Microbial identification

Main uses for accurate microbial identification are:

- **Healthcare** – accurate and fast identification of bacteria, fungi and parasites for correct and timely disease diagnosis, and appropriate treatment.

- **Epidemiology** – for the tracking and tracing of disease spread and outbreaks, as well as the identification of new isolates, e.g., antibiotic resistant isolates.

- **Pharmaceutical industry** – because microbes are a significant threat to sterility, accurate identification of environmental microbes is often a good manufacturing practice (GMP) requirement within the pharmaceutical industry.

- Additional scenarios – include **criminal investigation, microbial forensics** (the investigation of bioterrorism threats), and **environmental studies**.

Traditional vs. Modern Methods

- **Traditional methods** for microbial identification rely on **phenotypic identification** using staining, culturing and simple biochemical tests.

  Phenotype is the set of observable characteristics of an individual.

- Nowadays, newer and more powerful **molecular, immunological**, and **biochemical** analytical methods support and sometimes replace traditional methods.

Traditional Methods

Macroscopic Features

- Macroscopic features (visible by naked eye) encompass the **overall appearance** of growth of a microorganism **(colonial appearance)**, including its **shape, size, color** and **smell**.

- By examining the **gross** macroscopic features on an **agar culture**, the type of microorganism can be usually determined.

* Agar is solid gel like base used for most of solid media.

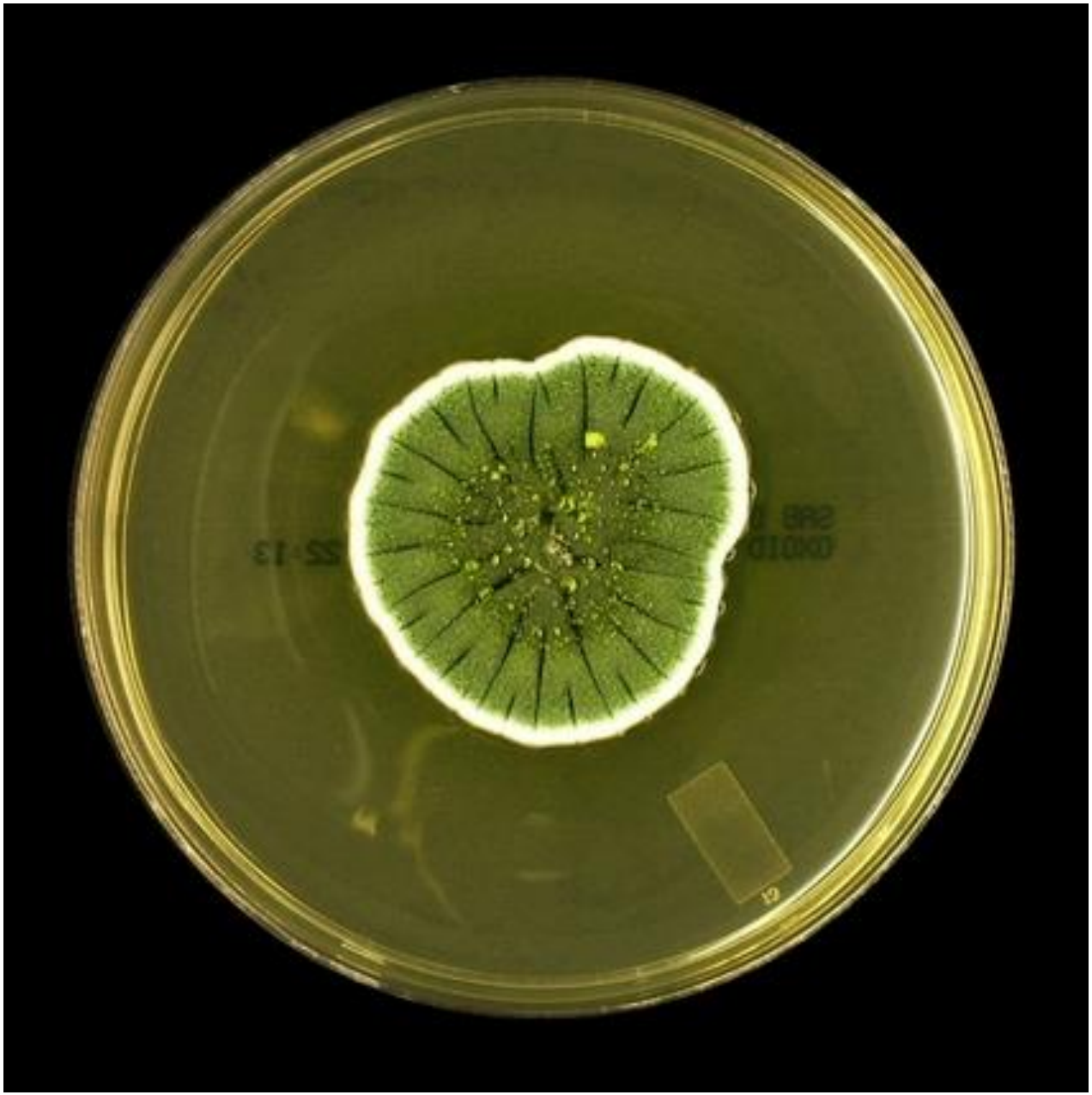
A General Guide to Examining Agar Cultures

