Course: MBIO 240
Laboratory Skills

Purification methods (bacteria & fungi)
and

Identification (culture characteristics)

Why purify cultures

What is a pure culture?

A pure culture contains only one species of microbe.

Why purify?

- To isolate individual species from a mixed culture, identify and characterize them
- To study the morphology and Physiology of individual isolated species (bacteria/fungi)

After isolating bacteria/fungi from different sources on an agar plate, we may see one type of colony that looks identical, or the plate is flooded with different colonies exhibiting various shapes, sizes, colours, and textures.

Therefore, Colonies are defined as **groups of microorganisms growing on a solid surface that can be seen with the naked eye**.

Mixed colonies

Colonies of bacteria isolated from unclean hands

Colonies isolated from the infected fruit surface

A mixed colony (culture) consists of multiple species of microorganisms

Pure colonies

Colonies of bacteria isolated from skin

Colony of fungi isolated from infected grapes

A pure colony is a single species of microorganism that has grown from a single cell, exhibiting distinct characteristics such as shape, margin, elevation and colour

- However, when mixed culture is obtained in the laboratory, they can be separated into pure cultures by using various culturing techniques.

- Upon culturing, the plates show only one kind of organism and can be easily identified, making them suitable for studying their cultural, morphological, and biochemical properties.

- To culture the mixed colonies, the mixture is first subjected to serial dilution until the various individual microorganisms become separated far enough apart on an agar surface.

- After incubation, they form visible colonies isolated from the colonies of other organisms, which can be aseptically transferred onto a new sterile medium to obtain a pure culture.
- or
- An isolated colony can be aseptically "picked off" and streaked to obtain a pure colony.
- After incubation, all organisms in the new culture will be descendants of the same organism, which is referred as a pure culture

Obtaining Pure Cultures

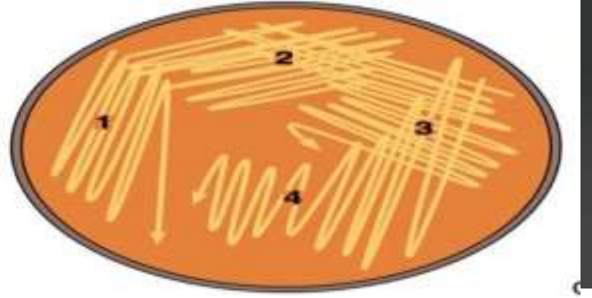
A pure culture contains only one species or strain of bacteria.

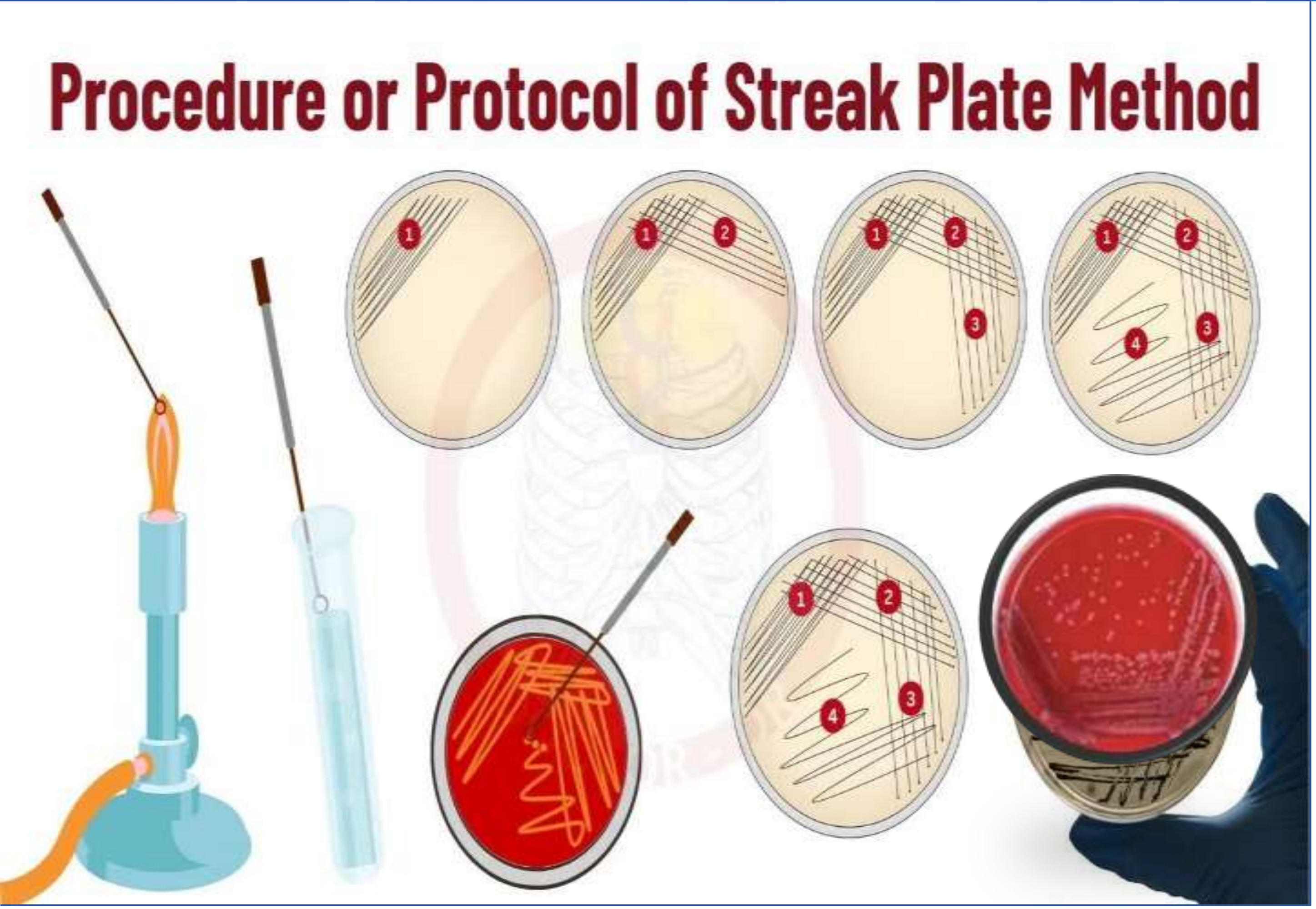
A colony is a population of cells arising from a single cell or spore or from a group of attached cells.

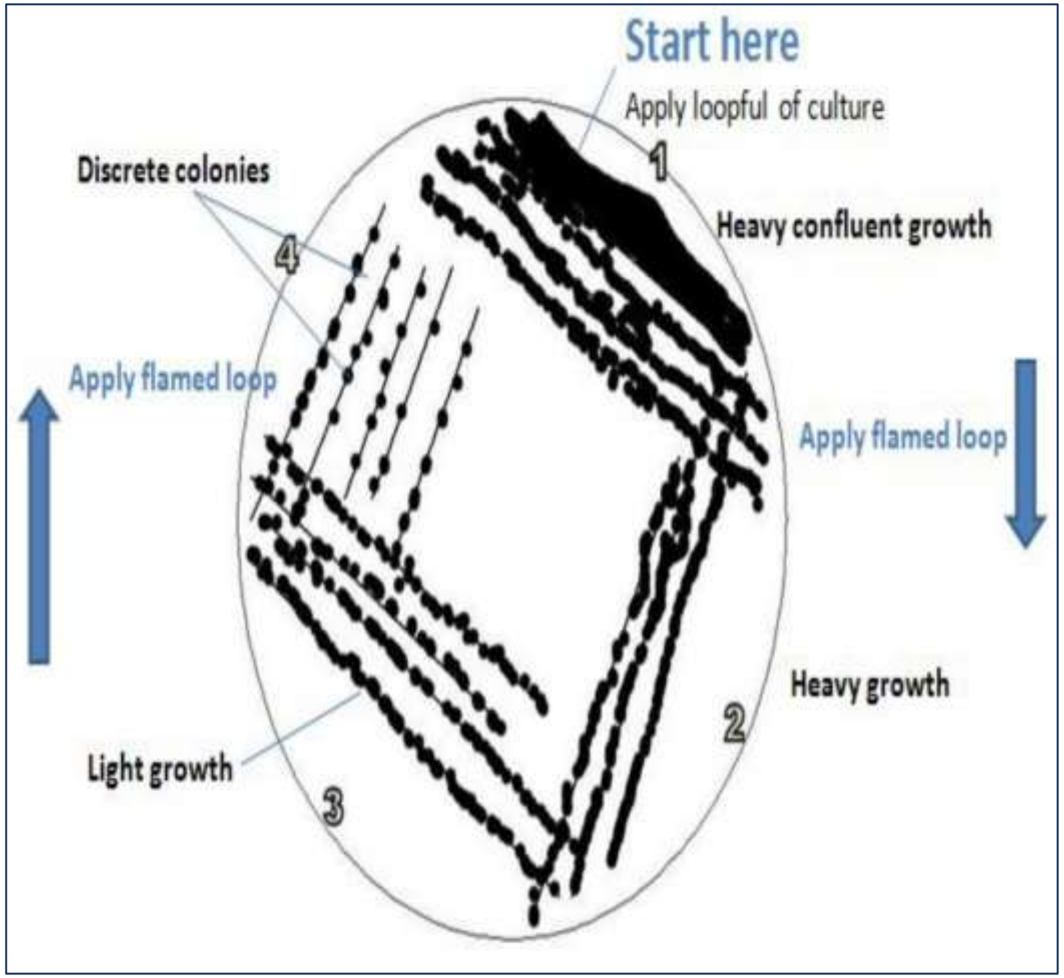
A colony is often called a colony-forming unit (CFU).

The streak plate method is used to isolate pure cultures.

Methods for culturing technique







The isolated colonies is the streaked across the agar surface, in different quadrants to obtain pure individual colonies

PROCEDURE FOR SPREAD AND POUR PALTE METHOD

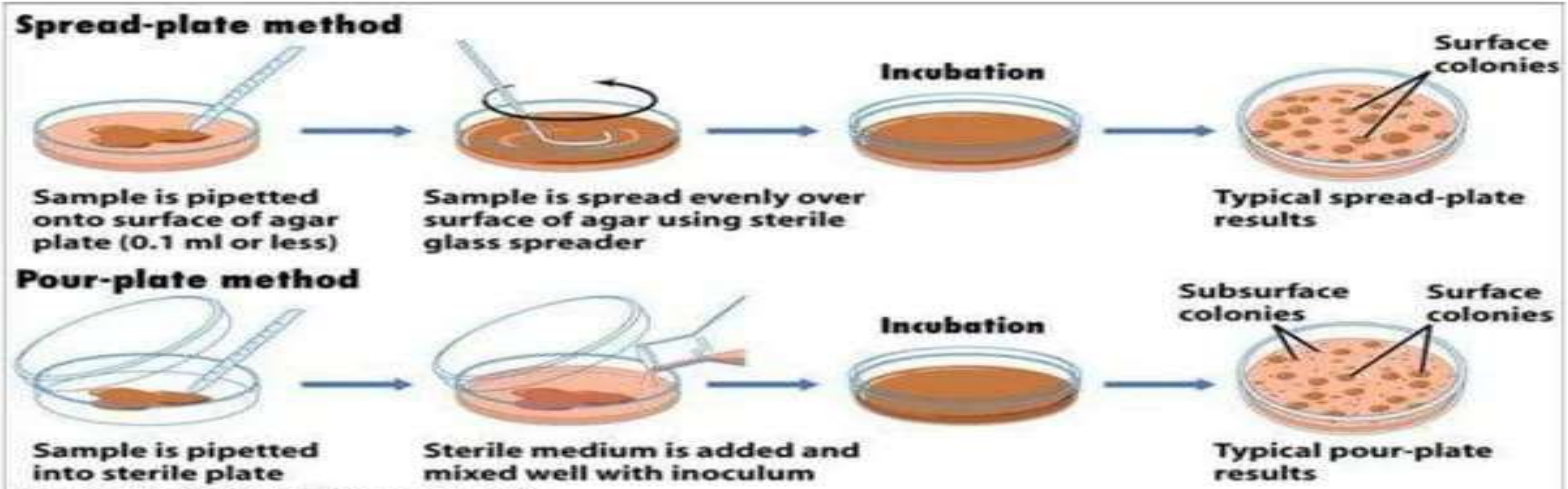
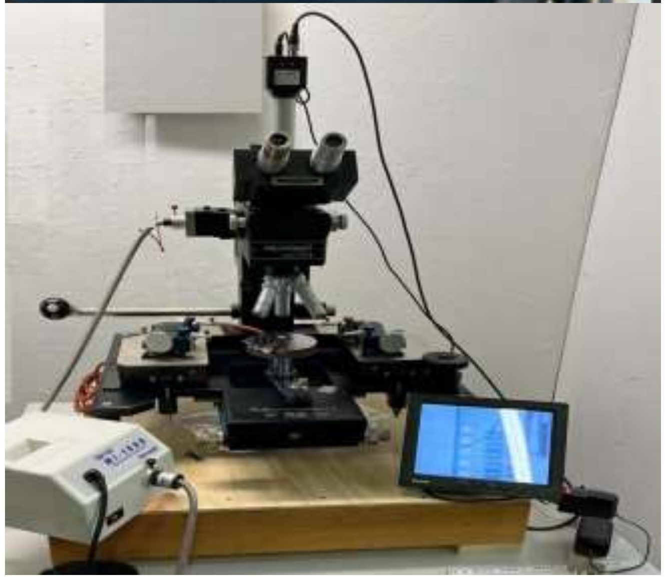


Figure 6-10 Brock Biology of Microorganisms 11/e

Micromanipulator

Micromanipulator

Micromanipulators have been built which permit one to pick out a single cell from a mixed culture. This instrument is used in conjugation with a microscope to pick a single cell (particularly bacterial cell) from a hanging drop preparation.



