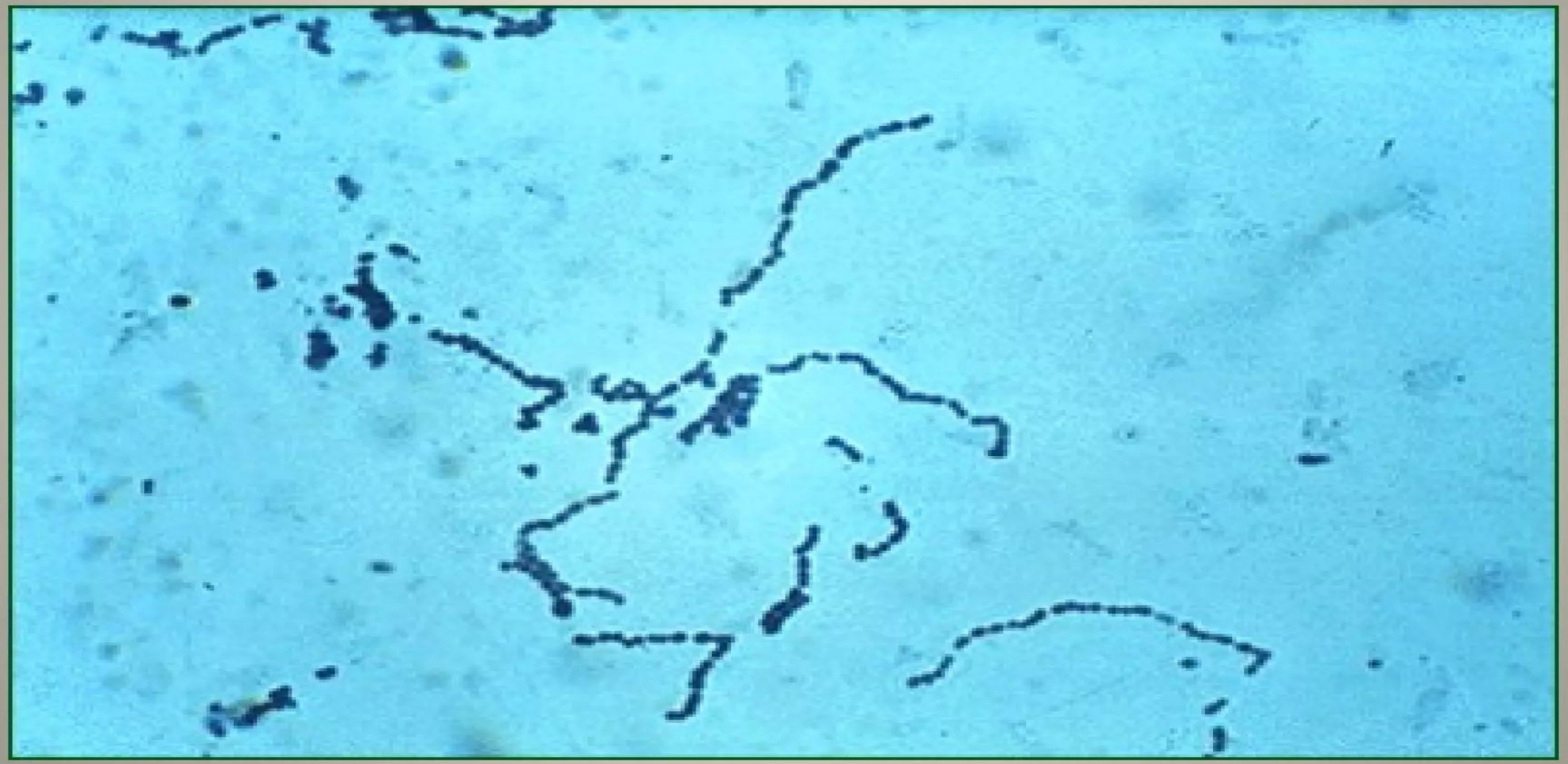
Cocci in clusters – staphylococci

Cocci in chains - streptococci

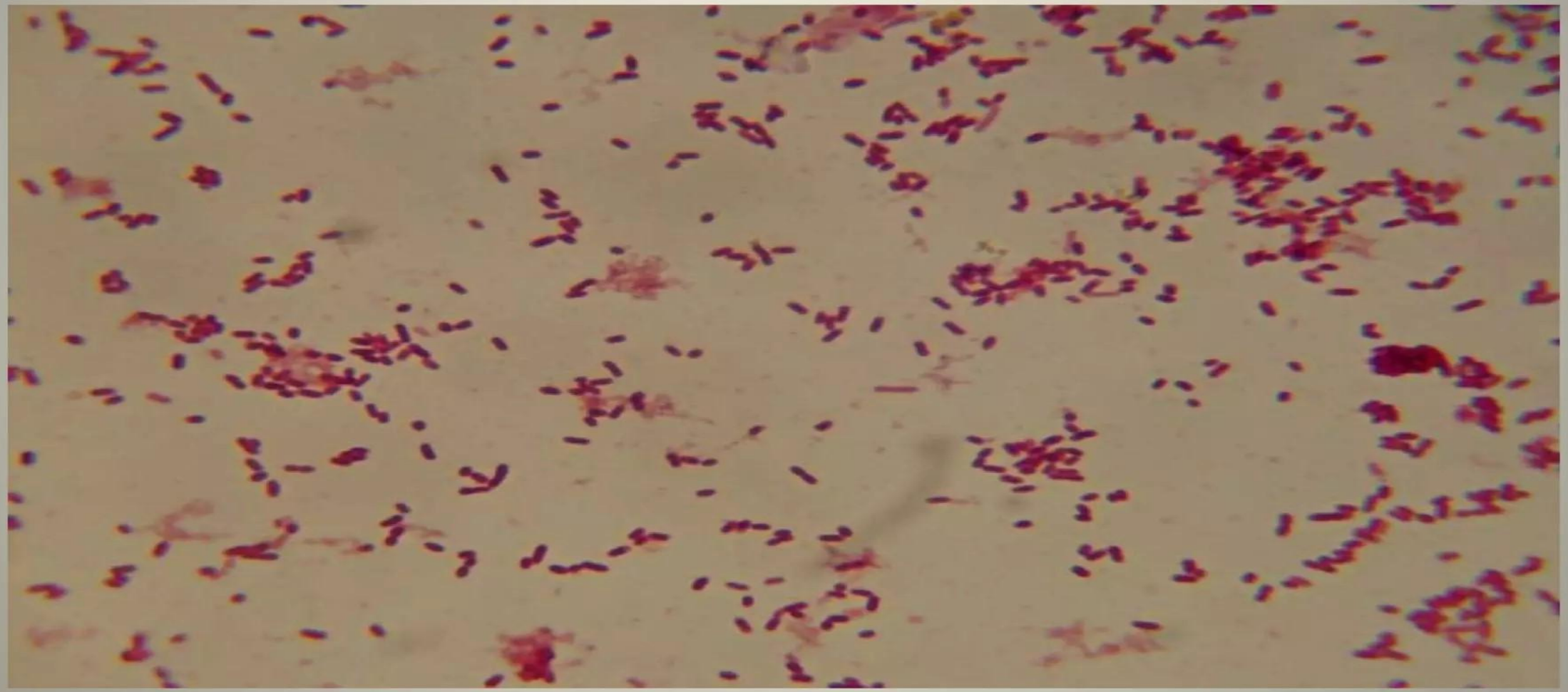
GRAM POSITIVE COCCI IN CLUSTERS



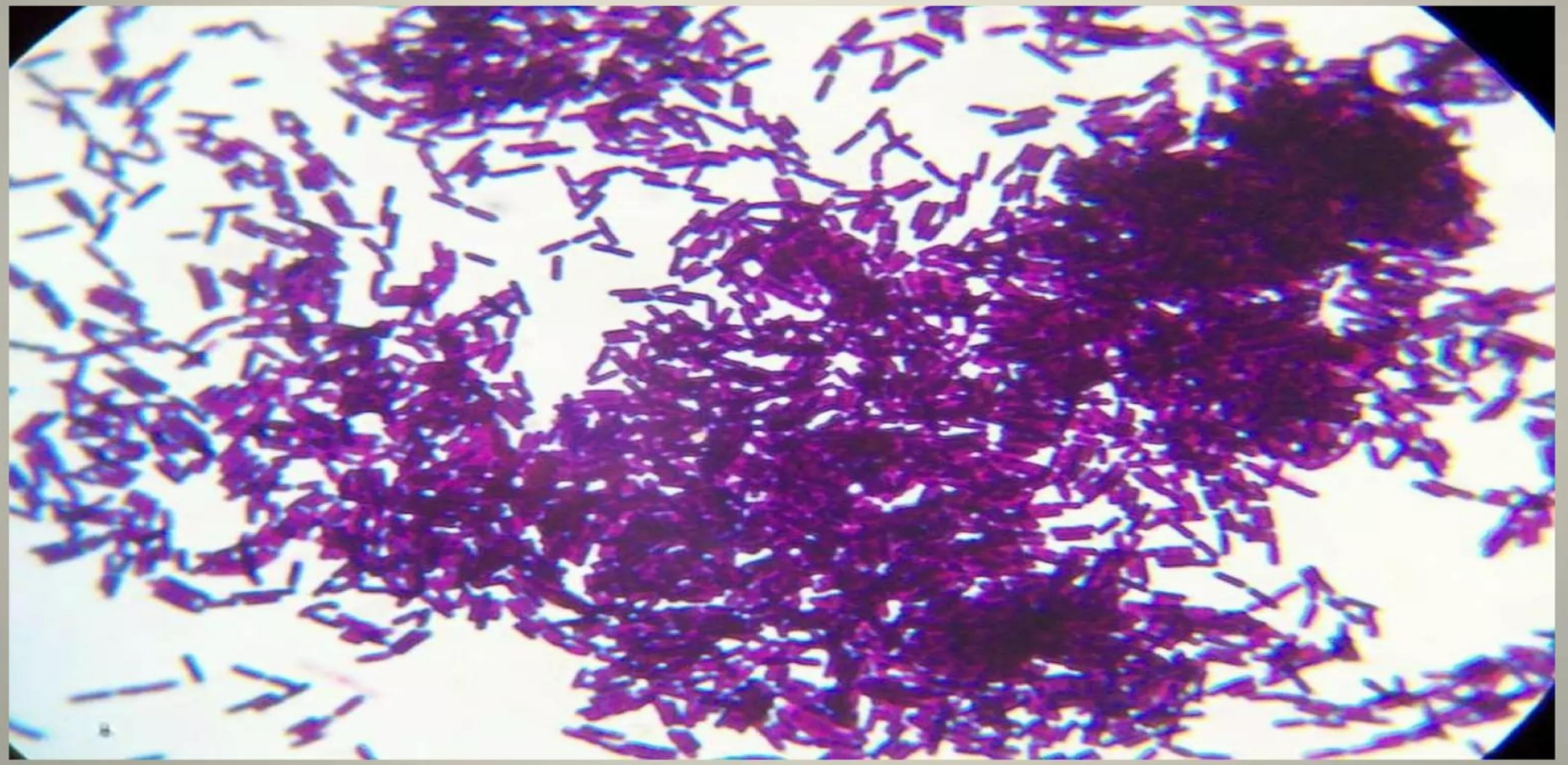
