

UNIT-II

Staining Techniques



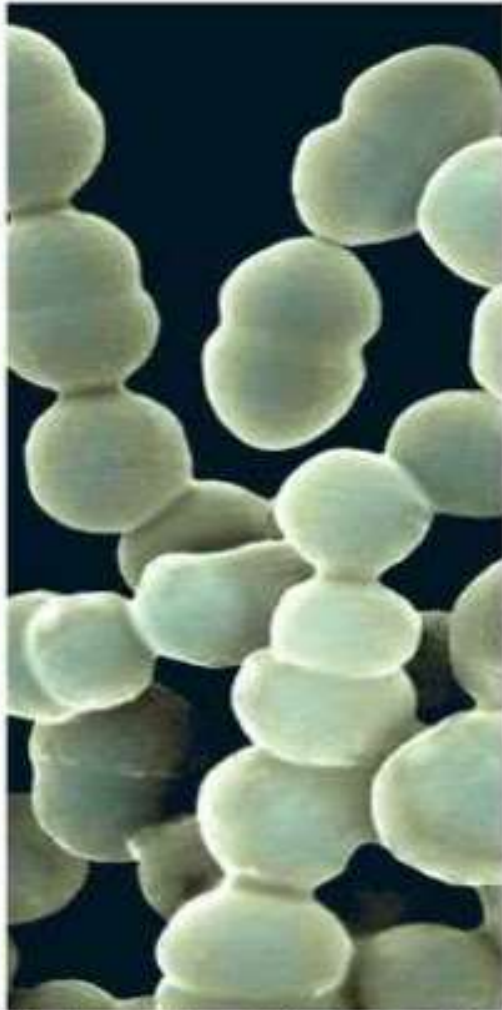
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RASMITA JENA
ASSISTANT PROFESSOR
SoPLS, CUTM, BBSR

Introduction

- **Staining** – Majority of the stains used for staining bacteria are of the basic type as nucleic acid of bacterial cells **attract the positive ions**, e.g. methylene blue, crystal violet. **Acidic** stains are used for **background staining**.

Microscopic Examination : Cell Shape



Spherical (cocci)

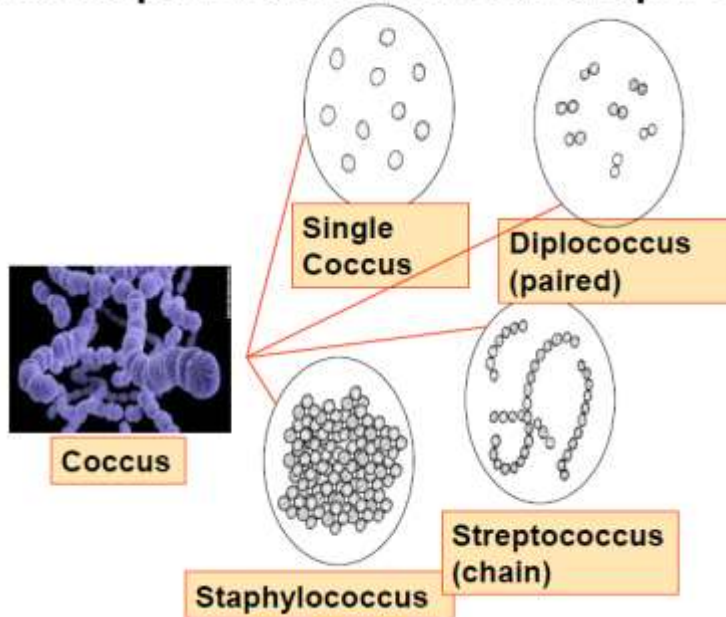


Rod-shaped (bacilli)

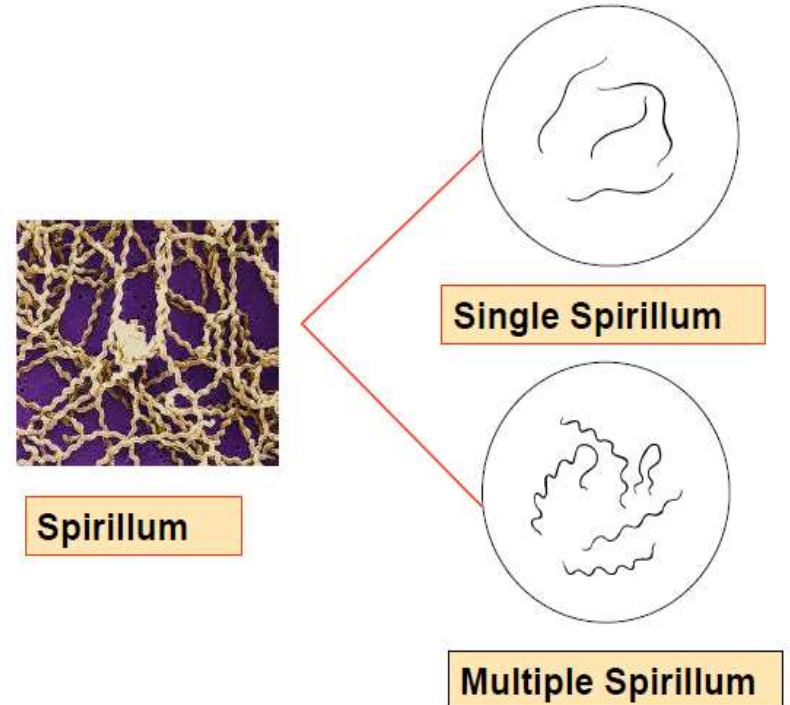


Spiral

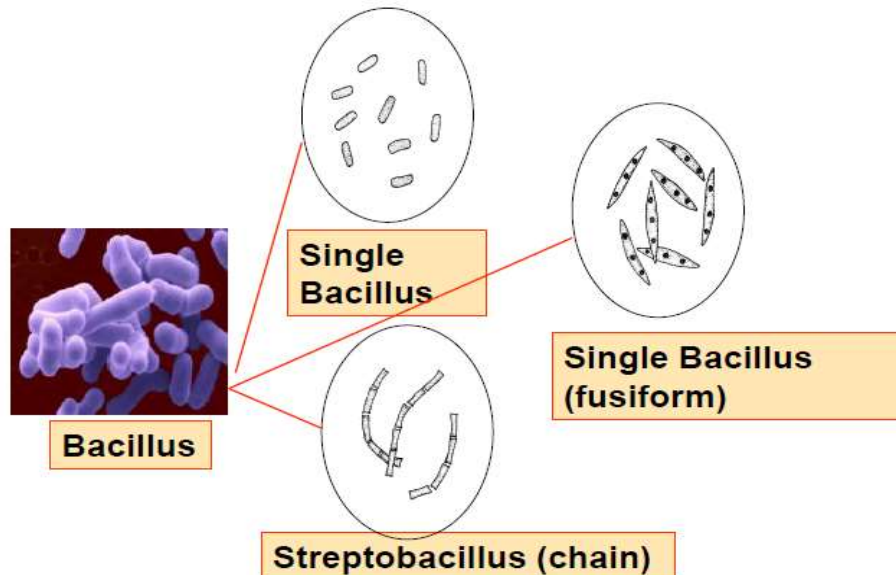
Microscopic Examination: Cell Shape: Coccus