Advantages of micromanipulator method

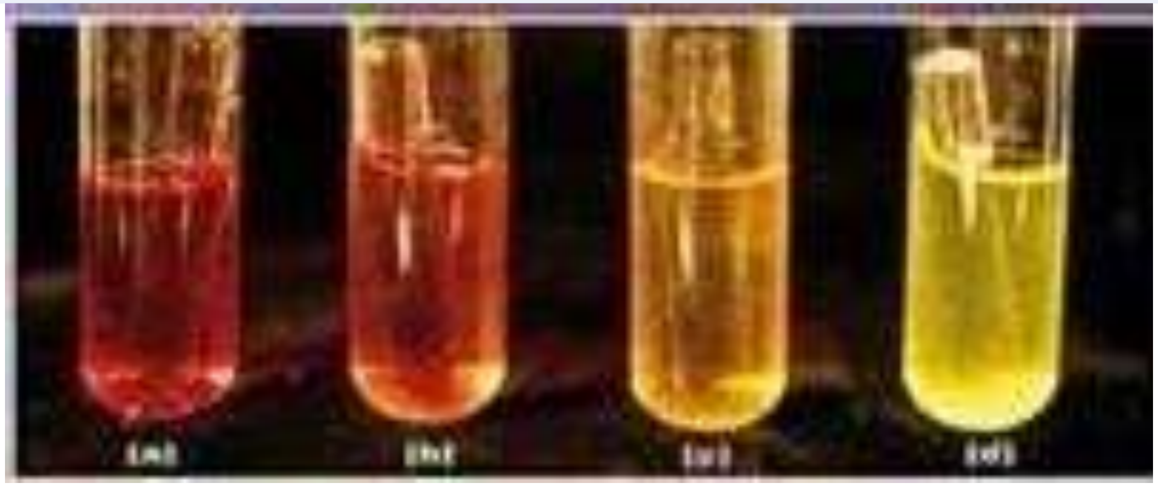
The advantage of this method is that one can be reasonably sure that the cultures come from a single cell and one can obtain strains within the species

Disadvantages

The disadvantages are that the equipment is expensive its manipulation is very tedious, and it requires a skilled operator.

Isolation by using selective enrichment media/ Enrichment method

- Enrichment media is a type of media that allows metabolically fastidious microorganisms to grow due to the addition of specific growth factors.
- It is the same as selective media, but enrichment media is liquid in consistency



Purification of fungal cultures

- The purification of fungi from mixed culture is needed for research and experimentation.
- Here are the main methods and considerations involved in the purification process:

❖ **Streak plating,**

❖ **Dilution methods**

❖ **Single spore isolation**

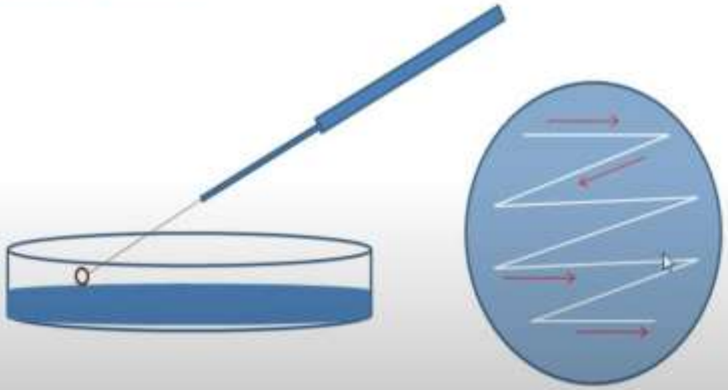
❖ **Direct transfer- needle, scalpel blade or cork borer**

Streaking- *Candida albicans*

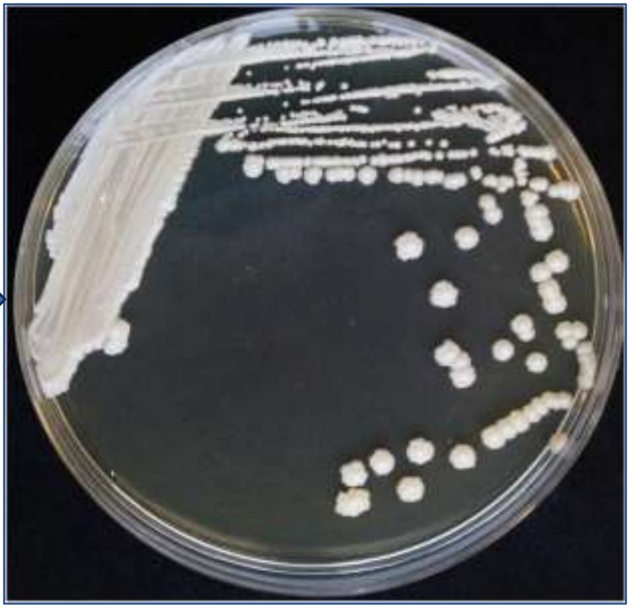
- Make a spore suspension in sterile water
- Take a loop full of spore suspension
- Streak over an agar surface in Petri dish

- Take a loop full of spore suspension

- Streak over an agar surface in Petri dish in a zig-zag manner



- Incubate the streaked plate at 25±2°C for 5-7 days or till the single-spore colonies appear along the streaks



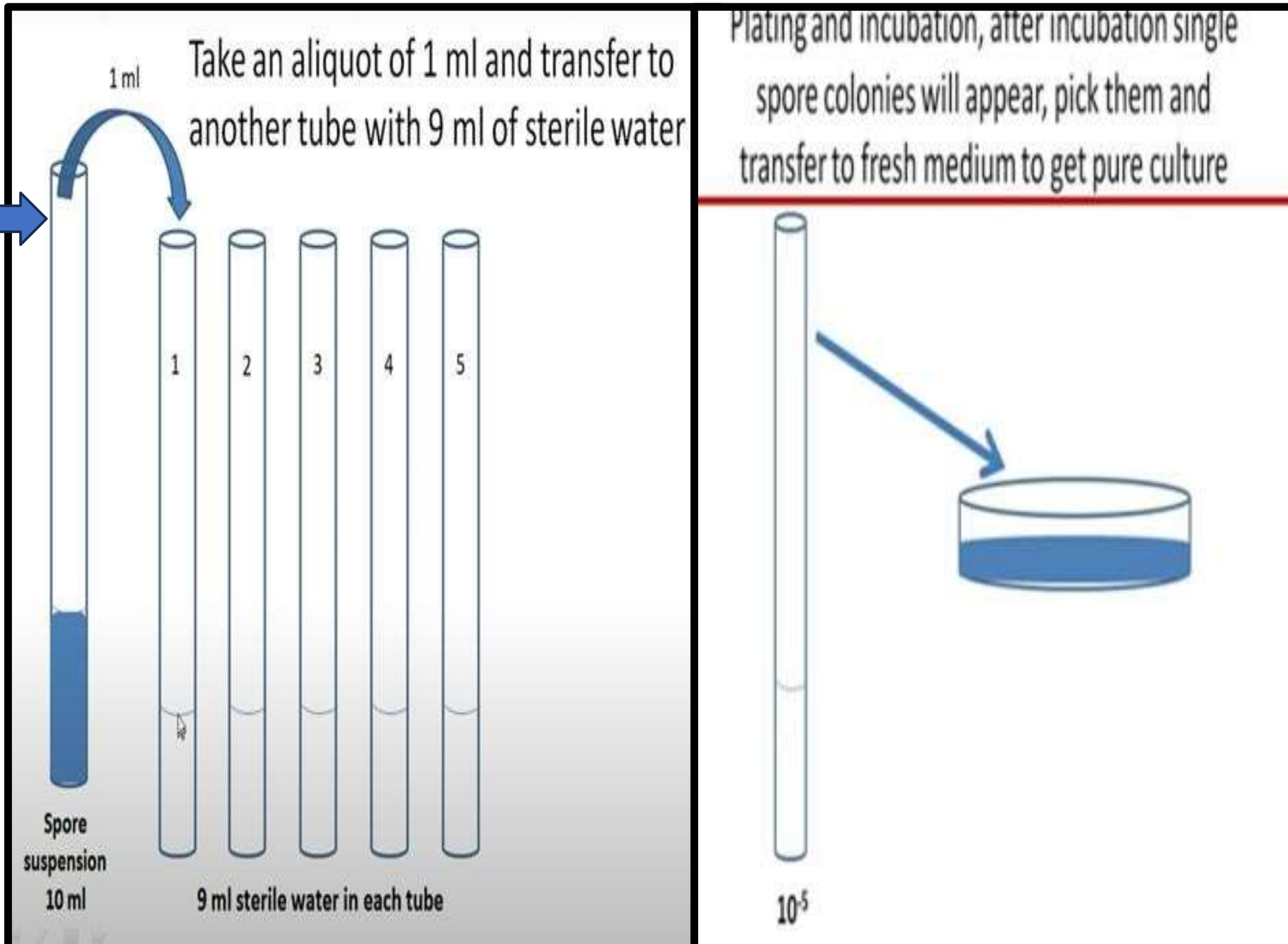
Colonies of Candida albicans obtained by streaking

- The single spore colonies can be picked aseptically and transferred to Petri dish or slant to get **pure culture**

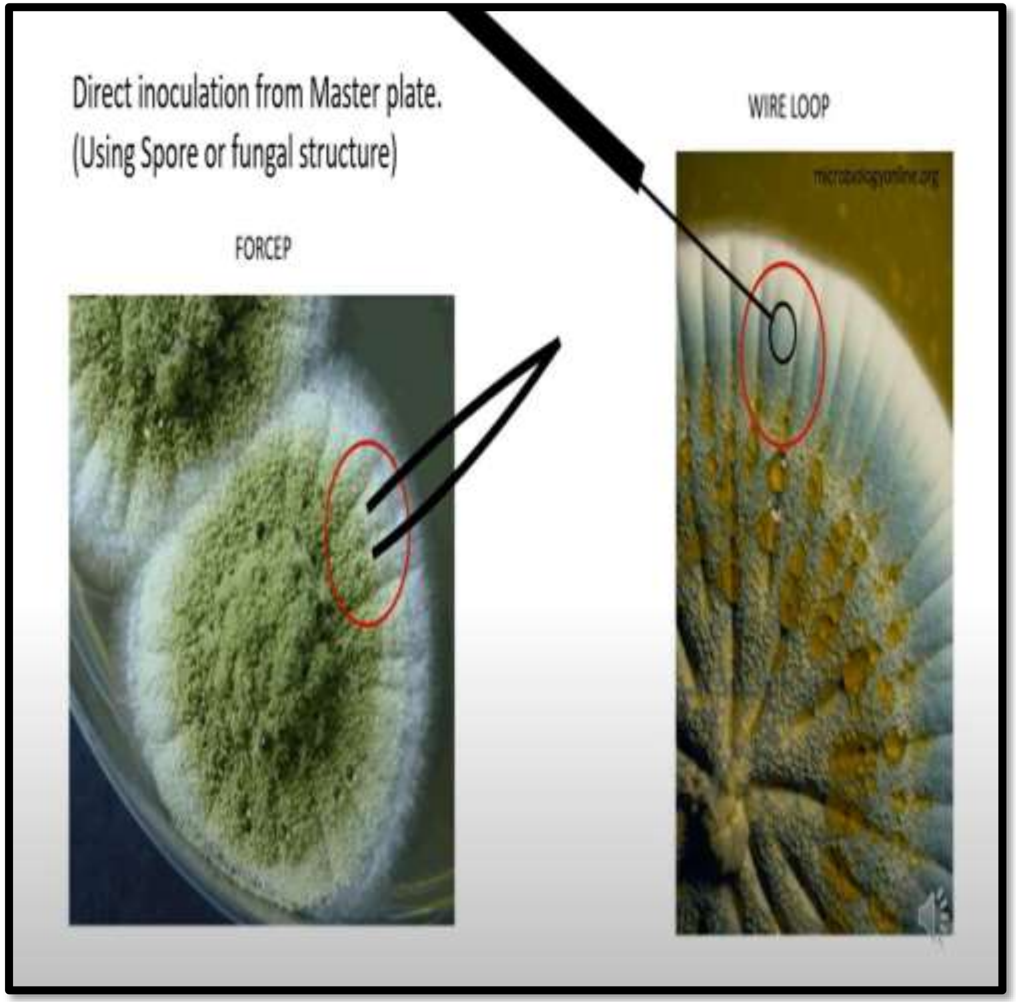
- **Aqueous Solutions**: Dilute spores in sterile water or liquifiable culture media. This can help in isolating single spores or hyphal tips from the mixed culture.