Fungi

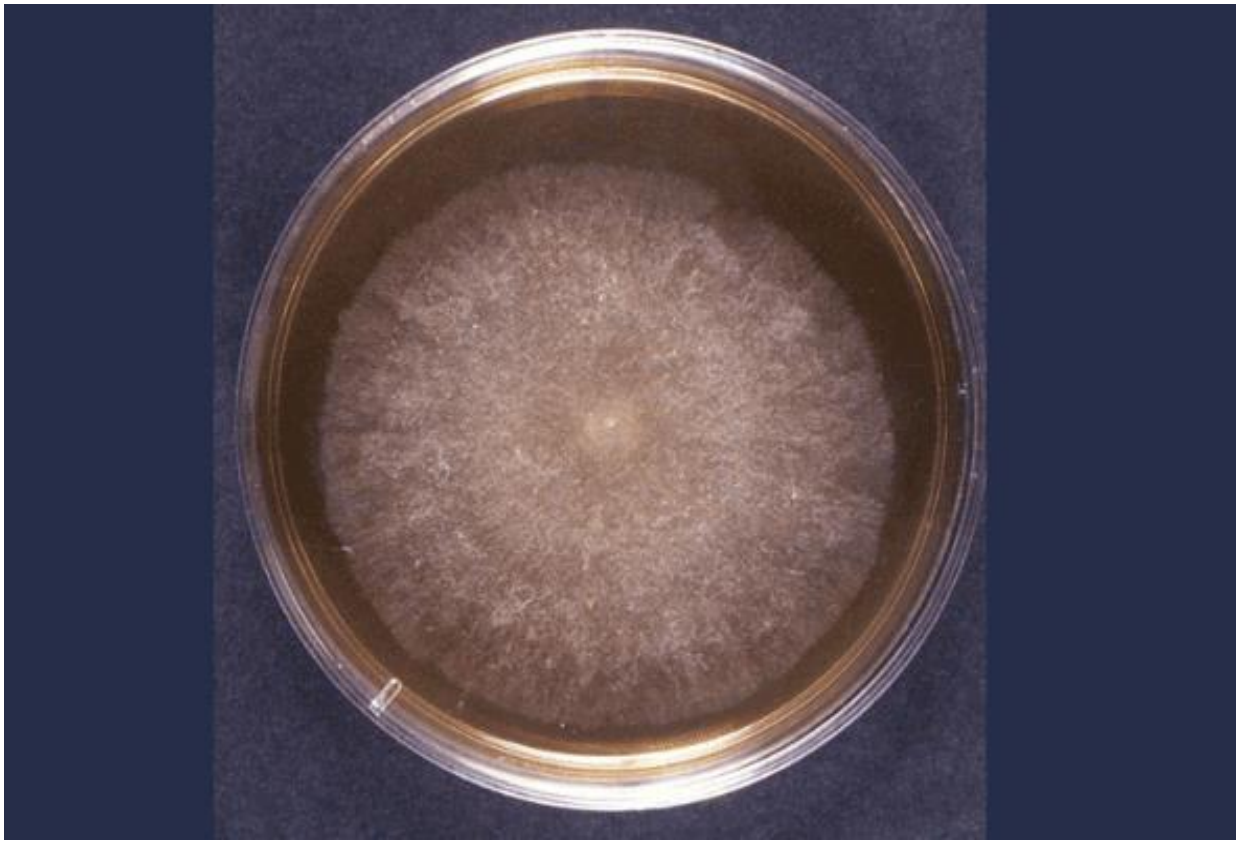
- **Filamentous fungi or molds** appear as **'hairy' irregularly shaped colonies** and often produce **visible spores** that may look powdery or dusty.

- Fungal colonies can contain **more than one color**, usually with a darker color in the center (often raised center) and a lighter color radiating from the center.

- Filamentous fungi **grow radially from the center** of an agar plate, with the youngest on the outside, and the older darker material (rich with spores) on the inside.



- Filamentous fungi may also grow as a **unicolored furry mat** with no obvious sign of spores.



Bacteria

- **Bacteria** often form **distinct colonies.**
- These colonies are usually smaller than fungal colonies.
- Texture can vary from **slimy to very dry**.
- They can be of any color.
- But colonies of **any one species** will always have **same color and appearance on a particular culture medium.**

- Bacteria often have a **strong odor** while filamentous fungi can be odorless or earthly smelling.

- **Yeast** can be the **trickiest to identify** based on macroscopic features, because colonies can often **look similar to bacterial colonies**, depending on the species and type of agar used.

Bacterial colonies

Yeast colonies

Microscopic Features

Staining

Most often, researchers apply stains to microbial samples for easier visualization under a microscope.

Gram Staining

- Gram staining is often the first test performed for bacterial identification.

- This purple stain, based on the **crystal violet dye**, is named after the Danish bacteriologist Hans Christian Gram, who developed it in 1884.

- Typical **Gram-positive bacteria** include ***Bacillus*, *Staphylococcus, Streptococcus*** and ***Clostridium* spp.**

- Typical **Gram-negative bacteria** include ***Escherichia*, *Helicobacter*** and ***Salmonella* spp**. are**.**

- Certain bacteria are **Gram-variable** and are, therefore, not suitable for identification by Gram-staining.

Principle of Gram staining

- When the bacteria is stained with **primary stain crystal violet** and **fixed by the mordant (iodine)**, some of the bacteria are able to **retain** the primary stain and some are **decolorized by alcohol.**

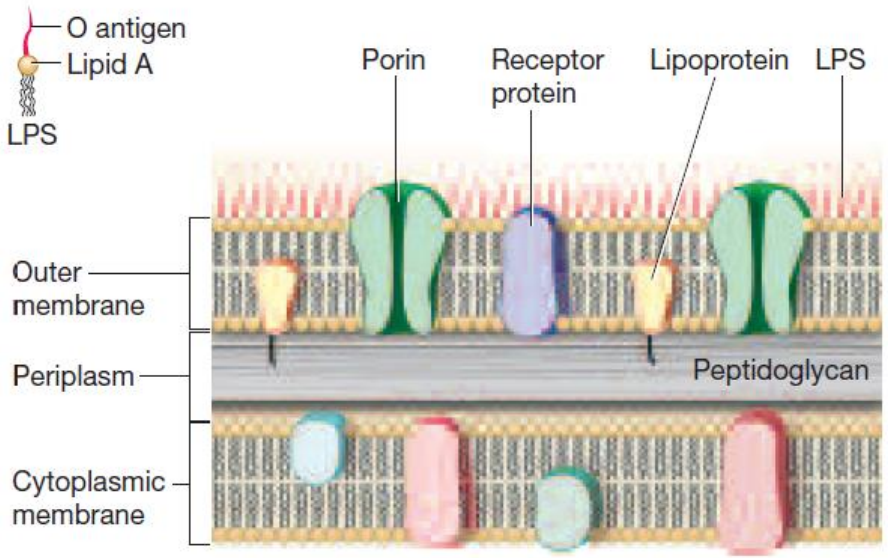
- The cell walls of **Gram positive** bacteria have a **thick layer of protein-sugar complexes called peptidoglycan** and lipid content is low.

- **Decolorizing** the cell causes this thick **cell wall to dehydrate and shrink**, which **closes the pores** in the cell wall and prevents the stain from exiting the cell.