Microscopic Examination: Cell Shape: Spirillum



Microscopic Examination: Cell Shape: Bacillus



Purpose of Staining

- **Bacteria cells are almost *colorless* and *transparent***
 - To render microscopic and semitransparent objects visible.
 - To reveal their shape and size.
 - To show the presence of various internal and external structures.
 - To produce specific physical or chemical reactions.

Types of staining techniques

Simple staining

(Use of of single stain)

Direct

(Positive)

Indirect

(Negative)

Differential staining

(Use of two contrasting stains)

Separation

into groups

1. Gram stain

2. Acid fast

stain

Visualization

of structures

1. Flagella stain

2. Capsule stain

3. Spore stain

SIMPLE STAINING

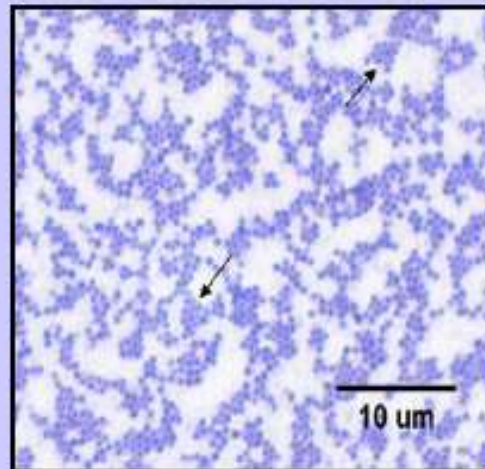
- The use of a single stain to colour a bacterial organism is commonly referred to as simple staining.
- All these dyes work well on bacteria as they have colour bearing ions (chromatophores) and are positively charged.
- The fact that bacteria **are slightly negatively** charged when the pH of the surrounding is near neutrality and produces a pronounced attraction between these **cationic chromatophores** and the organism so that the cell is stained.

- Such dyes are classified as **basic dyes**. **Crystal violet** and **Carbol fuchsin** are some other examples.
- Those dyes that have **anionic chromatophores** are called **acidic dyes**. **Eosine (sodium+eosine-)** is such a dye.
- The **anionic chromatophores, eosine- , will not stain bacteria** because of the **electrostatic repelling forces** that are involved.

- The staining times for most simple stains are relatively short, usually from **30 seconds to 2 minutes**, depending on the affinity of the dye.
- After a smear has been stained for the required time, it is washed off gently, blotted dry, and examined directly under oil immersion.
- Such a slide is useful in determining basic morphology and the presence or absence of certain kinds of granules.

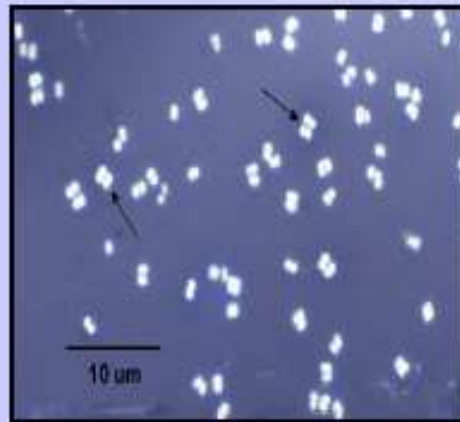
Direct staining (Positive staining)

- A simple staining technique that stains the bacterial cells in a single color.
- Many of the bacterial stains are basic chemicals; these basic dyes react with negatively charged bacterial cytoplasm (opposite charges attract) and the organism becomes directly stained
- Examples are methylene blue, crystal violet, and basic fuchsin.



Indirect staining (Negative staining)

- In this staining process, instead of cells background is stained.
- Here, an acidic dye like nigrosin or Indian ink is used. Acidic stain carries a negative charge and repelled by the bacteria, which also carry a negative charge on their surface. Hence, an acidic dye do not stain bacteria,
- Instead, it forms a deposit around the organism, leaving the organism itself colorless or transparent upon examination.



Bacteria have a slight (-) charge.

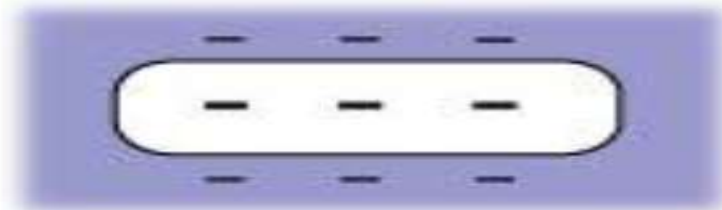


**basic dye
color portion is (+)**

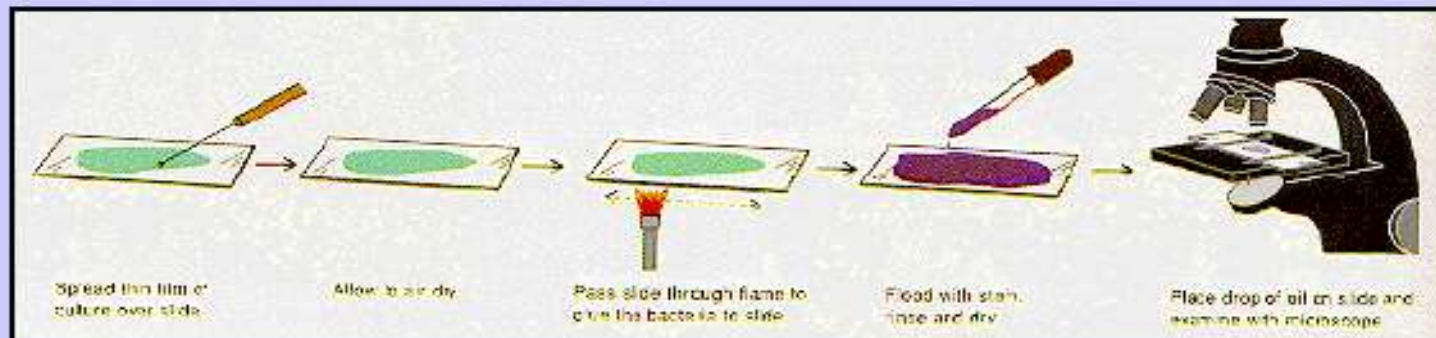
**acidic dye
color portion is (-)**



direct stain



indirect stain



Spread thin film of culture over slide

Allow to air dry

Pass slide through flame to cure the bacteria to slide

Flood with stain, rinse and dry

Place drop of oil on slide and examine with microscope

SMEAR

AIR DRY

HEAT FIX

STAIN

LOOK

Differential Staining

Gram staining :