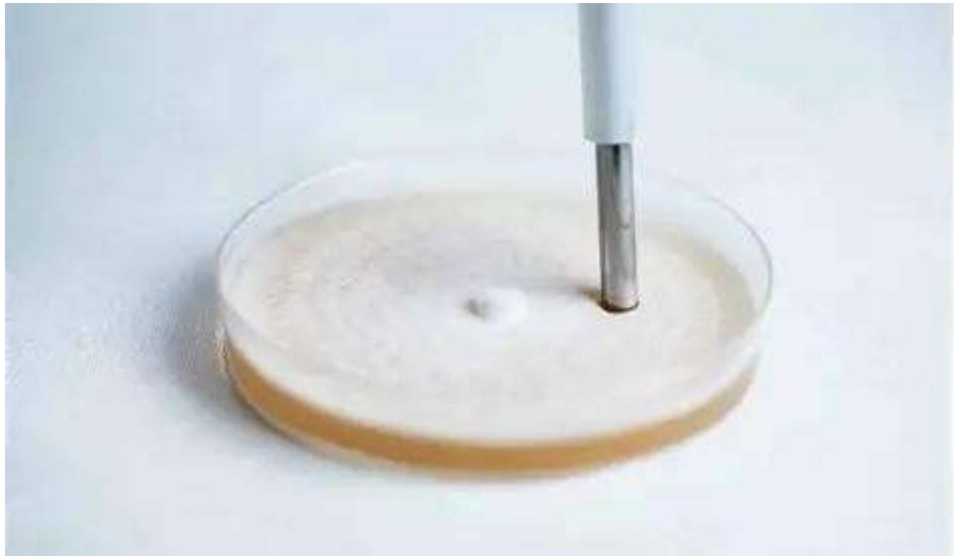
Dilution with sterile water

- Prepare a spore suspension 10 ml
- Take an aliquot of 1 ml and transfer to another tube with 9 ml of sterile water (10^{-1} dilution)
- Make 10^{-2} dilution by transferring 1ml from 10^{-1} dilution to another tube with 9ml sterile water
- Thus, make dilution of 10^{-3}, 10^{-4}, 10^{-5} and as much as required
- From the dilution of choice take 1 ml and do plating on agar surface in Petri dish.



Direct transfer



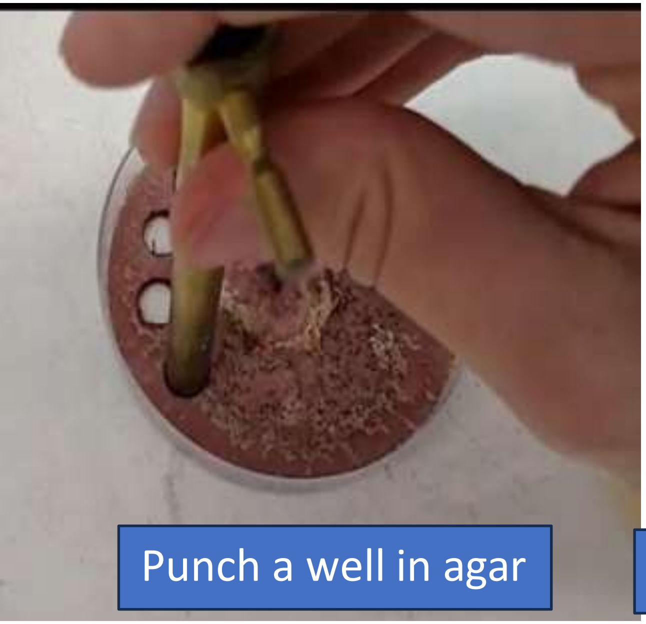


Transfer with the help of a cork borer

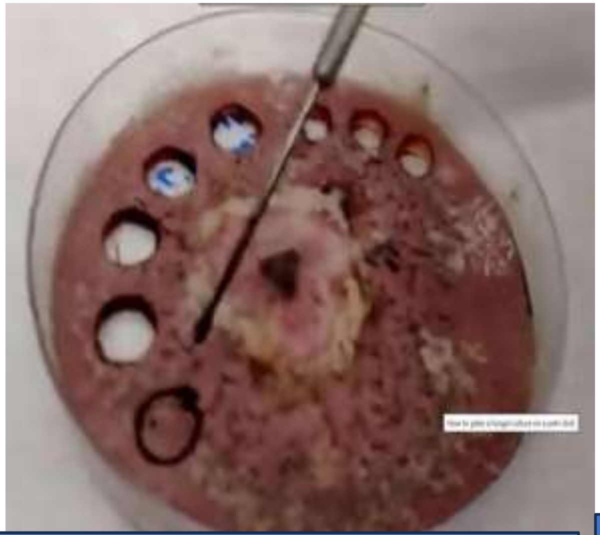
Cork borer

Dip in ethanol

Sterilize

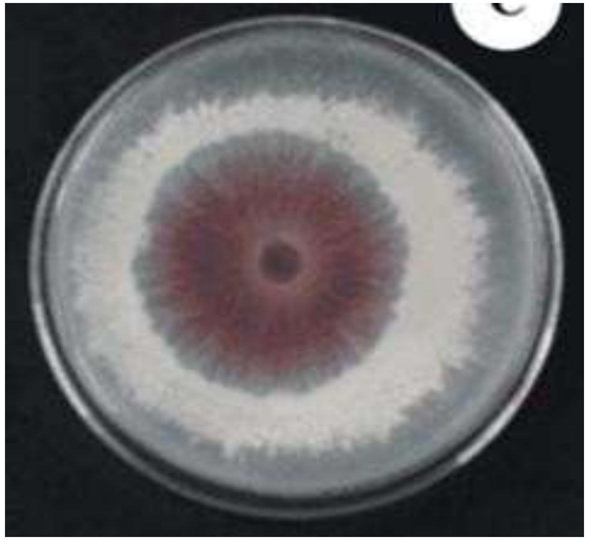


Punch a well in agar



Remove the mycelial disc

Place it upside down on a sterile agar plate



Incubate 25 °C for 5-7days

Transfer with a scalpel blade

Pure *Fusarium solani* culture growing from mycelial plugs

Bacterial and Fungal Preservation Methods

Preservation

- Culture preservation is a method/ technique used to maintain the viability and characteristics of microbial cells for an extended period of time.
- It also ensures **genetic stability** and **functionality**, allowing scientists to maintain strains for future experiments and applications

Once a microorganism has been isolated and cultured in pure form, they are refrigerated and subcultured periodically.

1. Short-term preservation	2. Mid-term preservation	3. Long-term preservation
Refrigeration	**Preservation in ampoules**	**Lyophilization**
Agar slants	**Preservation in glycerol**	**Cryopreservation**
Mineral oil immersion		
Paraffin method		

Preservation

Refrigeration

- Pure cultures can be successfully stored at 0-4°C either in refrigerators or in cold rooms.
- This method is applied for short duration (2-3 weeks for bacteria and 3-4 months for fungi) because the metabolic activities of the microorganisms are greatly slowed down but not stopped*.
- Regular subculturing is required (Why?)*

Agar slants

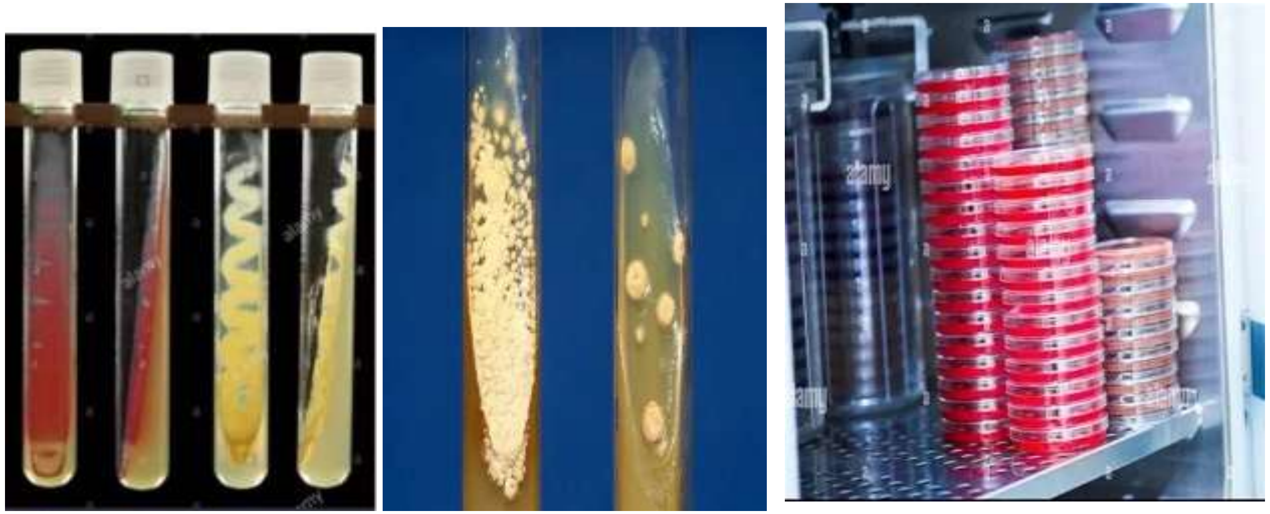
- All microbiology laboratories preserve microorganisms on agar slants.
- The agar slants are inoculated and incubated until good growth appears and stored in refrigerators.
- The slants are incubated for 24hr or more and are then stored in a refrigerator can be stored for few months.

Oil immersion

- The agar slants are inoculated and then covered with sterile mineral oil to a depth of 1 cm above the tip of slant surface.
- The slants are incubated for 24hr or more and are then stored in a refrigerator.
- These cultures are periodically transferred to fresh media.

Paraffin Method

- In this method, sterile liquid paraffin is poured over the slant (slope) of the culture and stored upright at room temperature.
- The layer of paraffin ensures anaerobic conditions,prevents dehydration of the medium, helps pure culture to remain in a dormant state and, therefore, the culture is preserved for several years.



Slants of bacteria and fungi

Oil immersion

Paraffin Method

Preservation

Preservation in Ampoules (80°C –112°F)

- Cultures are sealed in ampoules under sterile conditions. The ampoules are then sealed hermetically and can be stored for weeks to months.

Glycerol stocks (–80°C / –112°F)

Bacterial cultures are mixed with glycerol (typically 15-50%) and stored in cryovials at -80°C. Glycerol acts as a cryoprotective agent, allowing cultures to be preserved for several years.

Cryopreservation

- **Cryopreservation** involves preserving biological cultures at extremely **low temperatures (-196°C),** that process halts all biological activity and metabolic processes.
- **This process typically uses liquid nitrogen, in the stabilisers like Glycerol,** DMSO and sucrose, that prevent the formation of ice crystals and promote cell survival.

Lyophilization

- Lyophilisation, also known as freeze drying, involves freezing the culture and then reducing the surrounding pressure to allow the frozen water in the material to sublimate directly from ice to vapour.

This method does not typically require protectants as the process itself minimizes damage by removing water at low temperatures and pressures, thereby preventing ice crystal formation

Both methods are associated with proteins, but cryopreservation is more commonly used for preserving live cells and tissues, while lyophilisation is more cost-effective for large scale manufacturing of diagnostic tools

Aim

To determine the cultural characteristics of microorganisms and identify them

Identification of Bacteria

Besides, the identification of bacteria involves a combination of phenotypic and genotypic methods.

Phenotypic methods rely on the **physical characteristics of the bacteria, such as morphology, growth patterns, and biochemical reactions.**

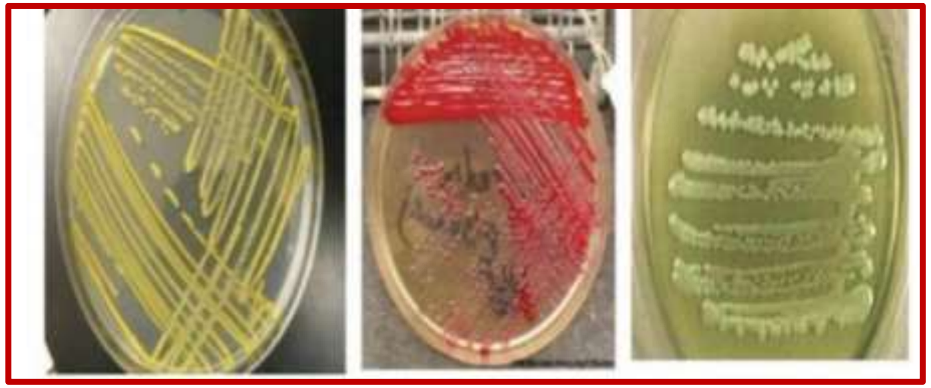
Genotypic methods, on the other hand, **use genetic information to identify bacteria**.