GRAM POSITIVE COCCI IN CHAINS



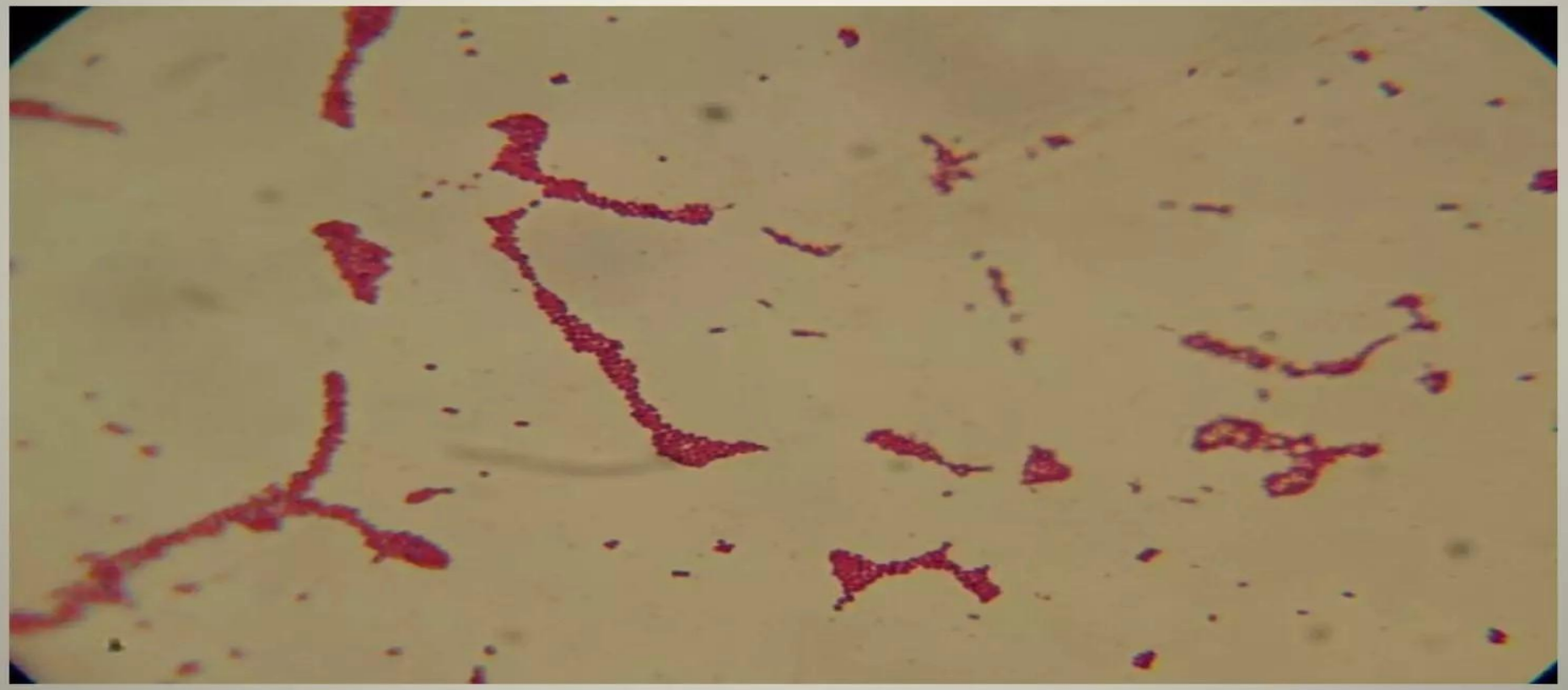
GRAM NEGATIVE BACILLI



GRAM POSITIVE BACILLI



GRAM NEGATIVE COCCI



The Gram stain is a differential stain commonly used in the microbiology laboratory that differentiates bacteria on the basis of their .cell wall structure

Most bacteria can be divided into two groups based on the composition :of their cell wall

1. Gram positive bacteria (thick layer of peptidoglycan-90% of cell wall and low content of lipid)- stains with purple

2. Gram negative bacteria (thin layer of peptidoglycan-10% of cell wall and high content of lipid) –stains with red/pink

The Cell wall