- Developed by **Christian Gram in 1884**; by using this procedure, bacteria are subdivided by their reaction to this stain into those which retain it, termed Gram-positive, and those which are decolourized, termed Gram-negative. Gram staining requires 4 different solutions.
 - (i) **A Basic Dye:** Discussed previously.
 - (ii) **A Mordant:** It increases the affinity or attraction between the cell and the dye, e.g. Iodine.
 - (iii) **A Decolorising agent:** It removes the dye from a stained cell, e.g., alcohol, acetone or ether.
 - (iv) **Counter stain:** It is a basic dye of a different colour than the initial one, e.g., Safranin.

Stain Preparation

- (i) **Crystal Violet:** Dissolve 2 g of crystal violet in 20 mL of 95% ethanol. Dissolve 0.8 g of ammonium oxalate in 80 mL of distilled water. Mix these 2 solutions; stand for 24 h and filter.
- (ii) **Gram's Iodine:** Dissolve 2 g of potassium iodide and 1 g of iodine in 300 mL of distilled water.
- (iii) **Safranin:** Grind 0.25 g of Safranin in a mortar with 10 mL of 95% ethanol.

Principle

- Gram-positive cell walls have a **thick peptidoglycan** layer beyond the plasma membrane.
- Characteristic polymers called **teichoic and lipoteichoic** acids stick **out above the peptidoglycan** and it is because of **their negative charge that the cell wall is overall negative.**
- These acids are also very important in the body's ability to recognize foreign bacteria. Gram-positive cell walls stain blue/purple with the Gram stain.

- Gram-negative cell walls are more complex. They have a thin peptidoglycan layer and an outer membrane beyond the plasma membrane.
- The space between the plasma membrane and the outer membrane is called the periplasmic space.
- The outer leaflet of the outer membrane is composed largely of a molecule called lipopolysaccharide (LPS). LPS is an endotoxin that is important in triggering the body's immune response and contributing to the overall negative charge of the cell.
- Spanning the outer membrane are porin proteins that enable the passage of small molecules.
- Lipoproteins join the outer membrane and the thin peptidoglycan layer. Gram-negative cells will stain pink with the Gram stain.

Procedure

1. Prepare smear on a clean slide.
2. Stain with crystal violet for 30 seconds.
3. Rinse with water.
4. Flood the film with Grams iodine and allow it to act for 30 sec.
5. Rinse with water.
6. Decolorise with 95% alcohol.
7. Rinse with water.
8. Counter stain with safranin for 20-30 sec.
9. Rinse with water and blot dry.
10. Examine under oil immersion objective.

Table 6.1 Steps in Gram Stain

Step	Process	Results	
		Gram+	Gram-
Initial stain	CV for 30 sec.	Stains purple	Stains purple
Mordant	12 for 30 sec.	Remains purple	Remains purple
Decolorisation	95% alcohol for 10-20 sec.	Remains purple	Becomes colourless
Counterstain	Safranin for 20-30 sec.	Remains purple	Stains pink

Acid fast stain

- The **Ziehl Neelsen** method employs **carbol fuchsin**, alcohol and a blue or green counter stain.
- Acid fastness is a phenomenon that is found in certain types of bacteria where they resist the process of de-colorization that occurs when acid is used to wash a sample that contains these bacteria.
- These bacteria also resist staining and it may require heat and concentrated staining to colorize them.

- Once this colorization has taken place, it becomes difficult to decolorize using acid, or stain them with another color unless the heat and concentration technique is used.
- This is why the term given to them is 'acid fast' just like one would use the term 'color fast' for cloth that does not leak color when washed.

ACID-FAST STAIN

Procedure	Reagent	Cell Color	
		Acid-fast Bacteria	Nonacid-fast Bacteria
Primary dye	Carbolfuchsin	RED	RED
Decolorizer	Acid-alcohol	RED	COLORLESS
Counterstain	Methylene blue	RED	BLUE

Stain Preparation

(i) Carbol fuchsin

1. Basic fuchsin 58 g
 2. Crystalline phenol 25 g
 3. 95% alcohol 50 mL
 4. Distilled water to 500 mL
- Dissolve the fuchsin and phenol in alcohol over a warm water bath, and then add water. Filter, before use.