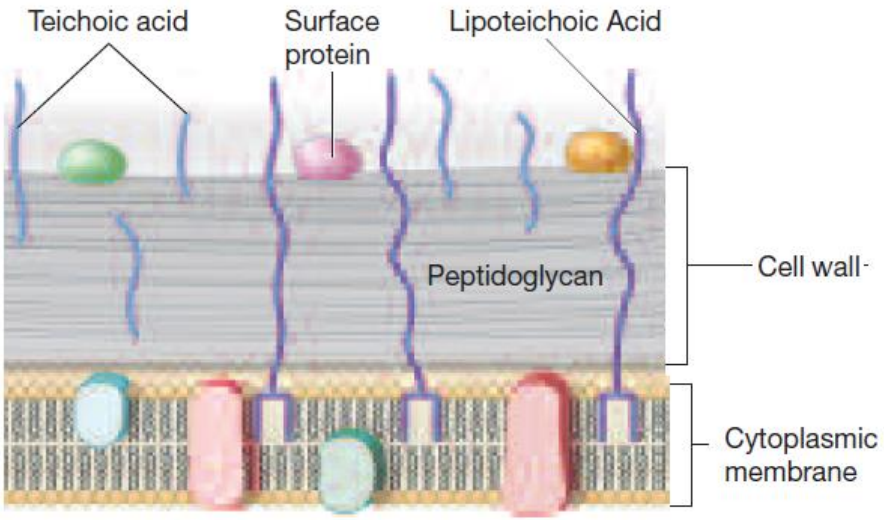
- So the **ethanol cannot remove the crystal violet-iodine complex** that is bound to the thick layer of peptidoglycan of Gram positive bacteria and they appear **purple** in colour.

- In case of Gram negative bacteria, cell wall also takes up the cv-iodine complex but due to the **thin layer of peptidoglycan and thick outer layer which is formed of lipids**, cv-iodine complex gets **washed off**.

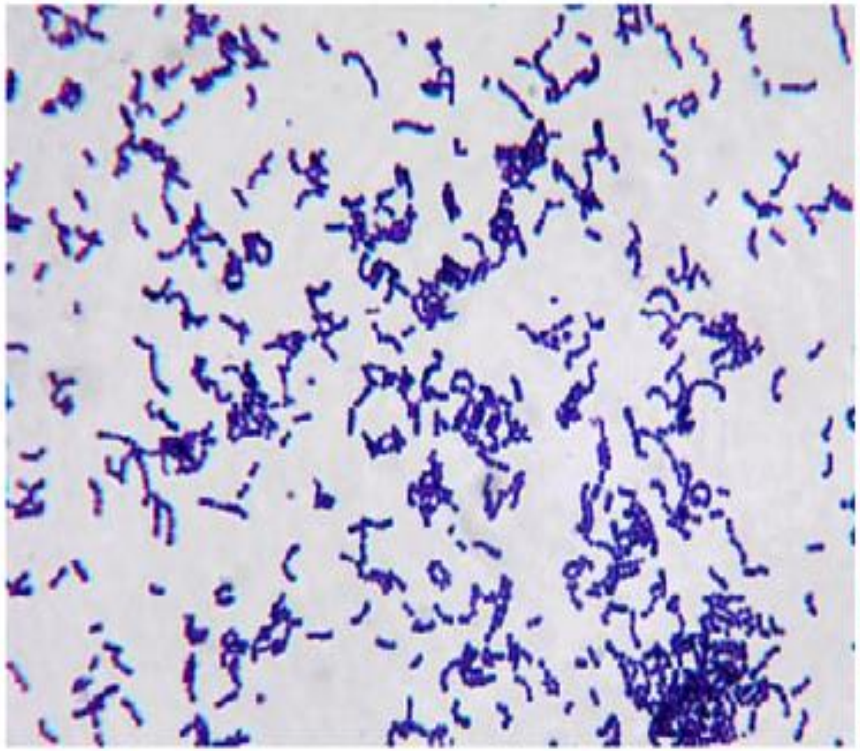
- When they are exposed to alcohol, **decolorizer dissolves the lipids** in the cell walls, which allows the crystal violet-iodine complex to leach out of the cells.