Methods used for identification include:

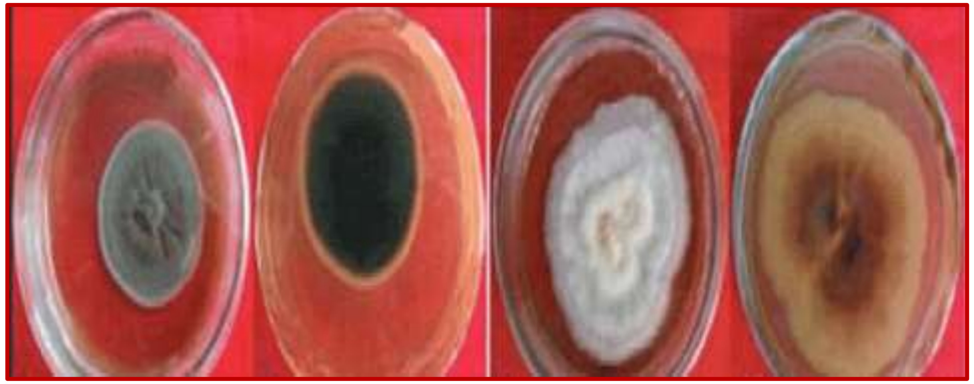
- **Gram Staining**: A simple staining technique that differentiates bacteria based on their cell wall structure.
- **Culture Growth**: Culturing bacteria on specific media to observe their growth patterns and characteristics.
- **Biochemical Tests**: Tests that measure the metabolic capabilities of bacteria, such **as enzyme production and sugar fermentation**.
- **Serological Tests**: Tests that **detect specific antigens on the surface of bacteria, such as O, H, and K antigens**.
- **Molecular Techniques:** Techniques like PCR, sequencing, and mass spectrometry that provide a more **detailed genetic profile** of the bacteria.

Colony and Cultural characteristics

- Microorganisms grow from a single cell or spore (derived from the same mother cell and are genetically identical)
- When a spore/cell is introduced in a predetermined culture medium (NA/PDA/SDA, etc) under controlled laboratory conditions, it reproduces to produce a **colony.**
- Hence, a microbiological **culture, or microbial culture**, is a method of multiplying **microorganisms.**
- The term culture can also refer to the microorganisms being grown.
- Therefore, the colony of each microorganisms exhibit particular specific morphological growth patterns like **colony colour, shape, texture, margin, and elevation, which are unique to bacteria and fungi.**
- These differences are called **cultural characteristics** or morphology.
- In other words, Colonial morphology, which can also be referred to as cultural characteristics, pertains mostly to the macroscopic appearance of a microorganism.
- Cultural characteristics or morphology may be used as an aid in identifying and classifying some organisms.



Colonies and the varied cultural characteristics of three different species of Bacteria



Colonies and the varied cultural characteristics of different species of fungi

Colony Morphology

Colonies with varied morphological features growing on a agar plate

Bacteria

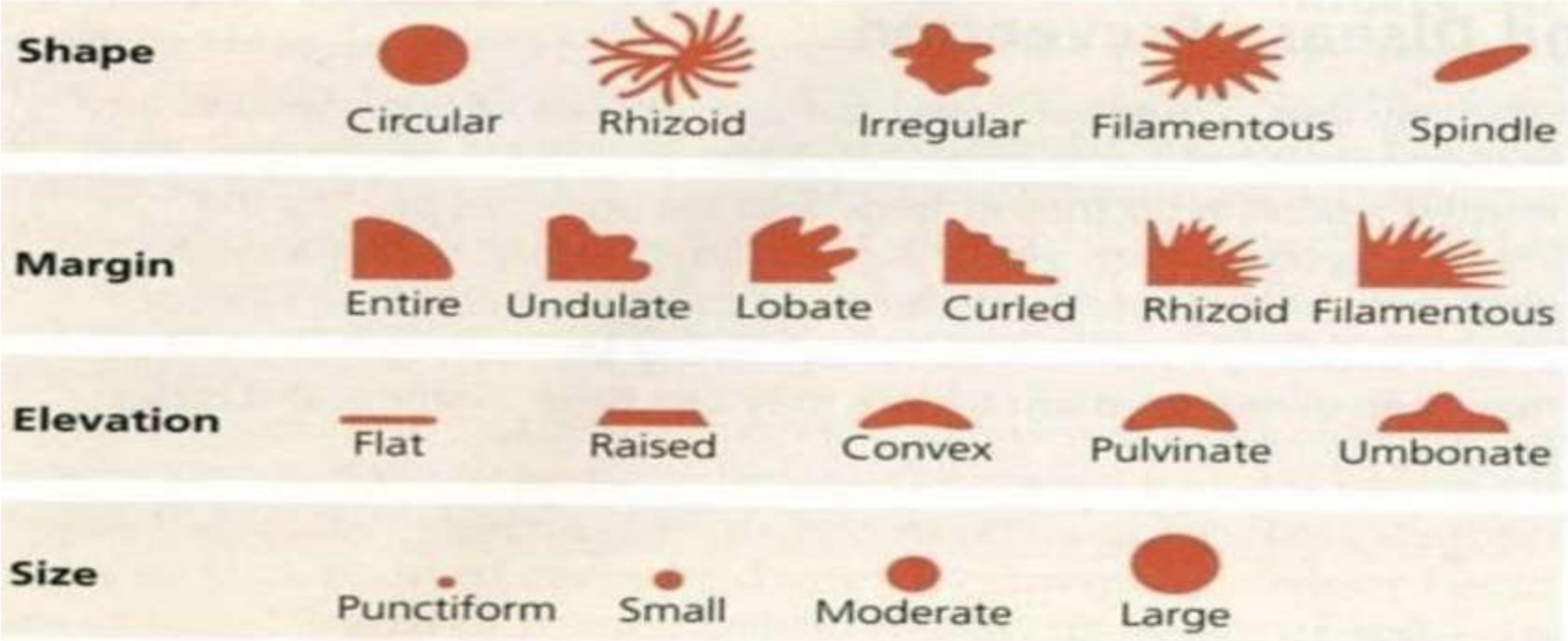
Fungi

These characteristics are crucial for distinguishing between different bacterial species and are used in microbiology to study and classify microorganisms

The key characteristics of bacterial colonies include:

- **Form**: The shape of the colony can vary from round to irregular to filamentous and rhizoid.
- **Elevation**: This describes how much the colony rises above the agar.
- **Margin**: **The edge** of the colony may be a vital characteristic in identifying organisms.
- **Size**: Colonies can vary in size, with tiny ones referred to as **punctiform (pin-point) and larger ones indicating motility.**
- **Colour**: The **pigmentation of the colony** can provide clues about the organism's identity.

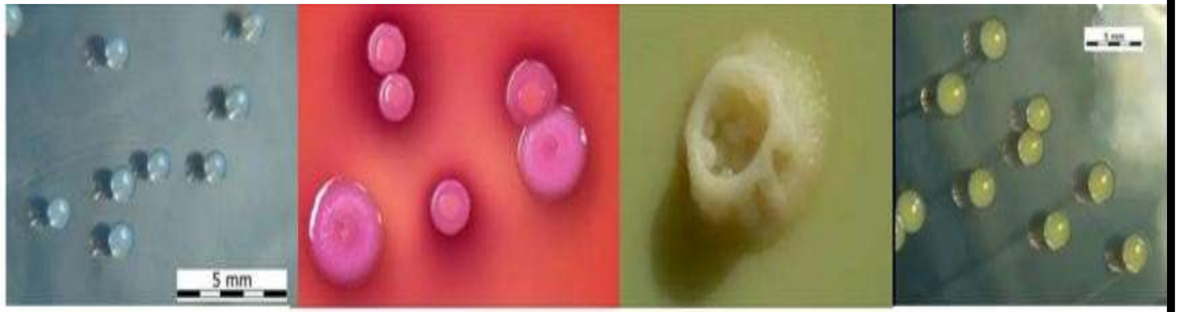
Surface Appearance: **The texture and surface characteristics** of the colony can also aid in identification.



Texture	Smooth or rough
Appearance	Glistening (shiny) or dull
Pigmentation	Nonpigmented (e.g., cream, tan, white) Pigmented (e.g., purple, red, yellow)
Optical property	Opaque, translucent, transparent

A Circular, glistening, and yellow | A Irregular form, glistening, and white. | A. Rhizod, veined and rough, yellow | A. Filamentous, rough, and white