(ii) 95% ethyl alcohol 970 mL

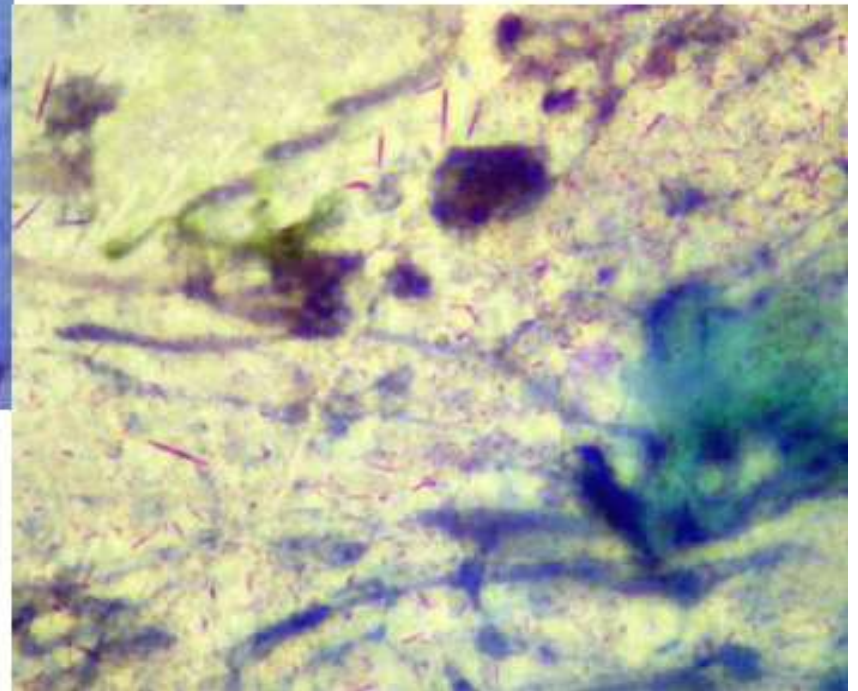
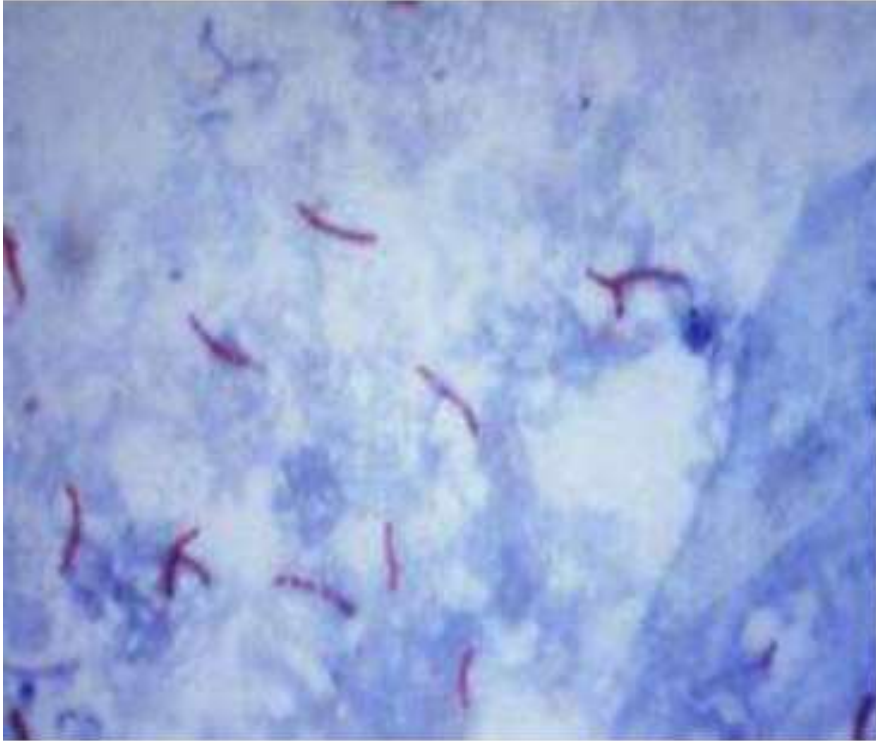
- Concentrated HCl 30 mL

(iii) 0.5% methylene blue or malachite green in distilled water

Procedure

- Pour **carbol fuchsin** on the slide and heat carefully until steam rises.
- Stain for 3-5 min but do not allow to dry.
- Wash well with water.
- Decolorize using acid alcohol for 10-20 s.
- Changing twice and counter stain for 2 min with **methylene blue or malachite green**.
- Acid fast organisms, e.g. *Mycobacterium tuberculosis* and *Mycobacterium leprae* stain well .

Acid fast bacteria



Structural Staining

- **Endospore stain**
- **Capsule stain**
- **Flagella stain**

Endospore stain

- Species of bacteria, belonging principally to the genera *Bacillus* and *Clostridium* produce **extremely heat resistant structures** called **endospores**.
- In addition **to being heat-resistant, they are also resistant to many chemicals that destroy non-spore forming bacteria.**
- This resistance to heat and chemicals is due primarily to a thick, tough spore coat.
- They resist staining and, once stained they resist decolorisation and counter staining. Several methods are available that employ heat to provide stain penetration.
- However, the malachite green-Schaeffer and Fulton and Dorner methods are commonly used by most bacteriologists. Thus endospore stains green but rest of the cell or a cell without endospore stains light red

Endospore staining



The malachite green -Schaeffer and Fulton Method

1. Prepare a thin smear on a clean slide.
2. Place the slide on staining rack above boiling water.
3. Cover the smear with small pieces of paper towel, keep saturated with malachite green (5% aqueous solution) and continue heating for 5 min.
4. Wash gently with water.
5. Counter stain with safranin for 30 seconds.
6. Wash with water and blot dry.
7. Examine under oil immersion objective.

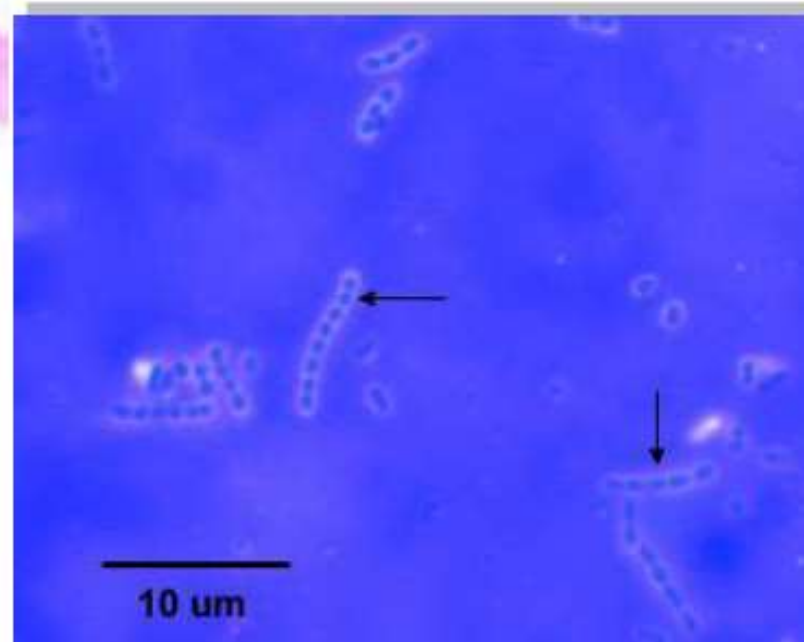
Dorner method

- The Dorner method for staining endospores produces a red spore within a colourless sporangium.
- Nigrosine is used to provide a dark background for contrast.
- The sporangium and endospore are stained during boiling in step 3, however, the sporangium is decolourized by the diffusion of safranin molecules into the nigrosine.
- The six steps involved in this technique are illustrated above