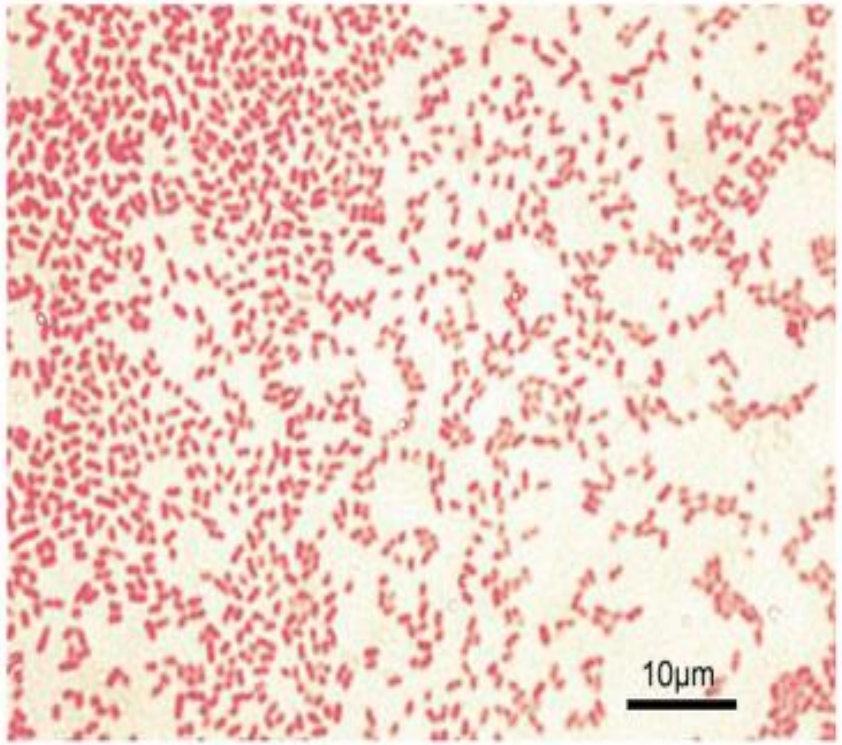
Cell Wall of Gram Negative Organism



Cell Wall of Gram Positive Organism



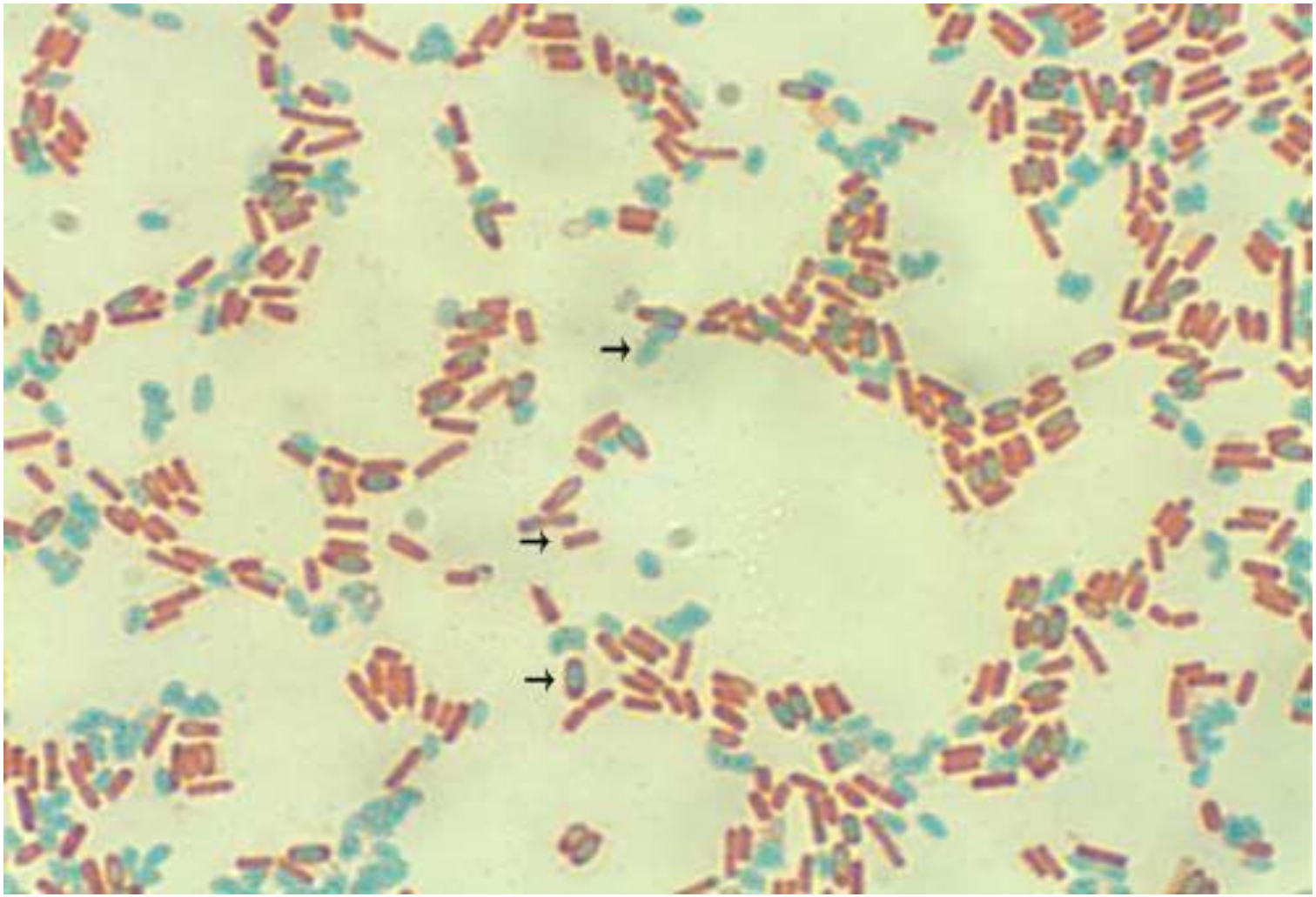
Gram Positive Bacteria



Gram Negative Bacteria

Endospore Staining

- This technique involves applying a stain to a bacterial sample to check for the **presence of spores**.
- Because **not all bacteria produce spores**, this information can be useful in identification.
- Several spore stains are available, and **malachite green** is probably the most popular one.

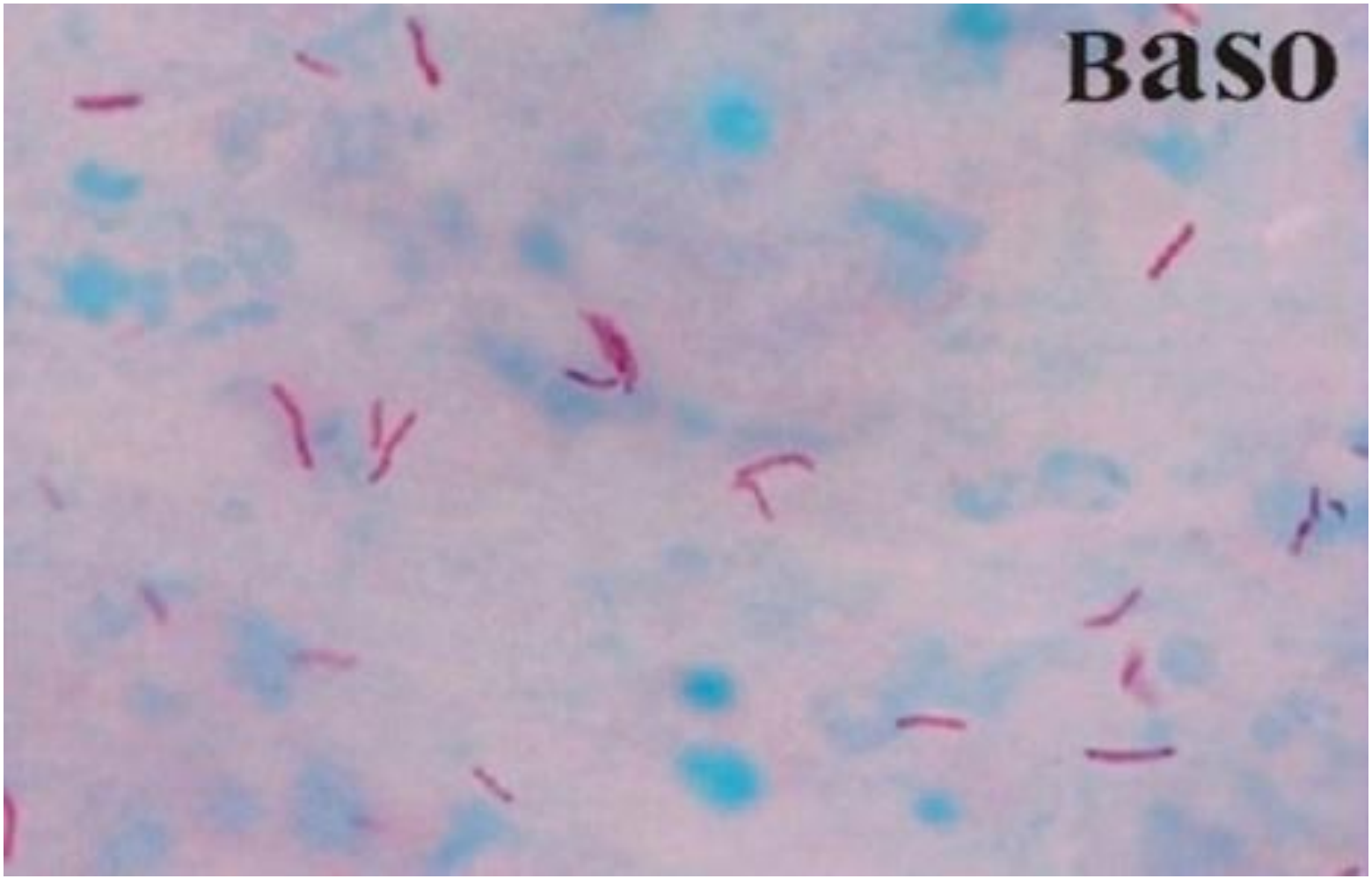


Ziehl-Neelsen Staining

- This is an important tool for the staining of *Mycobacterium tuberculosis* (TB), which **cannot be Gram-stained**.