Different Form of Colony

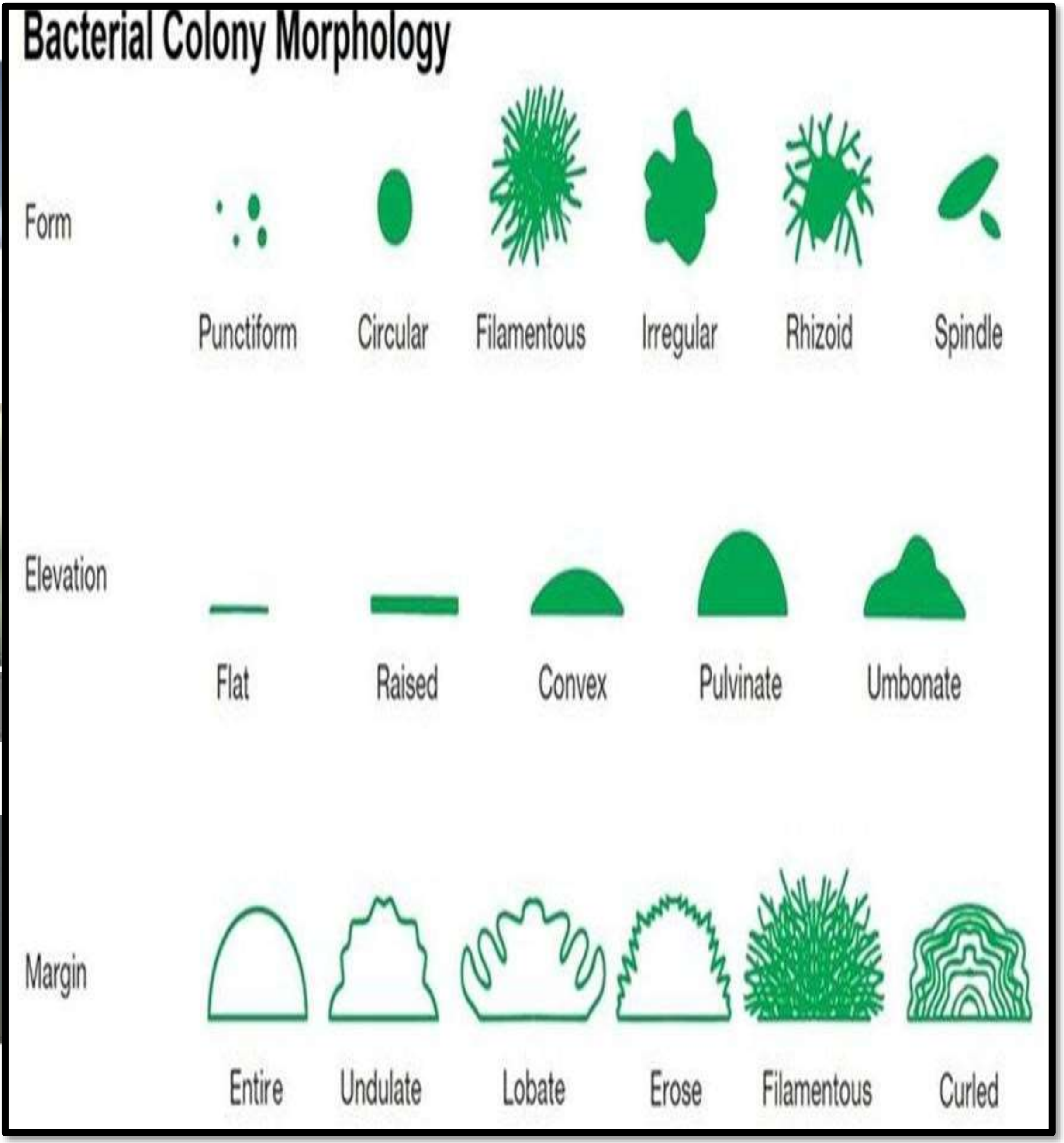


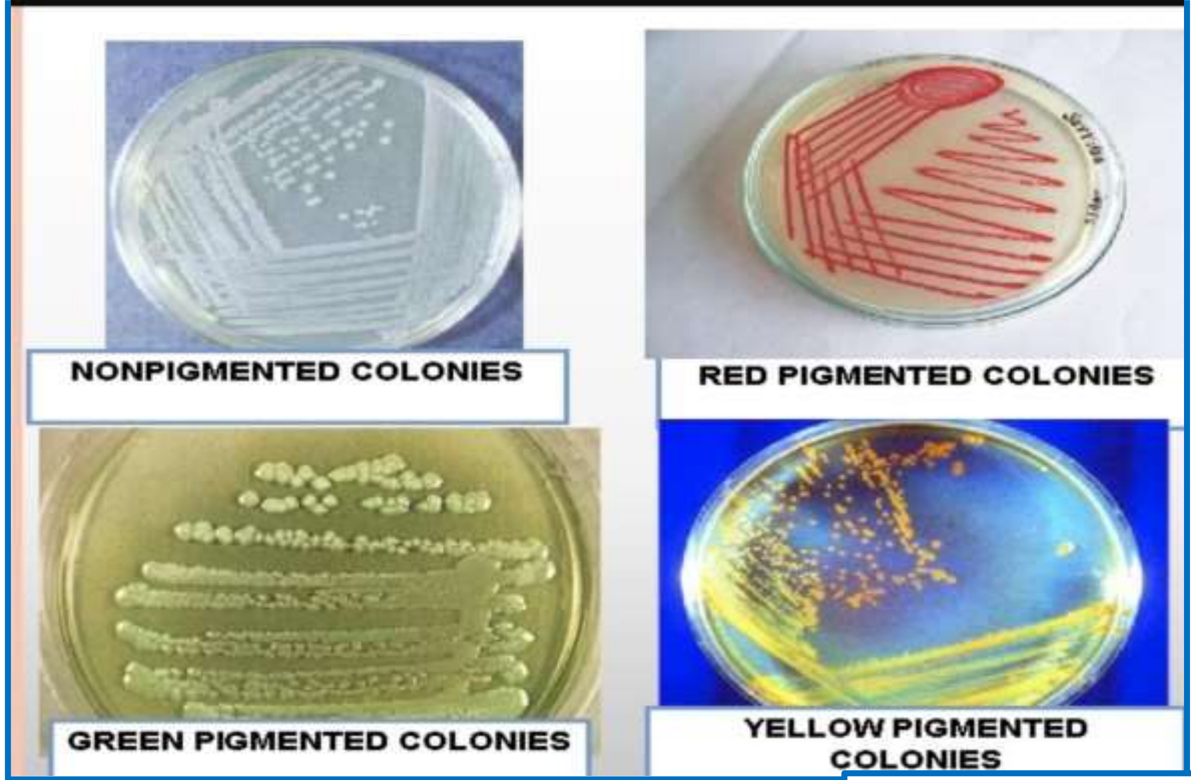
Raised	Umbonate	Crateriform	Convex

Elevation of Colony

A. Entire | A. Lobate | A. Curled | A. Undulate

Margin of Colony

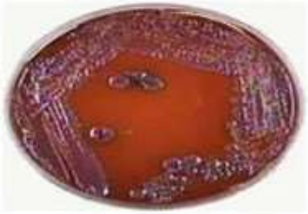




Pigmentation

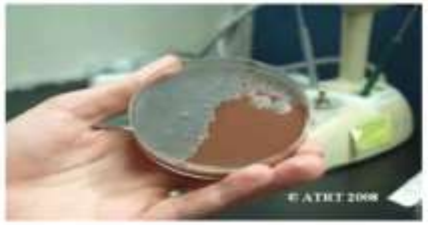
- **Opacity of the bacterial colony**

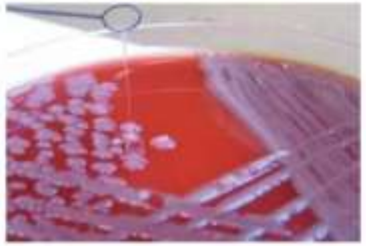
- Opaque (not clear)
- Translucent (clear)
- Iridescent(shine)



- **Texture of bacterial colony**

- Dry
- Moist
- Viscid (stick to loop)
- Mucoid (mucus-like)





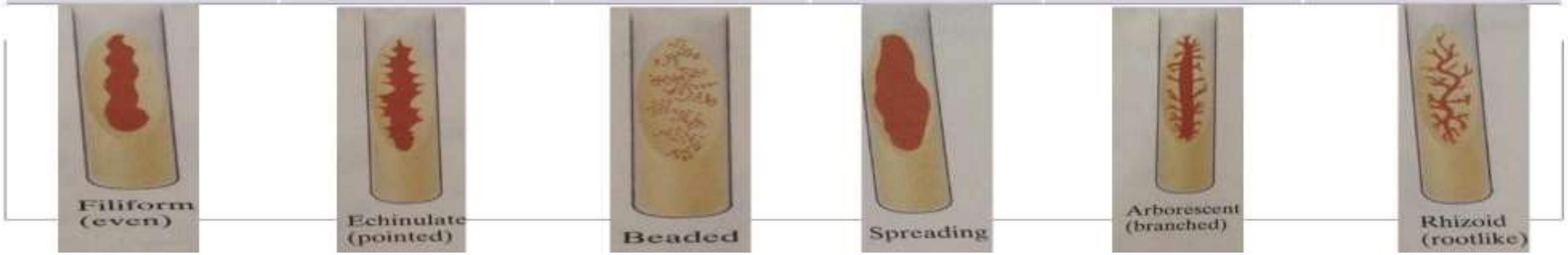
Growth In Nutrient Slants

1. **Growth** - the amount of growth is designated as **none, slight, moderate, or large**

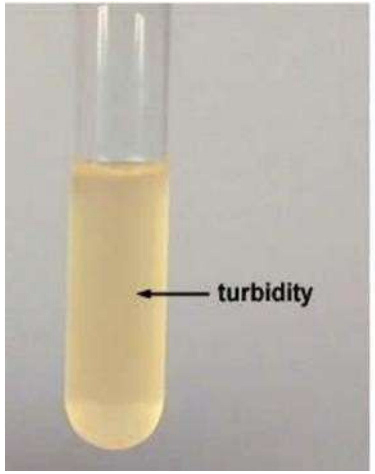
2. **Pigmentation** – chromogenic bacteria may produce intracellular pigments that are responsible for the color of the colonies on the agar surface.
3. **Optical characteristics** - these characteristics are based on the amount of light transmitted through the growth: opaque (no light transmitted), translucent (partial transmission), or transparent (full transmission).
4. **Form** – the appearance of the single line streak of growth on the agar slant.

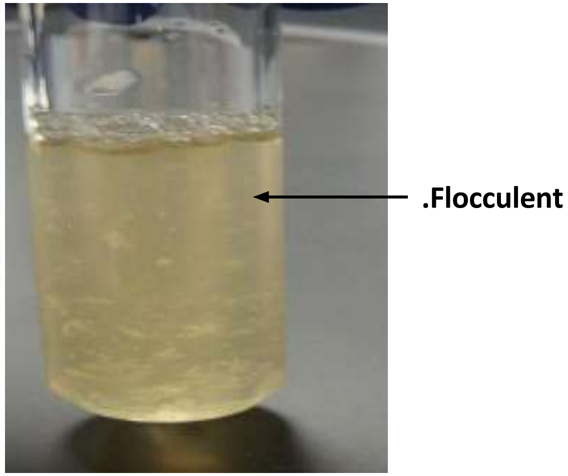
Filiform ; echinulate; beaded; effuse; arborescent ; rhizoid

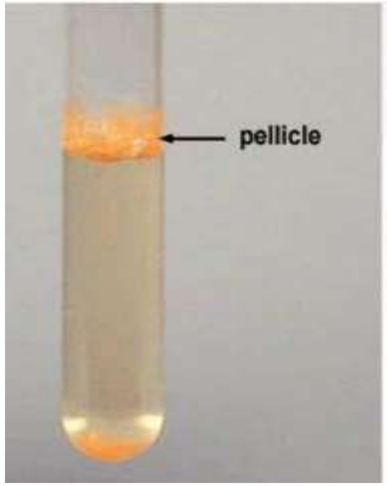
Filiform	Echinulate	Beaded	Effuse	Arborescent	Rhizoid
Continuous, threadlike growth with smooth edges.	Continuous threadlike growth with irregular edges.	Non confluent to semi confluent colonies	Thin, spreading growth	Treelike growth	Rootlike growth

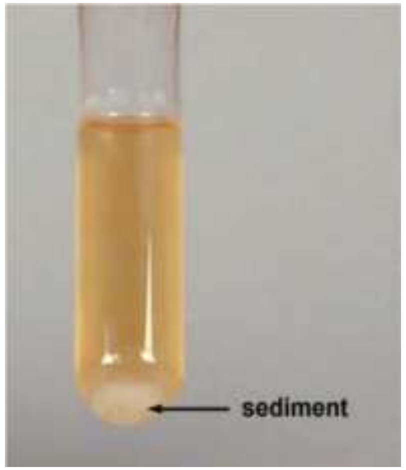


Bacterial growth on Nutrient broth









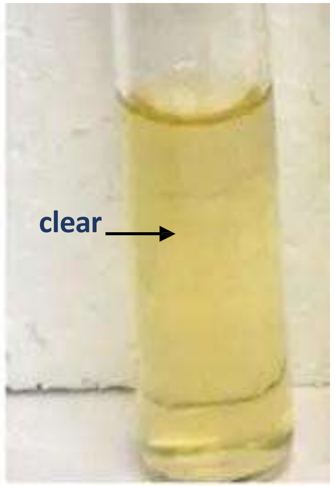
1. **Uniform fine turbidity –** **finely dispersed growth throughout (cloudy)**

2. **Flocculent** **– flaxy aggregates dispersed throughout**

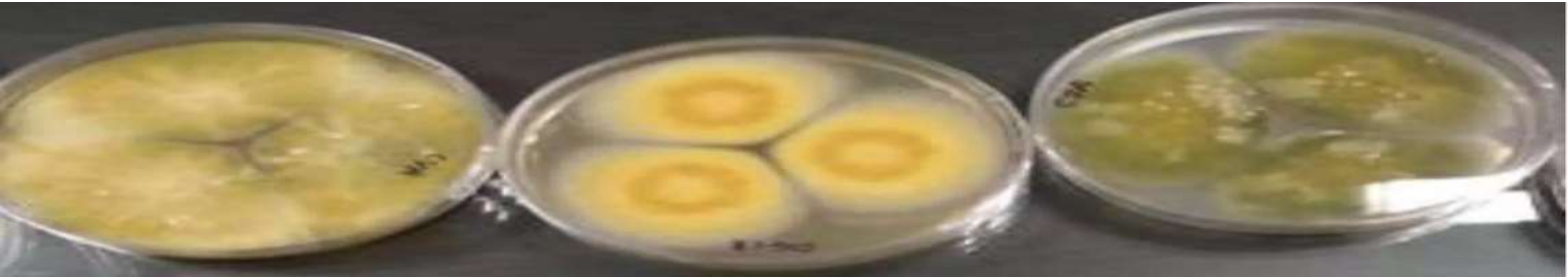
3. **Pellicle** **– thick, padlike growth on the surface**

4. **Sediment** **– concentration of growth at the bottom of the broth culture may be granular, flaxy, or flocculent**

5. **Ring formation** **– a ring of growth on the surface**



Fungal Identification and colony characteristics



Identification of fungi and bacteria

- Fungi and bacteria can infect a wide range of organisms, including humans, plants, animals, birds and other microorganisms
- Their identification plays a vital role in diagnosing infections for accurate and prompt treatment, in research, the pharmaceutical industry, epidemiological studies and for food and environmental safety, ensuring safety, health, and regulatory compliance.
- Microbiologists use various methods to identify fungal and bacterial species.
- Firstly, conventional techniques rely on morphology, culture characteristics (growth patterns), and biochemical reactions.
- Additionally, molecular assays enhance detection accuracy.
- Researchers now employ advanced tools to expedite identification.
- Moreover, timely identification improves patient treatment outcomes.
- Laboratories integrate both traditional and modern techniques routinely.,

Fungal Identification

- Identification of fungal infection is based on morphological and physiological properties of fungi, also clinical information of the patient
- Laboratory approaches used on diagnosis of fungal infection includes;

  Direct microscopic examination, **Histopathological methods**, **culture of the organism, serologic tests, PCR and other molecular methods**

Fungal identification

- **Fungi identification methods include a combination of the following approaches, strategies**

- Macroscopic Examination: This involves observing the growth form, colony characteristics, fruiting body structure, and spore print. it is particularly useful for identifying larger fungi like mushrooms, bracket fungi, and molds growing on surfaces.

- Microscopic Examination:

a) fungal sections stained with **lactophenol cotton blue**

b) wet mount preparations (skin, hair, nails, tissue) using potassium hydroxide or calcofluor white stain to visualise fungal structures.

c) Histopathological stains like Gomori methenamine-silver and periodic acid-Schiff are used to identify fungi in tissue sections.

- Culture Methods: Fungi can be cultivated on selective media such as Sabouraud Dextrose Agar (SDA), Potato Dextrose Agar (PDA), and Cornmeal Agar, which support the growth and differentiation of various fungal species.

- Molecular Methods: Techniques like PCR amplification of ribosomal DNA regions (e.g., ITS, LSU) followed by sequencing are highly recommended for accurate and rapid fungal identification.

- Biochemical Methods: These methods **involve biochemical tests** to identify the presence of **specific enzymes or metabolites associated with particular fungal species.**
- Immunological Methods: These methods **use antibodies to detect specific fungal antigens,** which can be used for identification in
- clinical settings.