Capsule stain

- Place a small drop of Indian ink on a clean slide. Mix into it a small loopful of bacterial culture. Drop a cover glass and blot off excess ink. Examine.
- For dry preparations mix one loopful of Indian ink with one loopful of suspension of organism in 5% dextrose solutions at one end of a slide.
- Allow to dry and pour a. few drops of methyl alcohol on it keep the slide over the flame to fix. Stain for a few sec. with 0.5.% aq. solution of methyl violet. The capsule will appear as haloes in blue cell of bacterium under microscope

Capsule Staining



Flagella stain

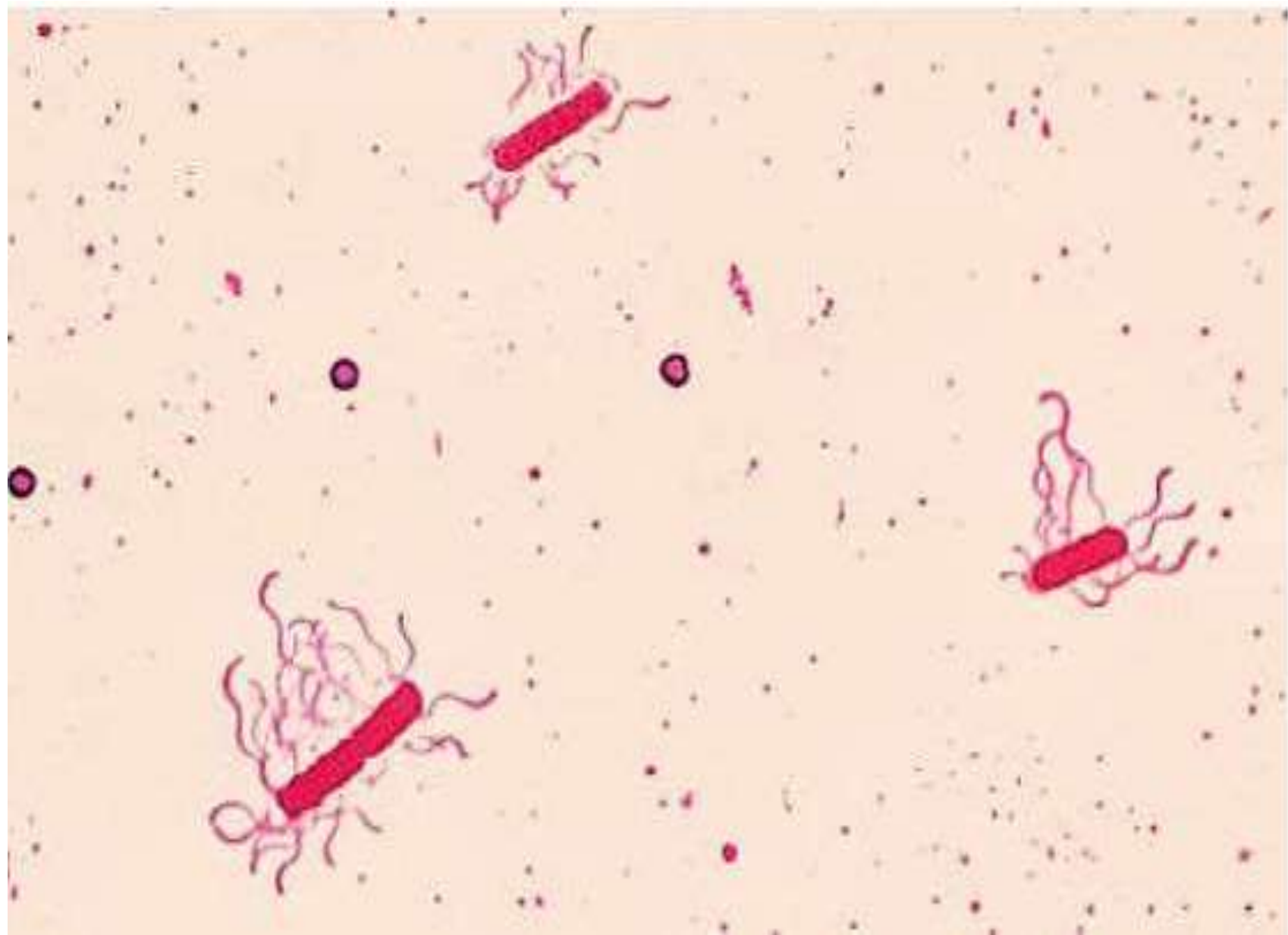
(i) Mordant

- 20% tannic acid solution 3 parts
- 5% tartar emetic solution 2 parts
- Distilled water 5 parts
- Mix and redissolve the heavy precipitate by boiling.

(ii) **Silver solution:** Dilute a saturated solution of silver sulphate with an equal volume of distilled water. Add 33% monoethylamine till the precipitate formed is re-dissolved. Solution can be stored for long term, heat gently before using .

Procedure

1. Take a clean slide and transfer a loopful of bacterial suspension holding the slide vertically. Allow the drop to run to the other end. Dry the film in air or in incubator.
2. Heat the mordant in a test tube to a boiling point and flood the slide with it wait for 2 minutes. Wash well in hot- water and finally in cold water. Allow to dry.
3. Cover the film with silver solution till thick portion of the film becomes dark to brown and metallic sheen appears on the edge.
4. Wash with water and blot dry.
5. For preparing a permanent slide dip the latter in a weak solution of gold chloride for 1 or 2 hr. Wash and mount in canada balasam.



IMViC tests

- IMViC reactions are a set of four useful reactions that are commonly employed in the identification of members of family **enterobacteriaceae**.
- The four reactions are: **Indole** test, **Methyl Red** test, **Voges Proskauer** test and **Citrate utilization** test.