- The red stain **carbol fuchsin** is first used and followed by a counter stain, such as **methylene blue**.

- *M. tuberculosis* **stain red** while other bacteria **stain blue**.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Principle of ZN staining

- Organisms such as *Mycobacteria* are extremely difficult to stain by ordinary methods like Gram stain because of **the high lipid content of the cell wall.**

- The phenolic compound **carbol fuchsin** is used as the primary stain because it is **lipid soluble and penetrates the waxy cell wall.**

- Staining by carbol fuchsin is further enhanced by **steam heating** the preparation to **melt the lipids** and allow the stain to move into the cell.

- **Acid** is used to decolorize nonacid-fast cells; **acid-fast cells resist this decolorization**.

- The ability of the bacteria to resist decolorization with acid confers **acid -fastness** to the bacterium.

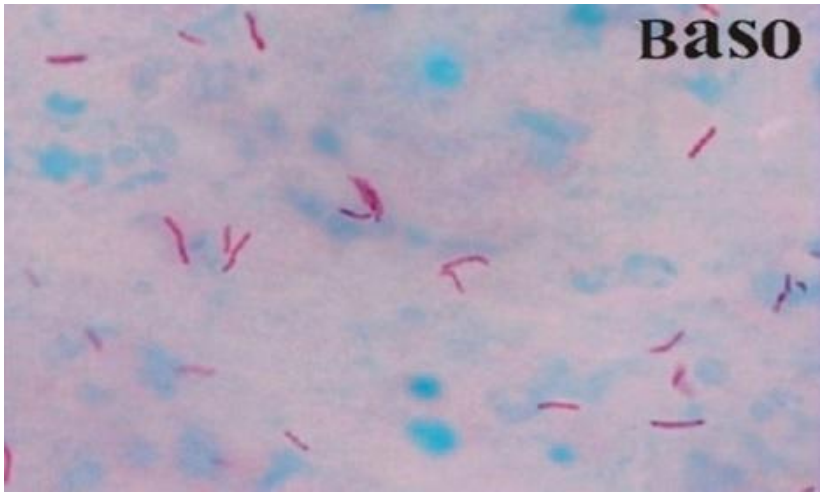
- As a result acid fast bacteria retain the **red** dye, carbol fuchsin.

- Acid fastness is due to the presence of **mycolic acid** which is present mainly in cell walls of *Mycobacteria*.

- Following decolorization, the smear is counterstained with **malachite green** or **methylene blue.**

- It **stains the background material**, providing a contrast colour against which the red AFB can be easily seen.

- **Acid alcohol** can also be used as decolorizing solution, resistant organisms are referred to as **Acid Fast Bacilli (AFB) or Acid Alcohol Fast Bacilli (AAFB).**



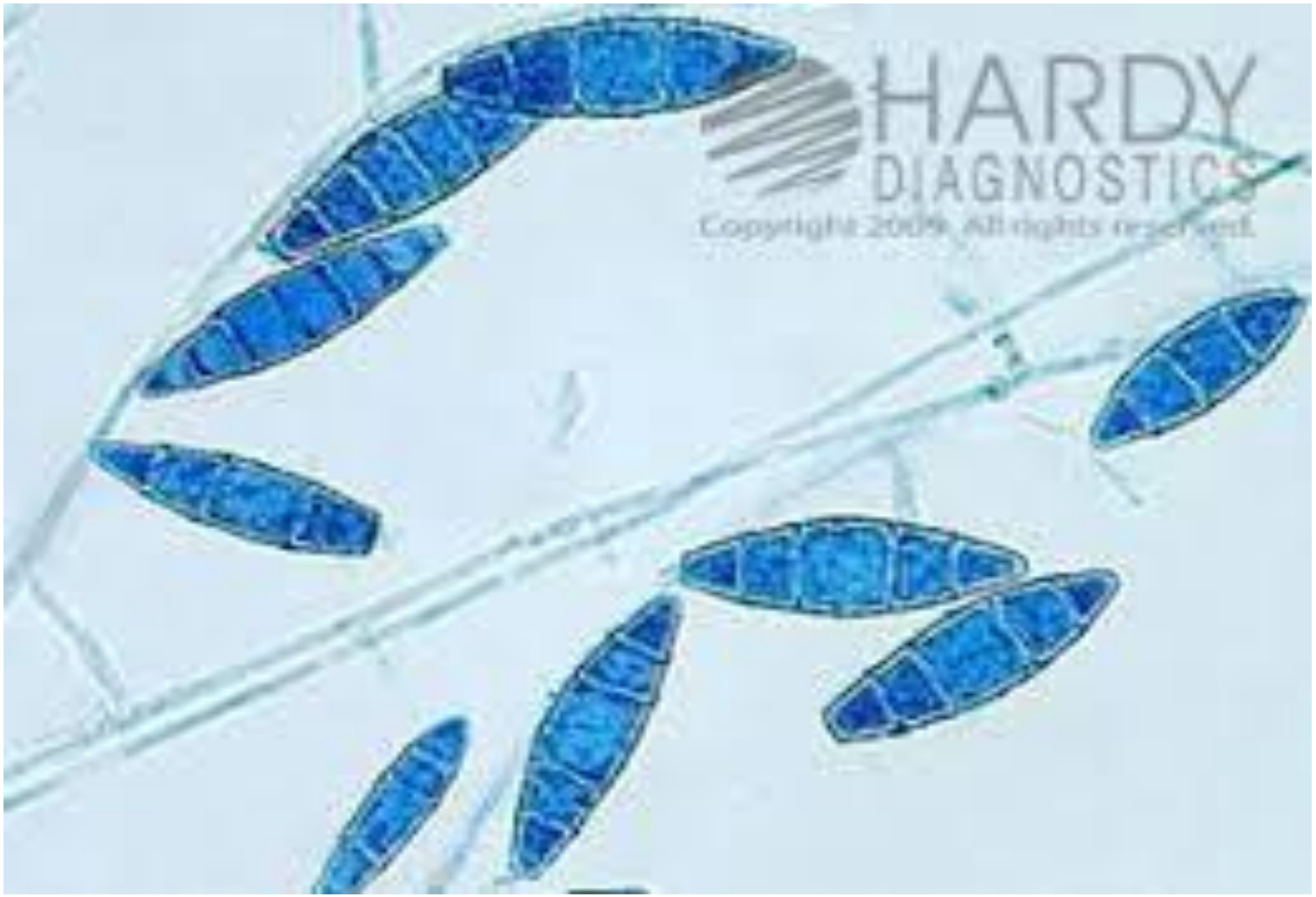
Stains for Fungi and Yeast

- Several fungal stains exist, although these are generally **non-specific** in nature.