Identification of fungal culture by colony morphology

a) **Texture**

b) **Colour**

c) **Reverse of the colony**

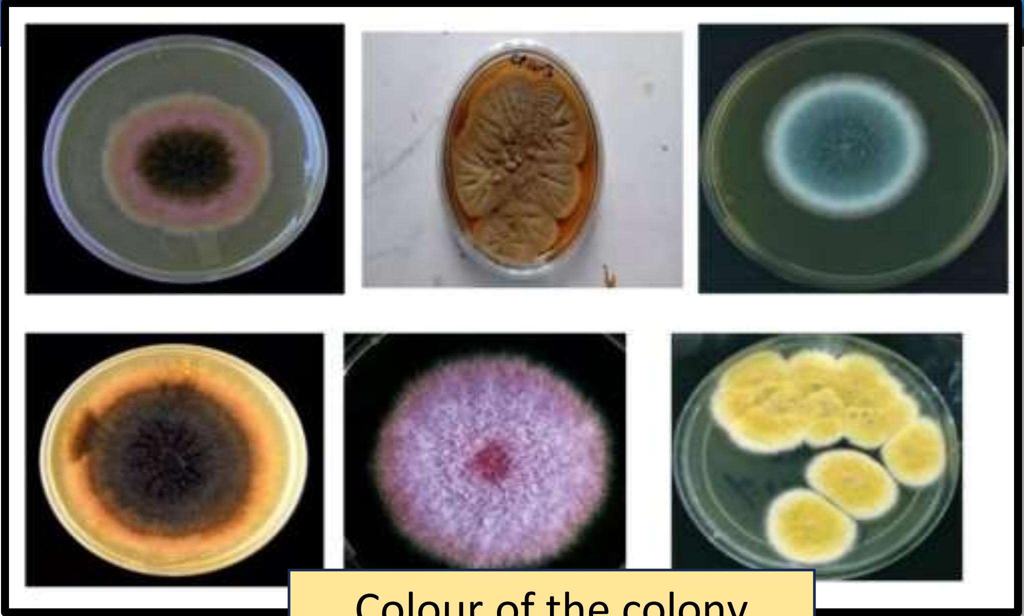
d) **Soluble pigments**

e) **Exudates**

f) **Center and margins of colony**

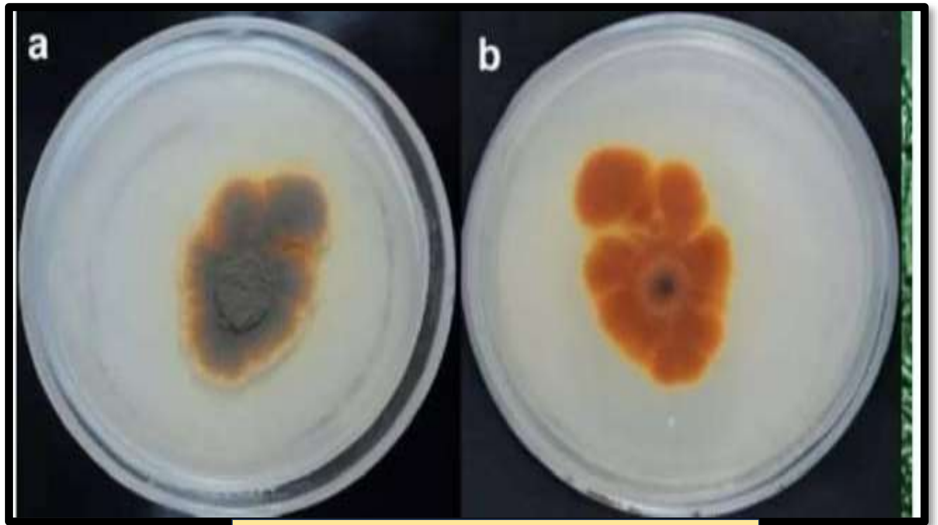
g) Grooves

h) **Colony measurements**

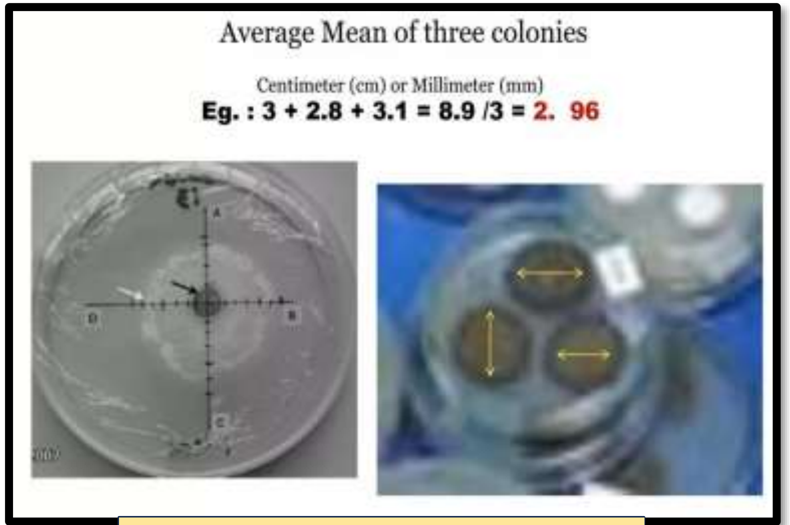


Colour of the colony

Soluble pigments



Reverse of the colony



Colony measurements

Colony morphology

Velvety Colony

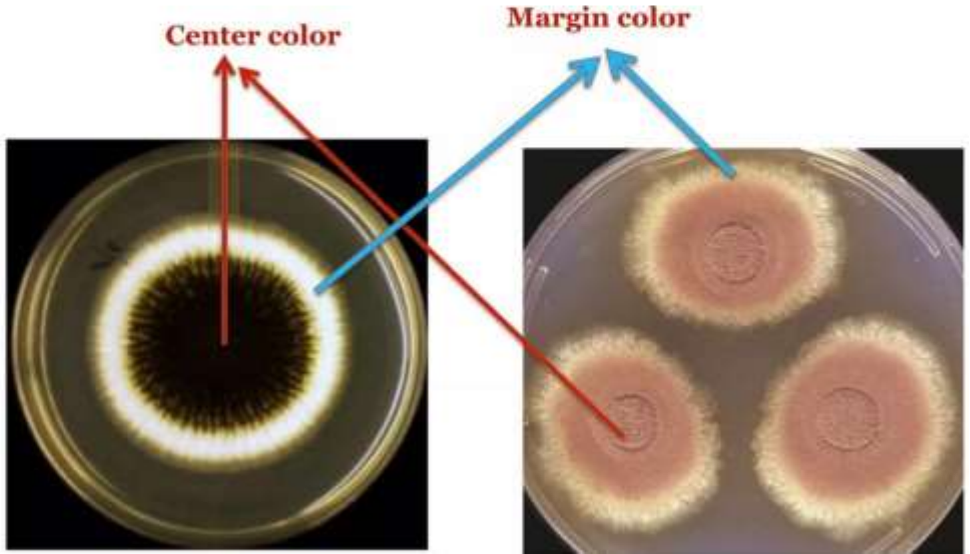
Cottony Colony

Powdery Colony

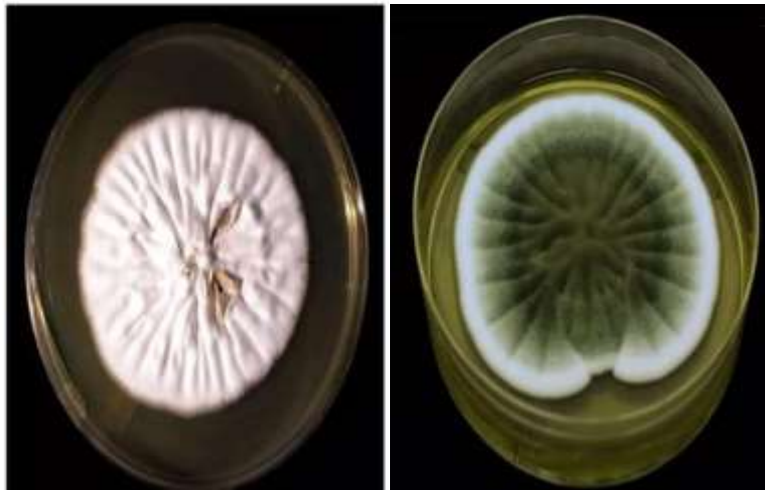
Hairy Colony

Granular Colony

Center and margins of colony



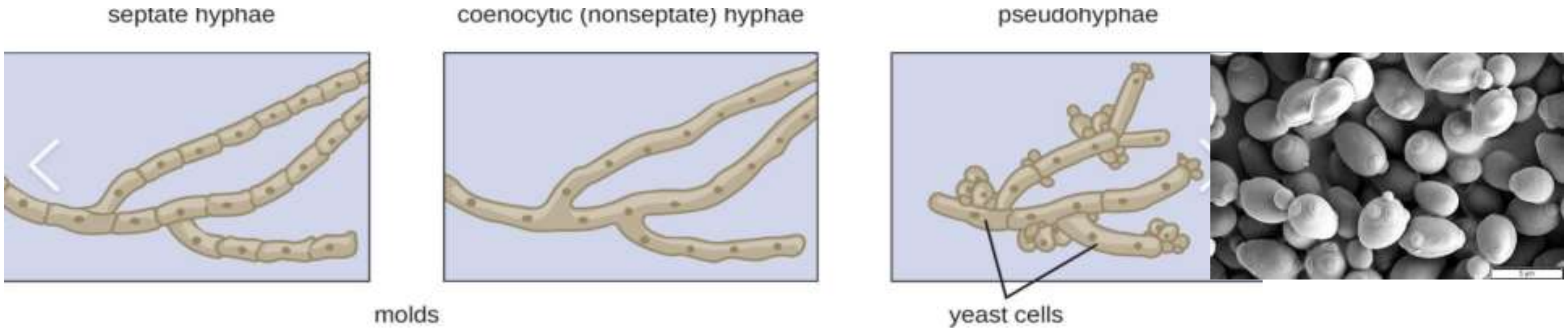
grooves



Exudates/secretion of liquid

Fungal identification

- Fungi (molds) are eukaryotic, multicellular organisms and are made up of filaments called **hyphae**.
- Hyphae can form a tangled network called a **mycelium** and form the **thallus** (body) of fleshy fungi.
- Hyphae that have walls between the cells are called **septate hyphae**; hyphae that lack walls and cell membranes between the cells are called nonseptate or **coenocytic hyphae**.
- In contrast to molds, yeasts are unicellular fungi that reproduce by budding and may result in daughter cells sticking together as a short chain or **pseudohypha** (Figure 1).
- Fungi infect plants and agricultural crops; besides, there are dermatophytes which infect humans, birds, and animals.
- The following media are most commonly used –**Potato dextrose agar**, **Sabouraud's agar, Rose Bengal agar, and Czepek Yeast extract agar.**



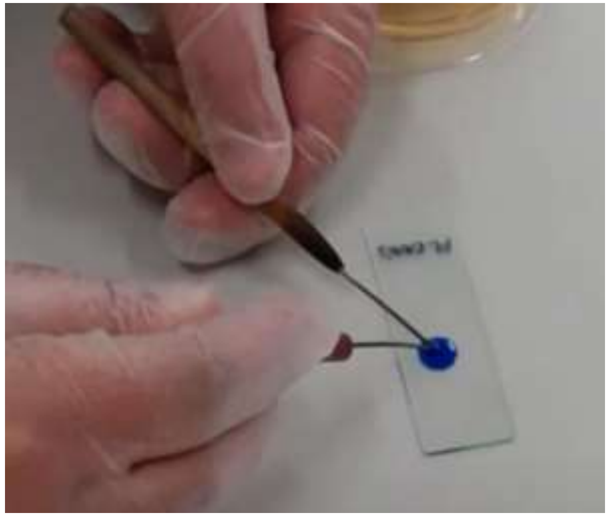
Identification by morphology

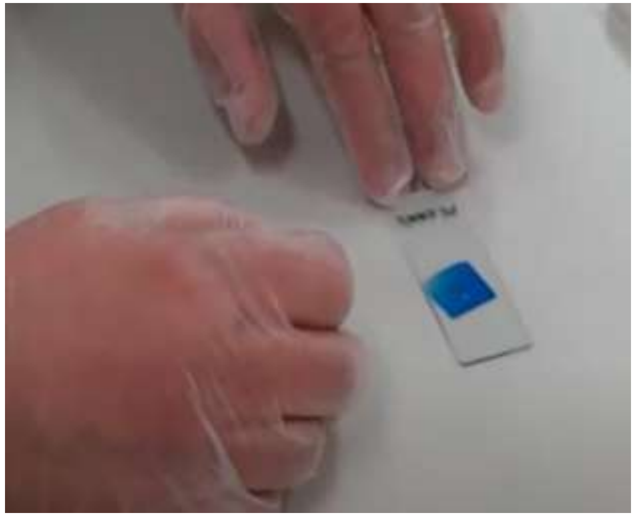
Microscopic Observation

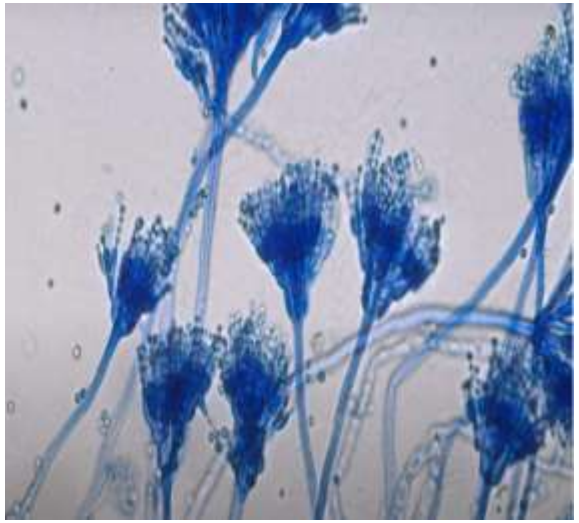
Using Stereo Microscope & Compound Light Microscope