IMViC Tests

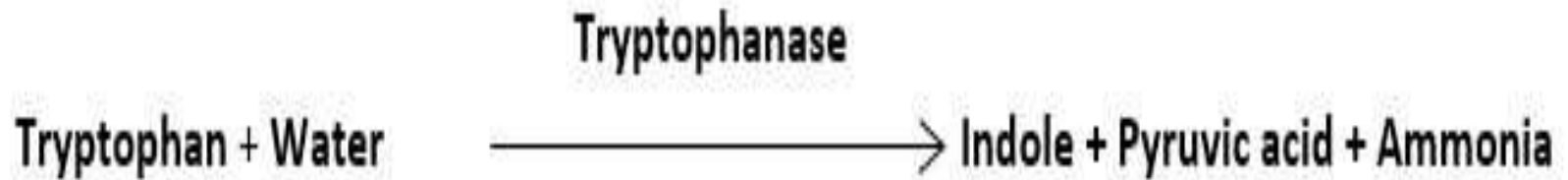


Objectives of IMViC Test

- To study some biochemical properties – **indole** production, **acid** production, **acetyl methylcarbinol (acetoin)** production, and **citrate** utilization – of isolated unknown bacteria in order to characterize and identify them.
- To selectively **differentiate and identify** members of the *Enterobacteriaceae* family.

Indole test

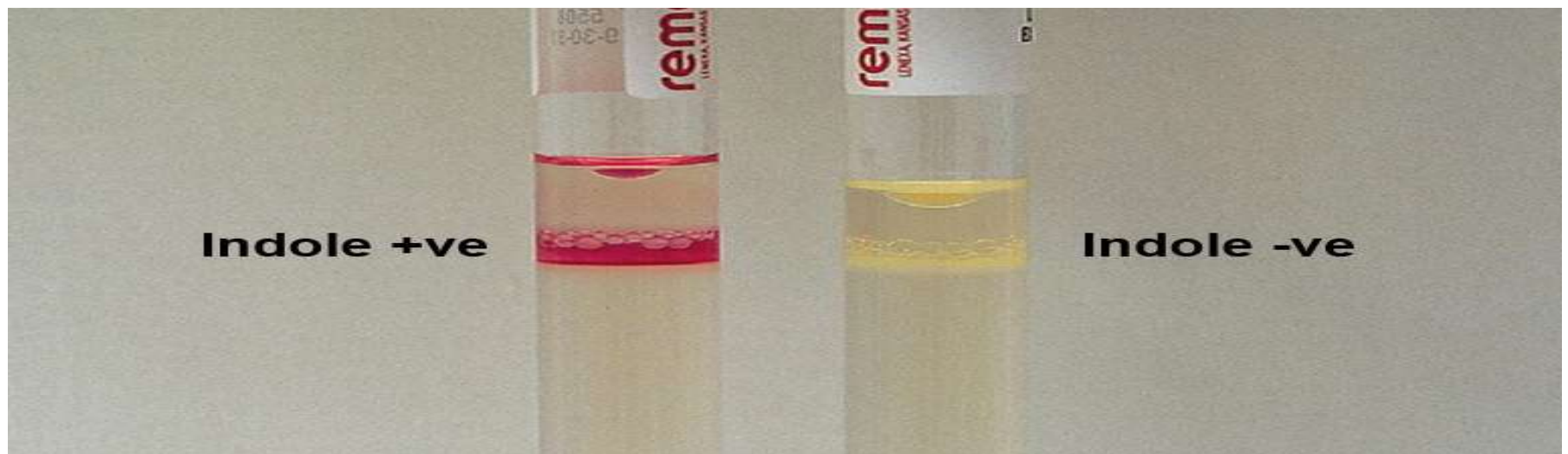
- The indole test is a biochemical test in the IMViC test series which detects **the ability of organisms (bacteria) to produce indole** as a metabolic product utilizing tryptophan.



Principle of Indole Test

- Some bacteria can produce an enzyme called '**tryptophanase**' which helps them to metabolize the amino acid '**tryptophan**' into '**indole, pyruvic acid, and ammonia**'.
- When the indole reagent is added to a medium with a bacterial culture that has produced indole; then **indole** combines with the **aldehyde** present in the reagent to give a **distinctive color (red)**.
- If **benzaldehyde** is present in the reagent, a **pink to a violet-red** quinoidal compound is formed, hence; a **pink to red color ring** is formed.
- If **cinnamaldehyde** is present in the reagent, a **blue to the green** color compound is formed, hence; a **green to blue color ring** is formed.

- **Kovac's indole reagent** uses **amyl alcohol** and **benzaldehyde**. The **amyl alcohol** is **water-insoluble** and forms an **oily layer**, thus giving a **cherry-red or pink – red ring** on the top.
- Test bacteria are cultured in the **medium containing tryptophan** for **24 – 48 hours** and an **indole reagent** is added following the incubation to read the result.
- **A positive result** is indicated by the **formation of a pink to violet-red or green to blue color** ring according to the type of reagent used.
- **A negative result** is indicated by the formation of a **lack of color change or a slight yellowish color ring** at the top.

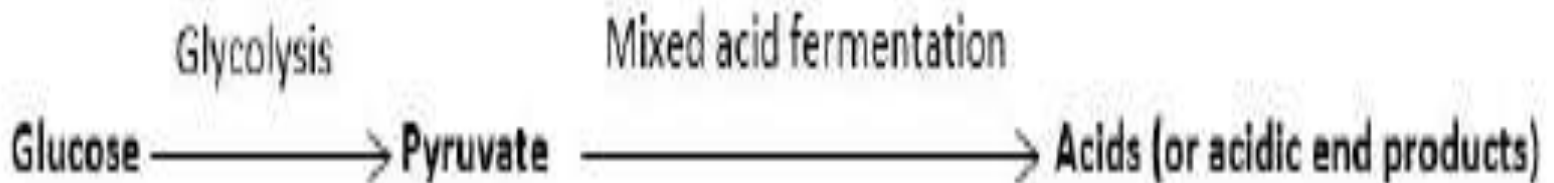


Indole Positive Bacteria: *Escherichia coli*, *Klebsiella oxytoca*, *V. cholerae*, *Proteus vulgaris*, *Porphyromonas asaccharolytica*, *Vibrio spp.*, *Flavobacterium spp.*, *Providencia spp.*, *Enterococcus faecalis*, *Haemophilus influenzae*, *Morganella morganii*, *Aeromonas spp.*, *Citrobacter koseri*,

Indole Negative Bacteria: *Klebsiella pneumoniae*, *Proteus mirabilis*, *Salmonella spp.*, *Shigella spp.*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, *Bacteroides fragilis*, *Staphylococcus aureus*,

Methyl red test

- Methyl Red (MR) Test is a biochemical test that detects the **ability of organisms**(bacteria) **to produce stable mixed acids as metabolic end products of glucose metabolism.**