- They help **visualize fungal elements** for identification **rather than differentiate** between fungal species.

- Examples of fungal stains include:

  Lactophenol cotton blue stain which stains **carbohydrates** present in fungal cell walls.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DIAGNOSTICS
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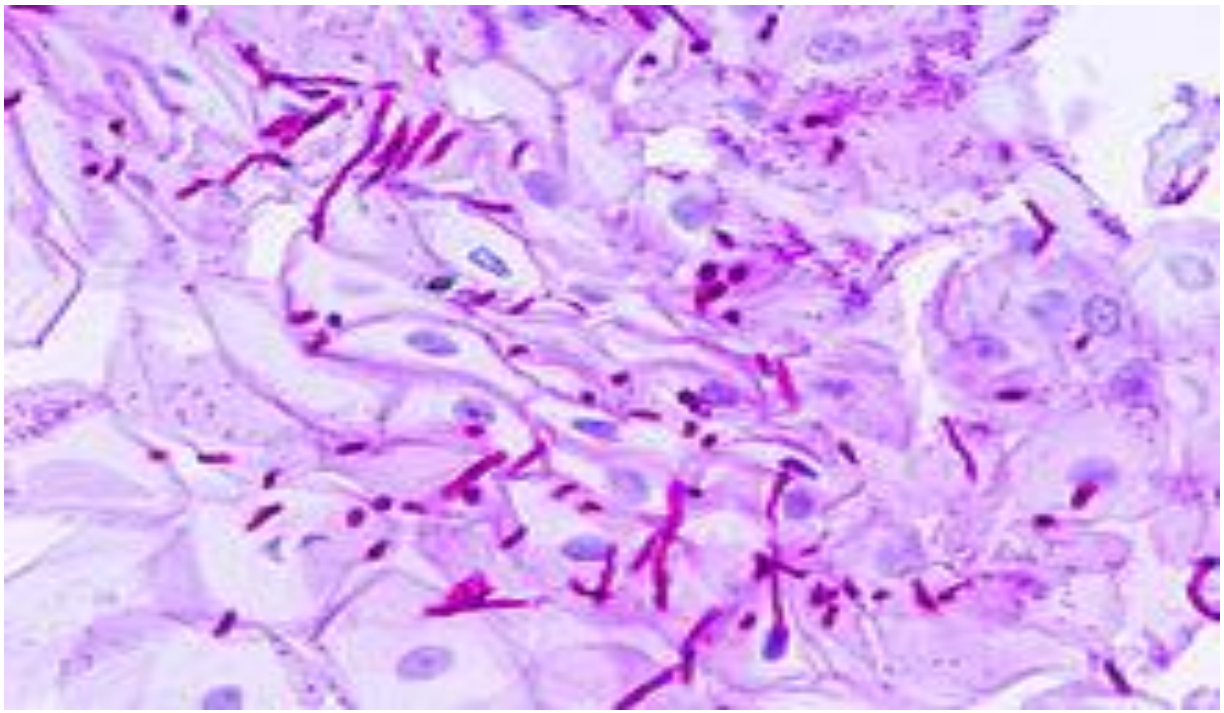
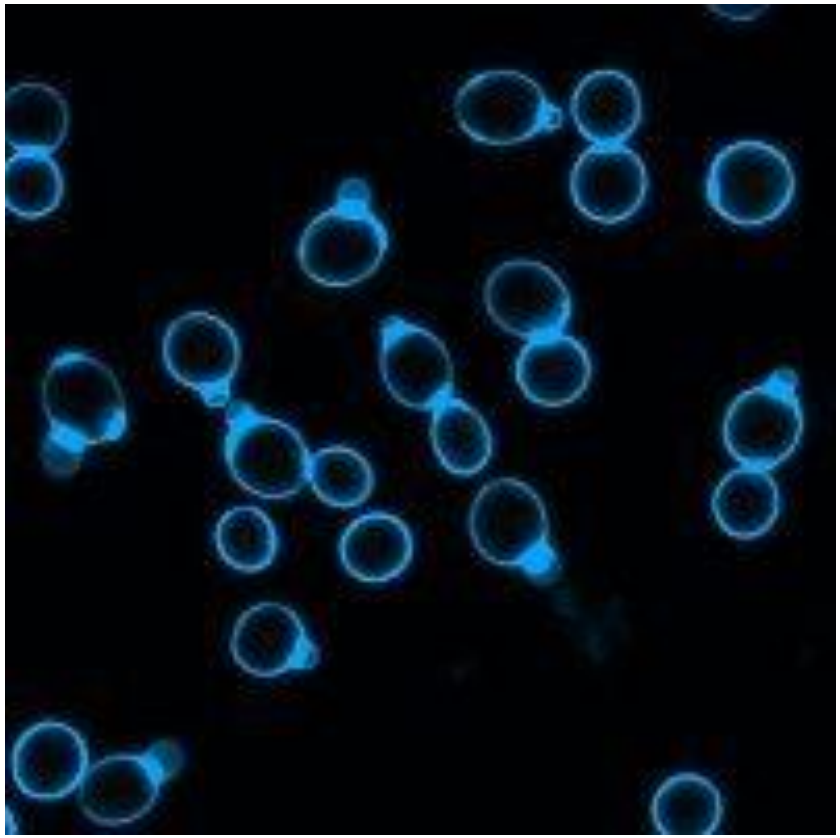
Periodic-acid Schiff stain (PAS stain) – this stains **carbohydrates** and other moieties and leads to a magenta (purplish-red)color in living fungi only.

This is used in infection diagnosis.



Trypan blue, analine blue, and **calcofluor white** are other commonly used fungal stains.