Lacto phenol Cotton Blue Staining (LPCB)

A portion of mycelial colony is picked with needle mounted on a slide , stained with LPCH and observed under a microscope after placing a cover slip carefully





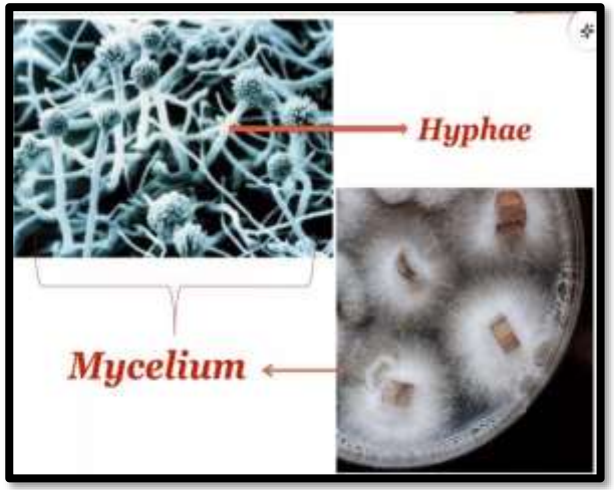


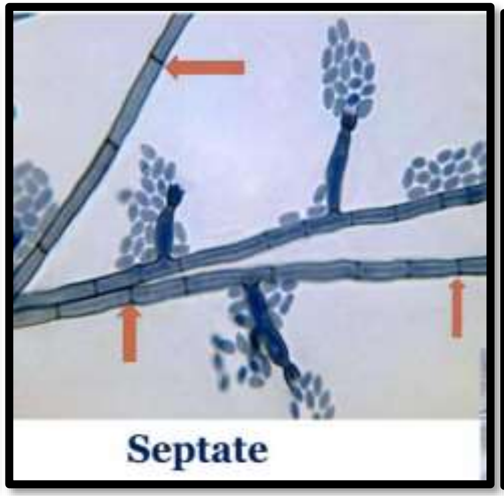
Slide preparation

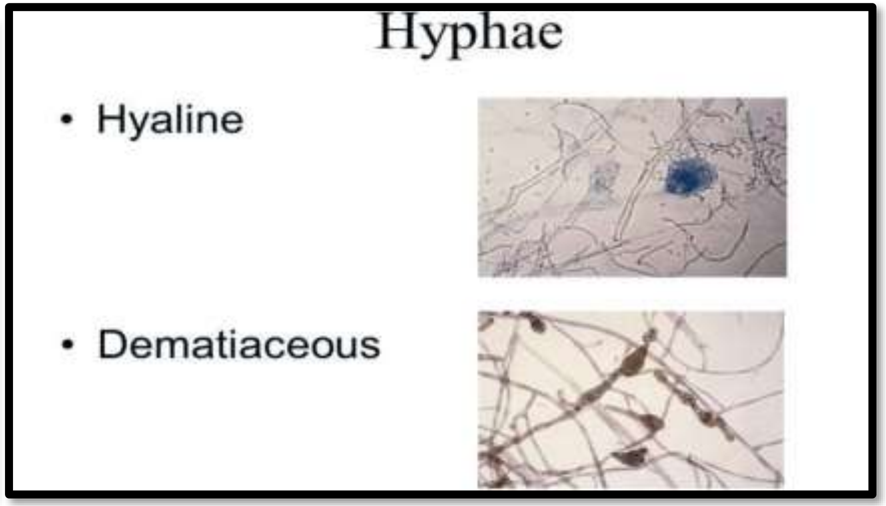
Microscopic observation

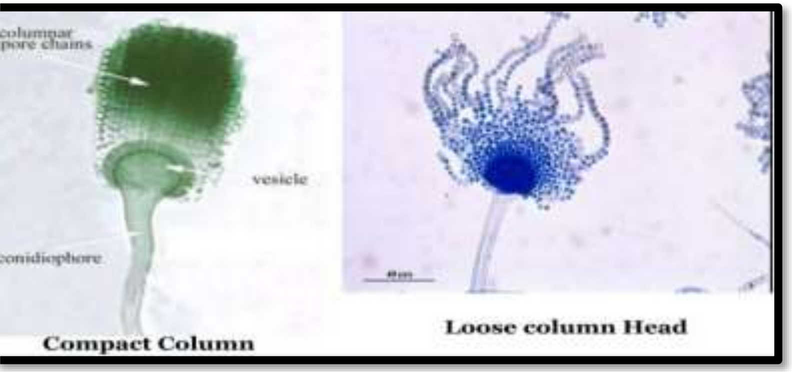
Micromorphology

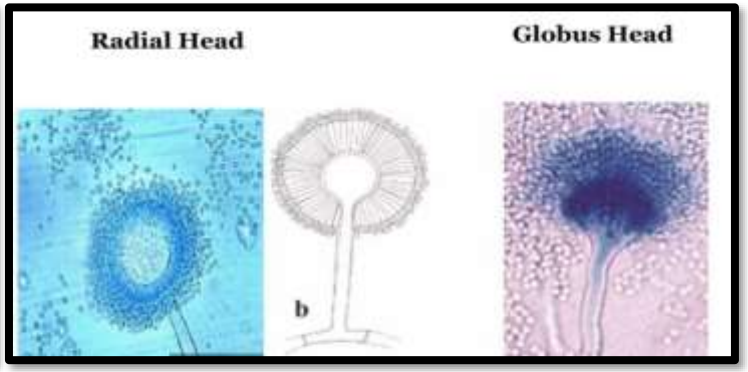
- The following structures are observed under a microscope.
  a)Unicellular or multicellular-(yeast/ mols)
  b) Hyphae-septate/ aseptate/pseudohyphae and branching
  c) Conidia/spores-- shape, size, colour and conidiophores
  d) Presence of macrospores, microspores, arthoconidia,
  f)Fruiting bodies



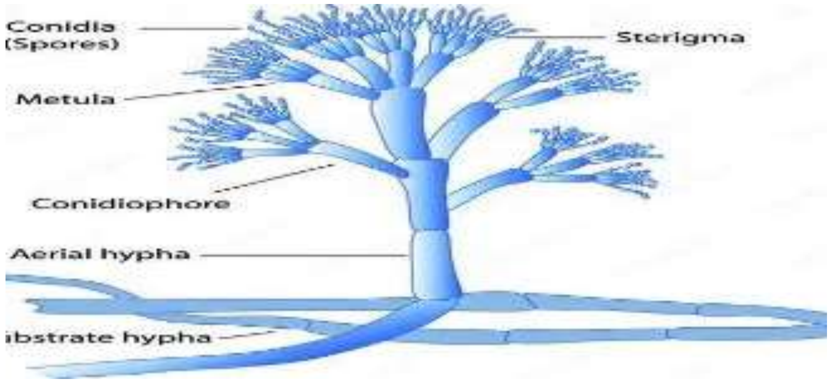








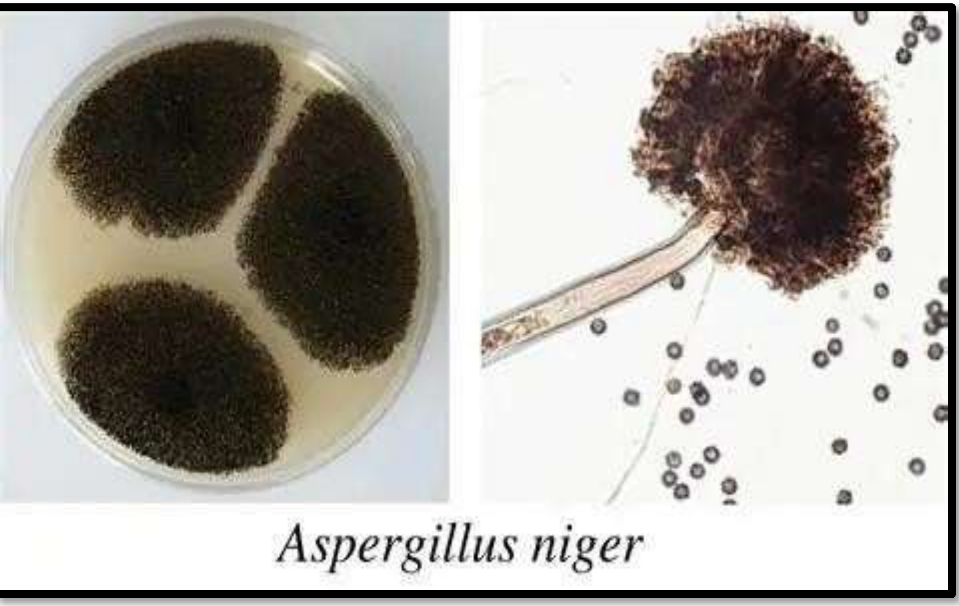
Conidia heads

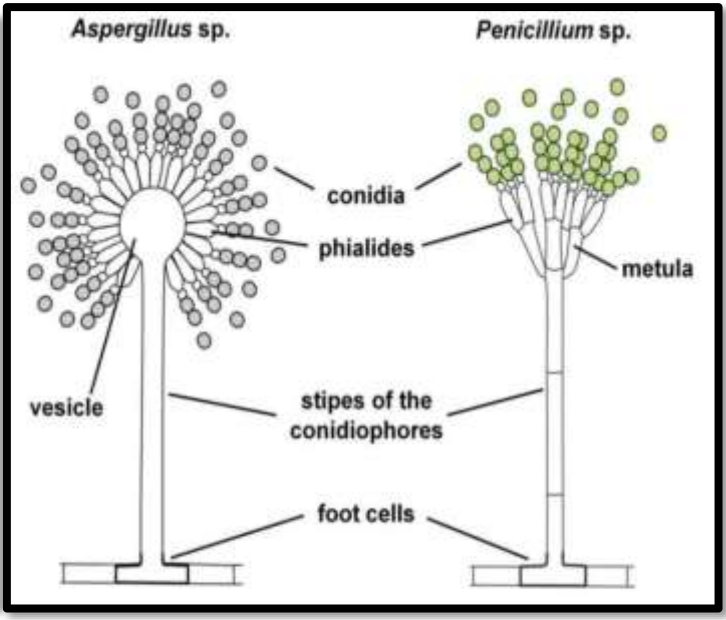


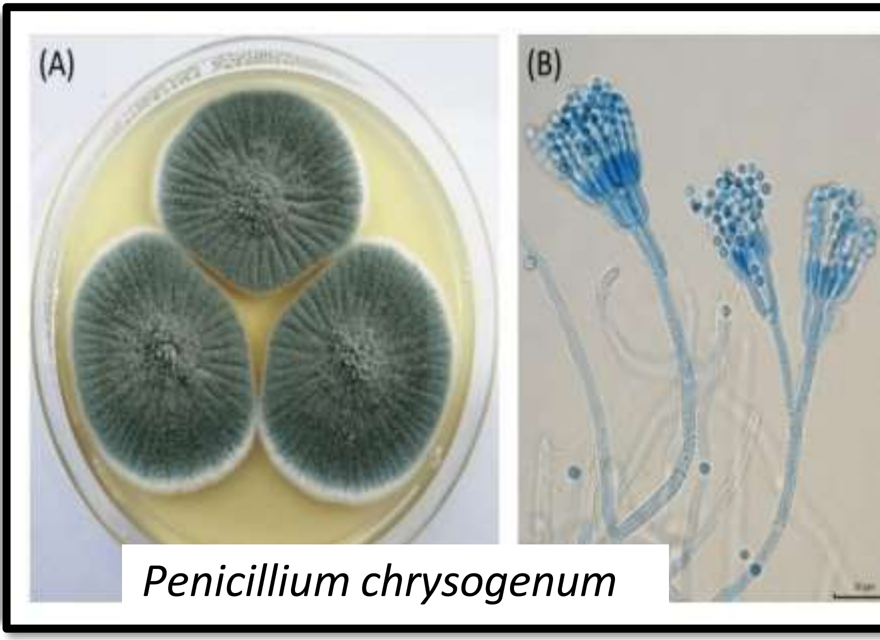
brush-like conidial arrangement in penicililum.

Conidiospores-Aspergillus and Penicillium sp.

- Fungi reproduce asexually by **conidiospores** and sporangiospores.