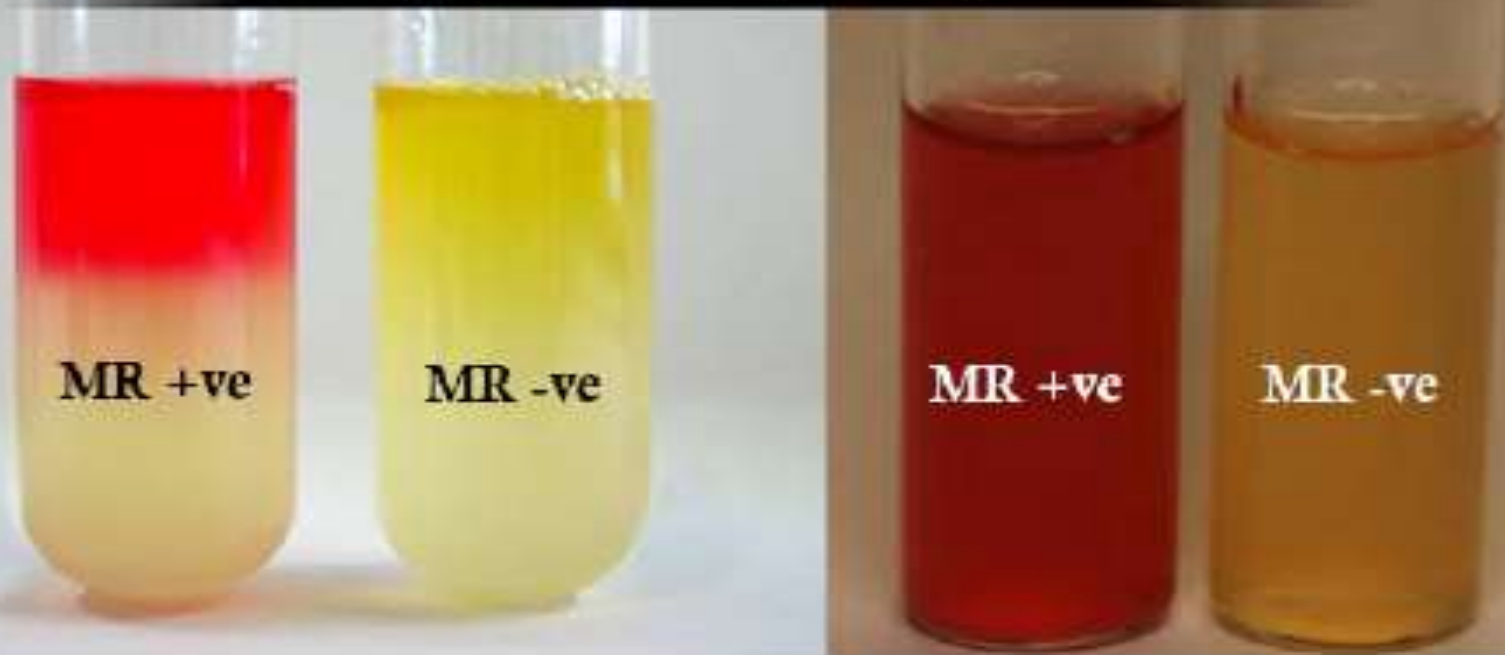


Principle of methyl red test

- Some species of bacteria use the mixed acid fermentation pathway as their glucose metabolism process.
- Following this metabolic pathway, they convert **pyruvate into stable mixed acids.**
- When such acid fermenters bacteria are grown in a **medium containing glucose** (or carbohydrate), they will release the acids, hence; **decreasing the pH of the medium to 4.4 or lower.**

- When **methyl red indicator** is added in a medium containing such acid fermenters, it will turn the **medium in to red colour.**”
- Following the **24-hour incubation** on the **MR-VP broth (glucose phosphate broth)**, a methyl red indicator is added to the broth.
- **A positive result** is indicated by the development of **red color** while a **negative result** is indicated by the development of **yellowish color**.

Methyl Red (MR) Test



MR Test.

MR Positive Bacteria: *Escherichia coli*, *Salmonella spp.*, *Shigella spp.*, *Citrobacter spp.*, *Proteus spp.*, *Yersinia spp.*, *Edwardsiella spp.*, *Staphylococcus aureus*,

MR Negative Bacteria: *Klebsiella pneumoniae*, *Enterobacter spp.*, *Hafnia spp.*, *Serratia marcescens*.