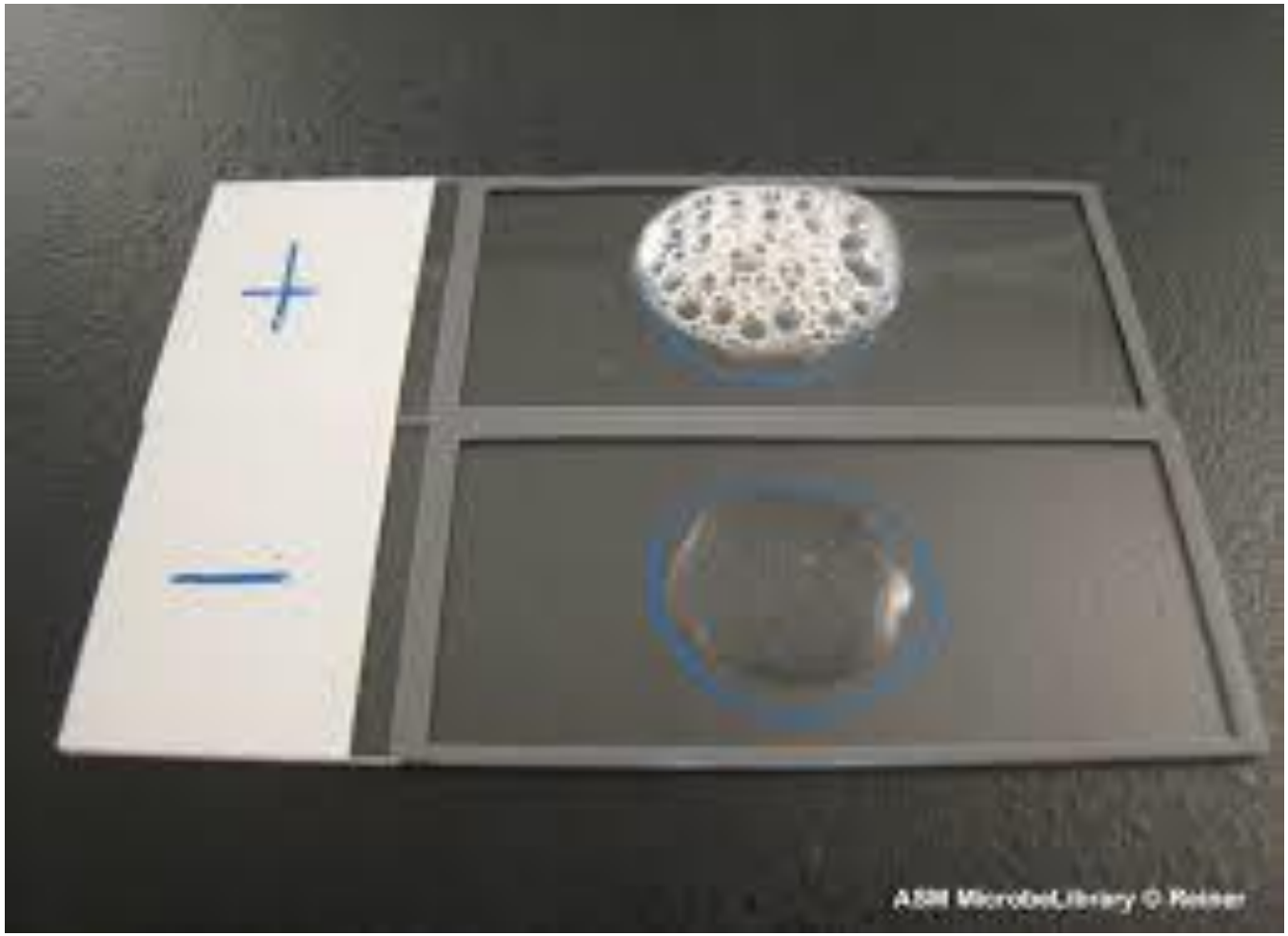
calcofluor white staining

Simple Biochemical Tests

Catalase Test

- If the unidentified bacterial species has catalase activity, **bubbles of oxygen** appear when **hydrogen peroxide** is added to a scraping of bacteria on a microscope slide.

- **Staphylococci, Micrococci, *E. coli*** and ***Salmonella*** spp. are amongst the most routinely encountered **catalase-positive** bacteria, while ***Streptococcus*** and ***Enterococcus*** bacteria are **catalase-negative.**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ASM MicrobeLibrary © Reimer

Oxidase Test

- The oxidase test identifies bacteria with **cytochrome c oxidase activity (CCO)**.

  This enzyme forms a part of the **bacterial electron transport chain.**

- When present, CCO **oxidizes the reagent** (tetramethyl-p-phenylenediamine) **to a purple-colored product**.

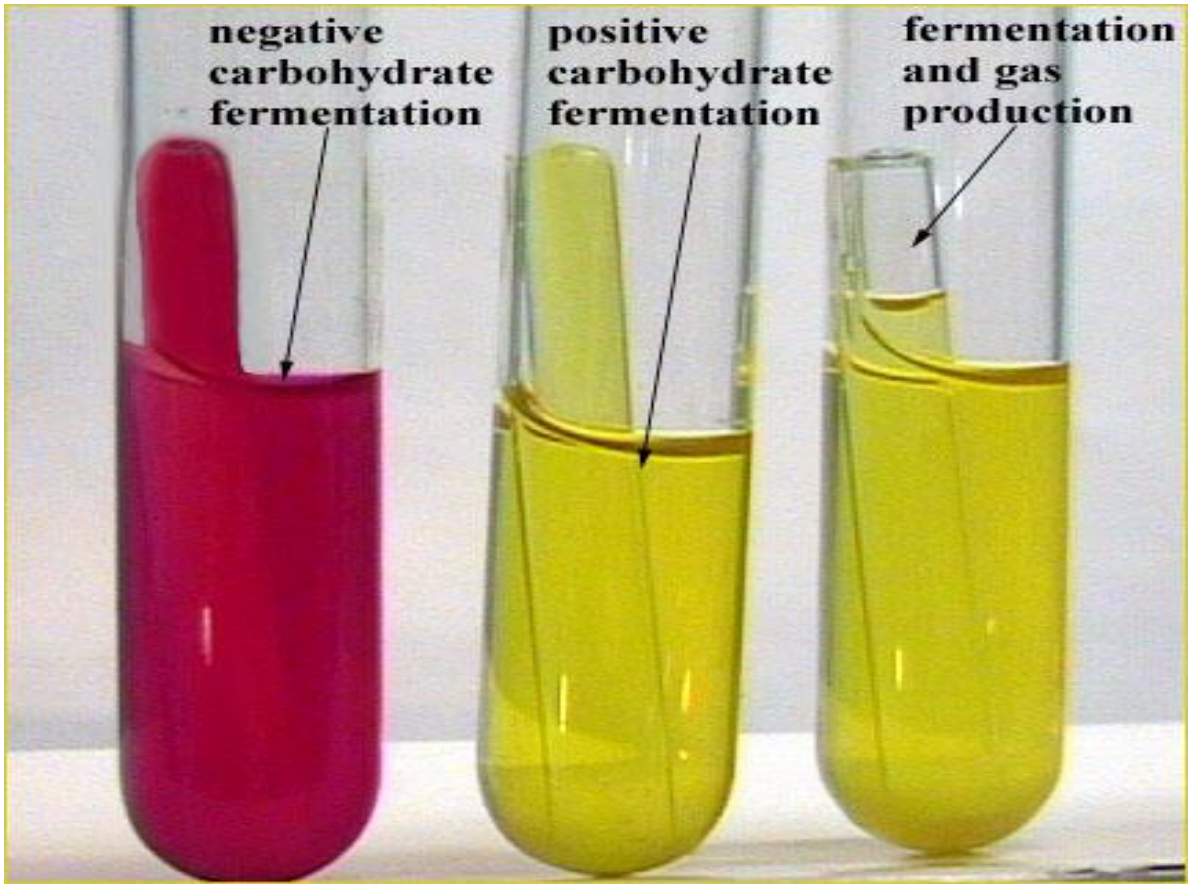
- When the enzyme is not present, the reagent remains in the reduced state and is colorless.

- While oxidase-positive bacteria are **aerobic**, they are not necessarily strict aerobes and may be capable of anaerobic respiration.

- Similarly, a **false-negative** result is obtained if bacterial species possesses an **oxidase incapable of reacting with the test reagent.**

Substrate Utilization Tests

- A range of **substrate utilization tests** for microbial identification is available commercially.
- These tests (usually for **bacterial species**) contain a panel of substrates, e.g., **different carbon and nitrogen sources**.
- Bacterial species use these substrates **differentially.**
- Fermentation tests using various sugars are important. During fermentation, **acid is produced** which indicated by **change in color** of an indicator.



From left:

a. No fermentation, color of indicator phenol red is unchanged
b. Fermentation, color of indicator phenol red is changed to yellow
c. Gas is also produced during fermentation.

- A record of the color changes in substrates after incubation with bacteria generates a **key (or pattern)** of substrate utilization.

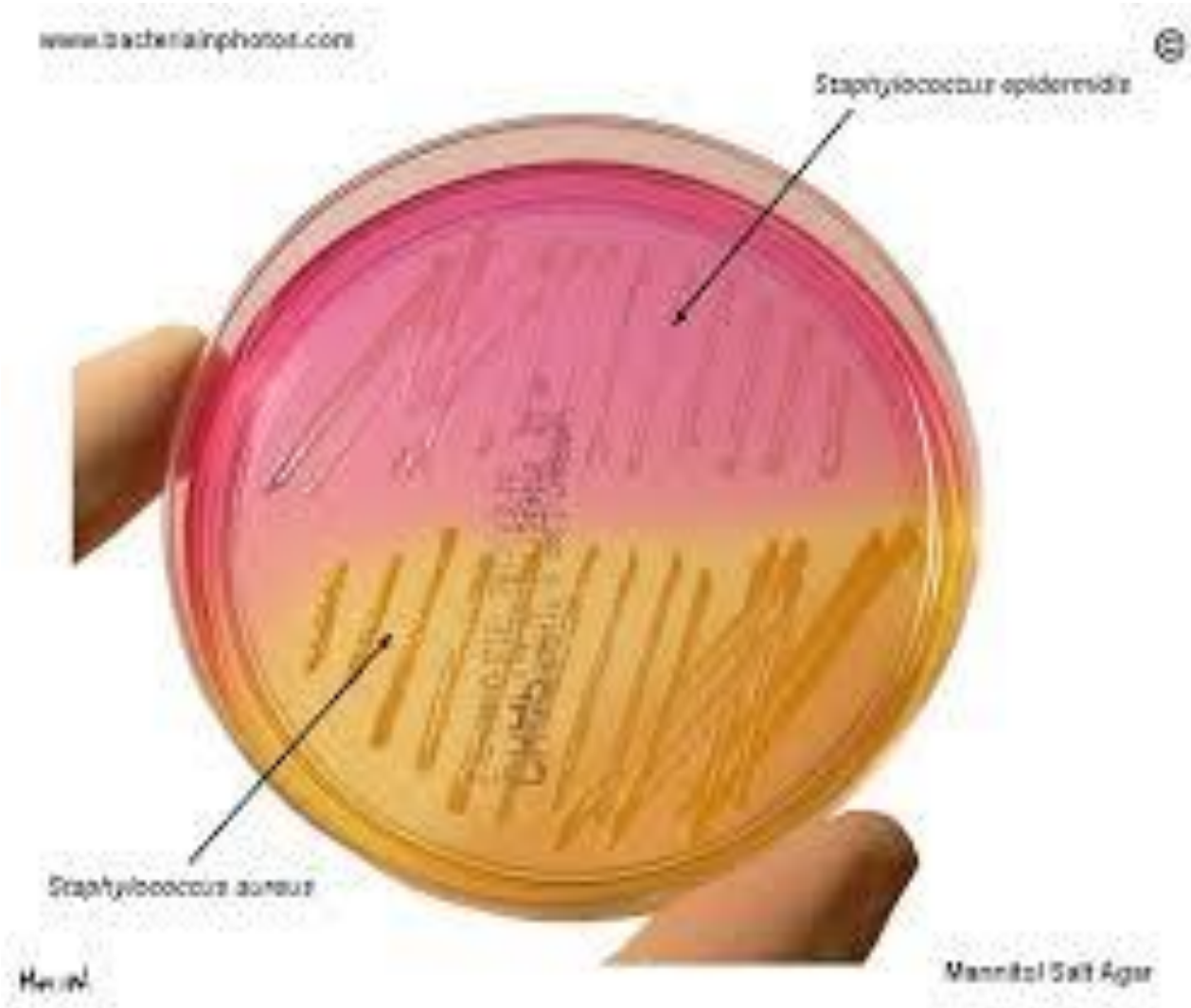
- This key is **compared** to the substrate utilization patterns of a **computerized list** of bacterial species.

- These tests are probably more reliable in **combination** with catalase/oxidase testing and microscopic examination.

- These tests overlap with the concept of **selective growth media.**

- A wide range of selective media exist for the isolation and identification of bacterial and fungal species.

MacConkey agar:
Left: Lactose fermenting pink colonies
Right: Non-lactose fermenting colorless colonies


Staphylococcus epidermidis
Staphylococcus aureus
Mannitol Salt Agar

Physiological Requirements for Growth

Addressing the following questions can help narrow down the list of possible species.

- **Does the organism require oxygen?**
- **In what temperature range does the organism grow best?**
- **Can the organism tolerate acidic media?**


Thank You!