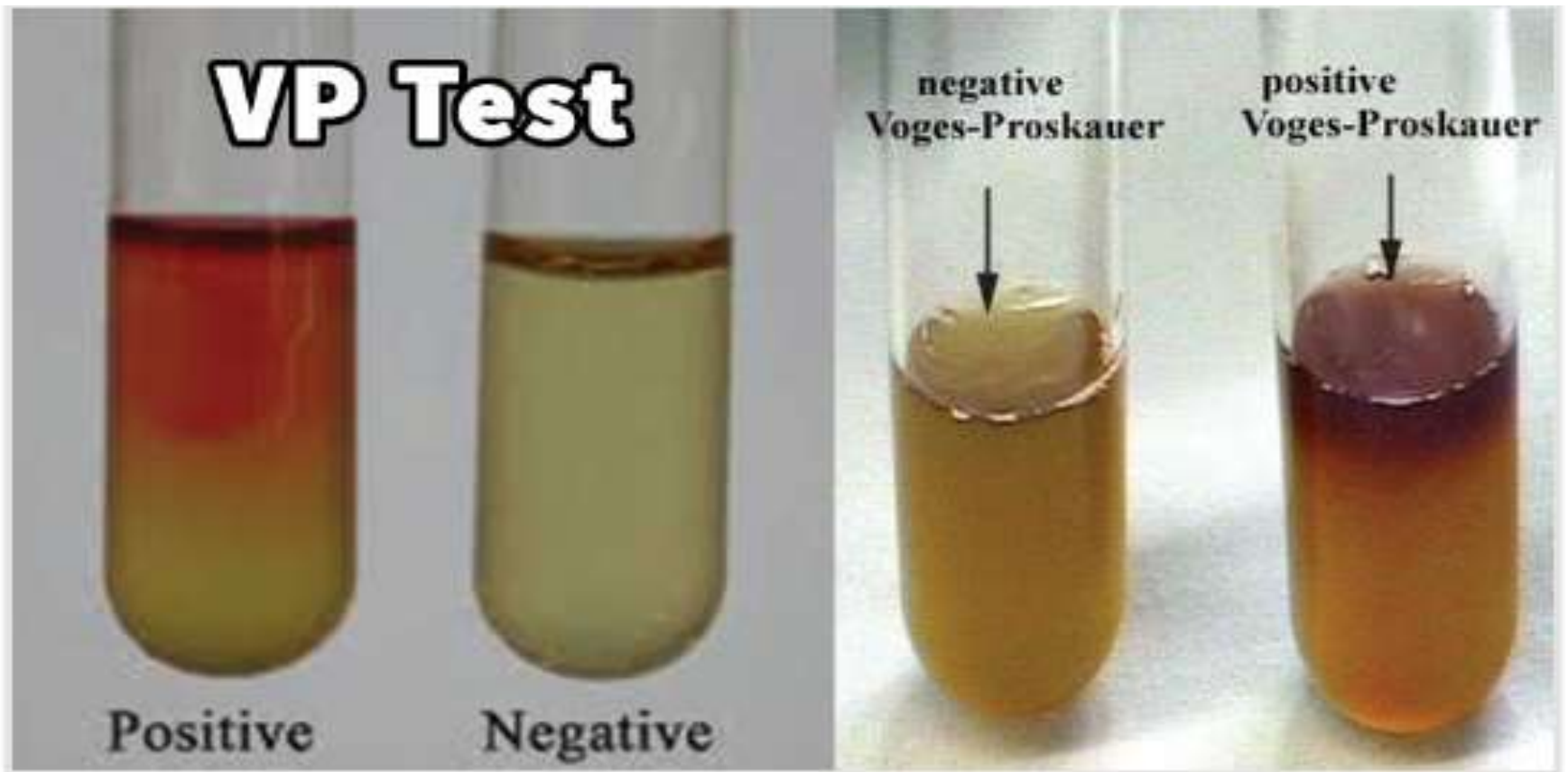
Voges- Proskauer test

- Voges-Proskauer (VP) Test is a biochemical test in the IMViC test series which detects the **ability of organisms (bacteria) to metabolize the pyruvate into a neutral intermediate product called 'acetyl methyl carbinol' or 'acetoin'**.

Principle:

- Pyruvate can be metabolized into a neutral intermediate product called 'acetyl methyl carbinol', commonly called the 'acetoin' during the **butanediol pathway of 2,3-butanediol production.**

- If **acetoin** is present in the media, it is oxidized readily to **diacetyl** in **presence of air and KOH**.
- Thus produced **diacetyl**, in the presence of α – **naphthol**, will react with the **guanidine component of peptone** forming a **pink to a red colored product**.
- Following the **48-hour aerobic incubation** on **MR-VP broth**, **VP reagents I and II** are added and the color change is observed within 30 minutes.
- **A positive result** is indicated by the **development of pink – red color at the top of the broth immediately or within 30minutes but not more than 1 hour**. **No change in color** represents a **negative VP test**.



VP Test.

VP Positive Bacteria: *Klebsiella spp.*, *Enterobacter spp.*, *Viridans Streptococci* (except *S. mitis*, and *S. vestibularis*), *Proteus mirabilis*, *Hafnia spp.*, *Serratia spp.*, *Staphylococcus aureus*,

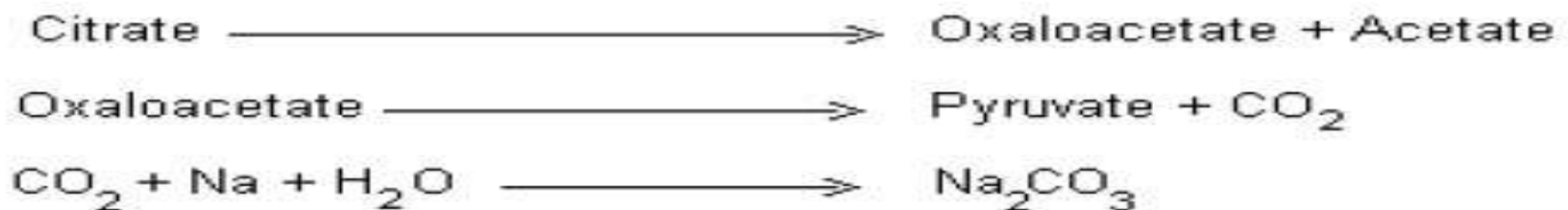
VP Negative Bacteria: *Escherichia spp.*, *Proteus vulgaris*, *Citrobacter freundii*,

Citrate utilization test

Citrate Utilization Test is a biochemical test in the IMViC test series which detects the ability of organisms (bacteria) to utilize citrate as a sole source of energy. It is indicated by the letter "C" of the IMViC.

Principle of Citrate Utilization Test

Some bacteria can utilize 'citrate' as their sole source of carbon. Such bacteria produce citrase enzymes which will break the citrate into oxaloacetic acid and acetic acid. The oxaloacetic acid will then be decarboxylated to produce pyruvate and CO₂.



Released CO_2 will combine with H_2O and excess sodium from sodium citrate to produce alkaline 'sodium carbonate'. The sodium carbonate will increase the pH of the medium.



Additionally, the released CO_2 will trigger the metabolism of ammonium salts. Utilization of the ammonium salts as a source of nitrogen will cause the production of ammonia (or ammonium hydroxide).



The combined effect of ammonium hydroxide and sodium carbonate will increase the pH of the media above 7.6. This increase in pH will turn the pH indicator bromothymol blue in the medium from deep forest green (at neutral pH) to Prussian blue.

Following the incubation of 24 – 48 hours (up to 4 days for some), bacterial growth and color change in the slant portion is observed. A positive result is indicated by growth and change in color of slant from green to intense blue. A negative result is indicated by no change in the color of the slant.

Citrate Positive Bacteria: *Klebsiella spp.*, *Citrobacter spp.*, *Serratia marcescens*, *Proteus mirabilis*, *Enterobacter spp.*, *Salmonella spp.* (except *Salmonella Typhi* and *Paratyphi A*), *Edwardsiella spp.*, *Providencia spp.*,

Citrate Variable Bacteria: *Proteus vulgaris*, *V. cholera*, *V. parahaemolyticus*.

Citrate Negative Bacteria: *Escherichia coli*, *Shigella spp.*, *Salmonella Typhi* and *Paratyphi A*, *Yersinia spp.*, *Morganella morganii*, *Staphylococcus aureus*, etc.



Shows citrate utilization **positive test result with blue color regarding *Klebsiella* spp. (left) negative result with green color regarding *E.coli* (right).**