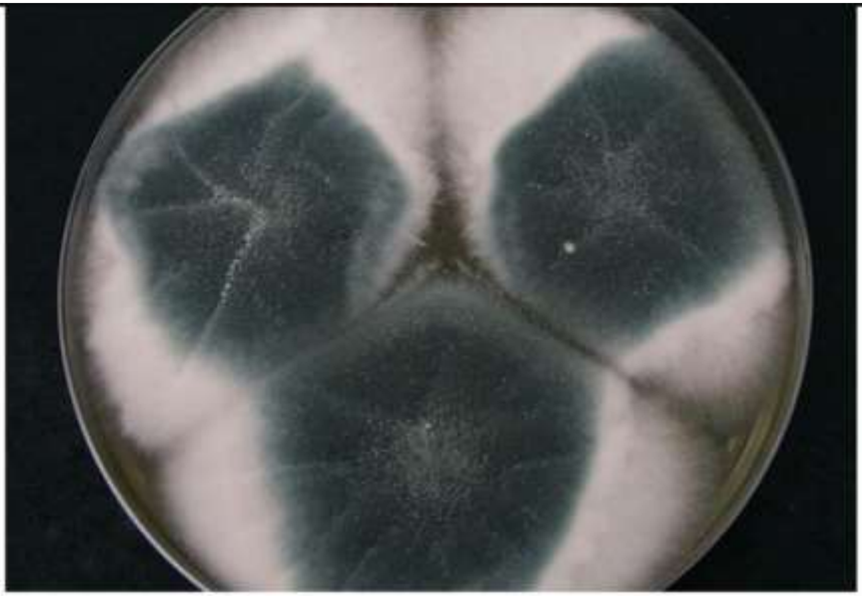


Aspergillus and Penicillium are common contaminant molds, and they reproduce by conidiospores

- **Aspergillus, although nonpathogenic, may become opportunistic and cause severe respiratory tract infection in a compromised host. They also produce mycotoxins, which cause serious health issues and cancer. The conidiophore terminates in a ball-like structure called a vesicle. Its conidiospores, which typically appear brown to black, are produced in chains on phialides coming off the vesicle**
- Penicillium is one of the most common household molds and is a frequent food contaminant.

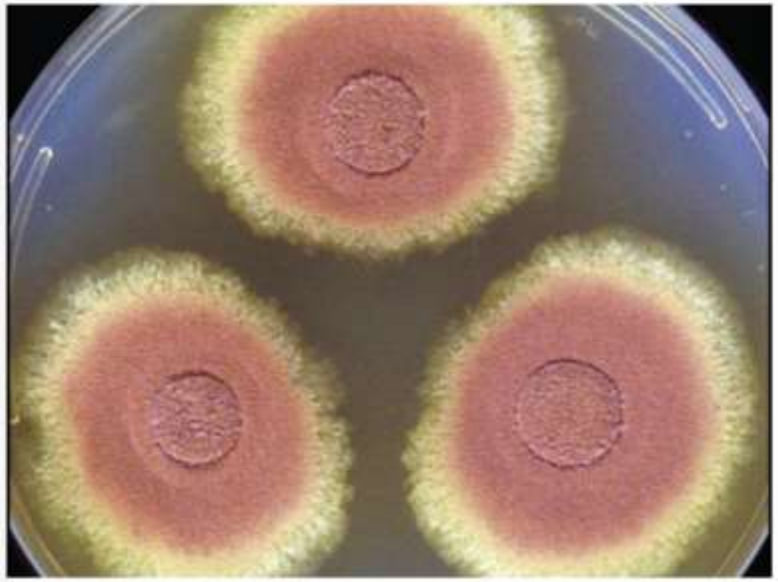
The conidiospores of Penicillium usually appear grey, green, or blue and are produced in chains on finger-like projections called phialides coming off the conidiophore.

Aspergillus flavus

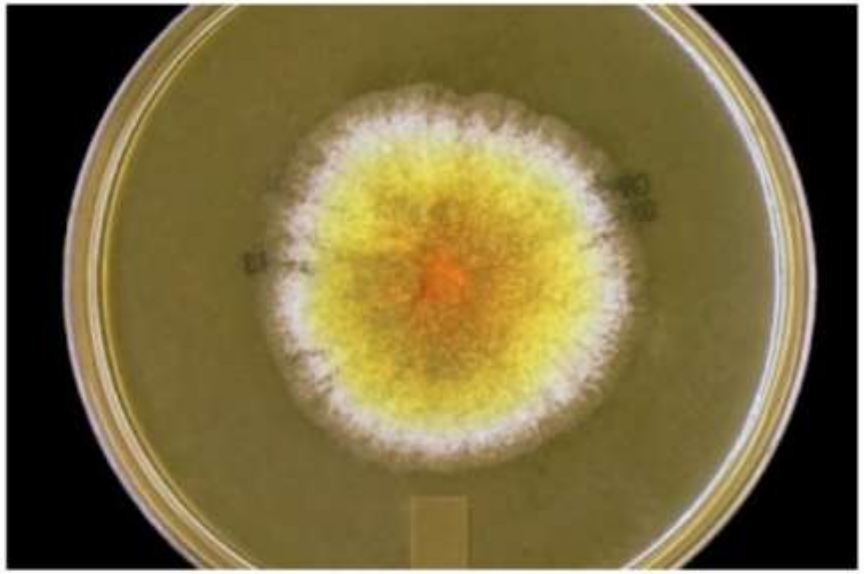


Aspergillus fumigatus

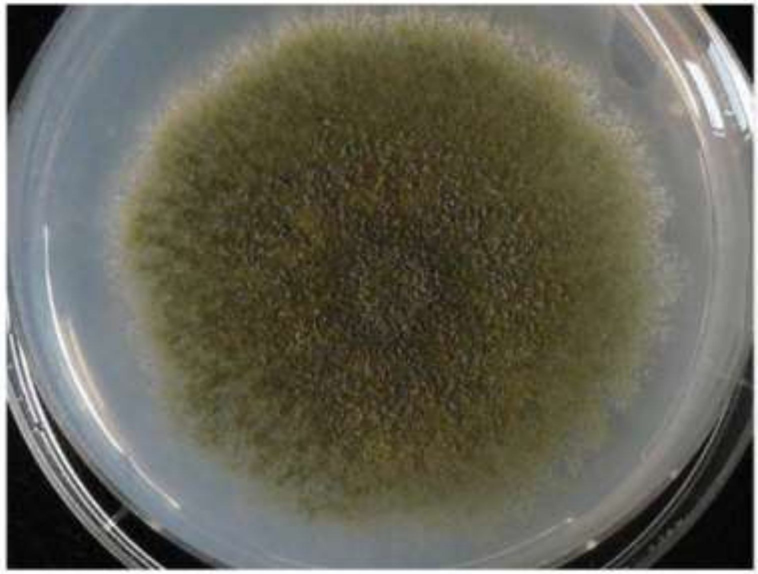
Aspergillus niger



Aspergillus terreus



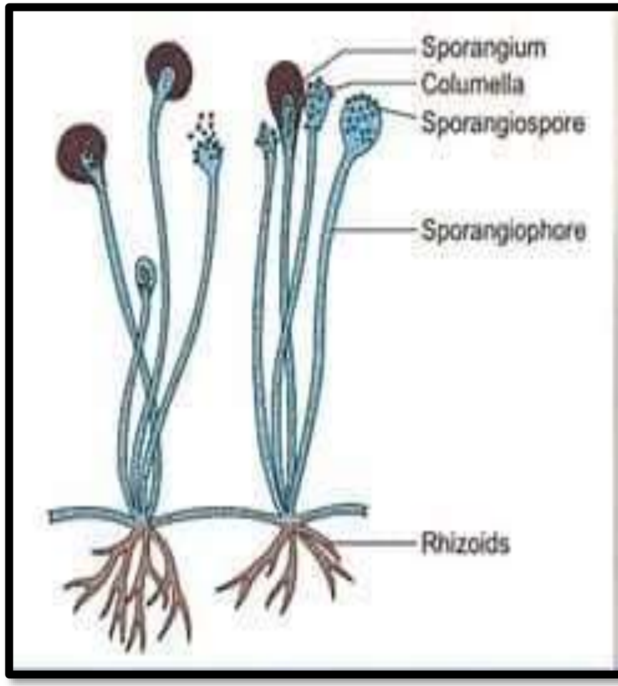
Aspergillus glaucus

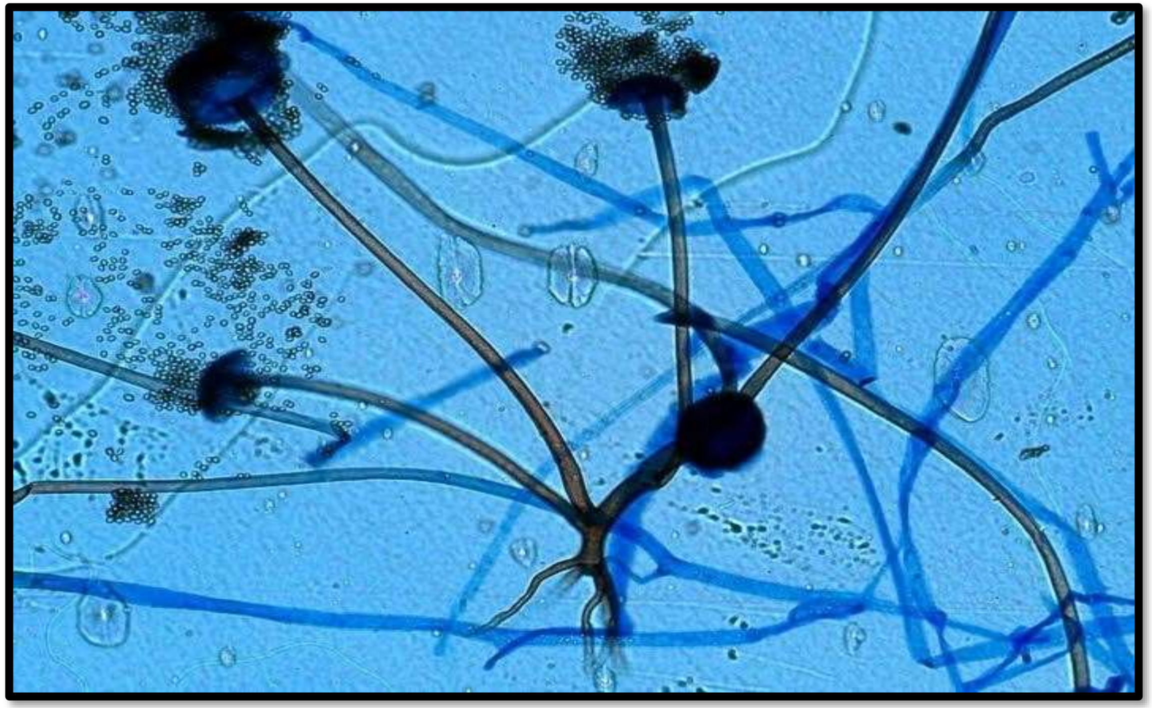


Aspergillus nidulans

Sporangiospores- *Rhizopus stolonifer*

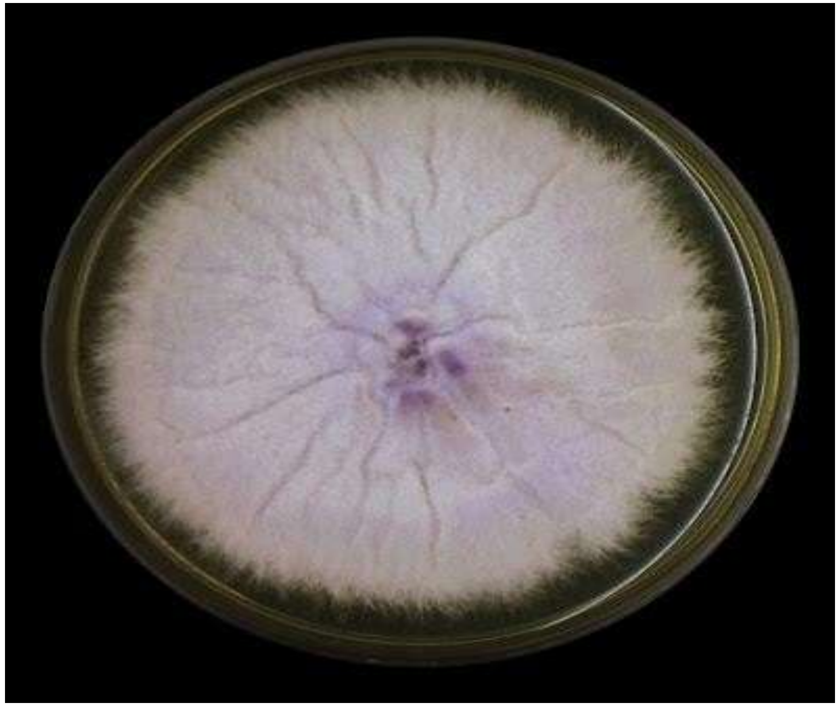
- Sporangiospores are produced within a sac or sporangium on an aerial hypha called a sporangiophore
- Rhizopus is an example of a mold that produces sporangiospores.
- Although usually nonpathogenic, it sometimes causes opportunistic wound and respiratory infections in the compromised host.
- At the end of its sporangiophore is dome-shaped end called a columella that extends into a sac-like structure called a sporangium. Its sporangiospores, typically brown or black, are produced within the sporangium .
- Anchoring structures called rhizoids are also produced on the vegetative hyphae.

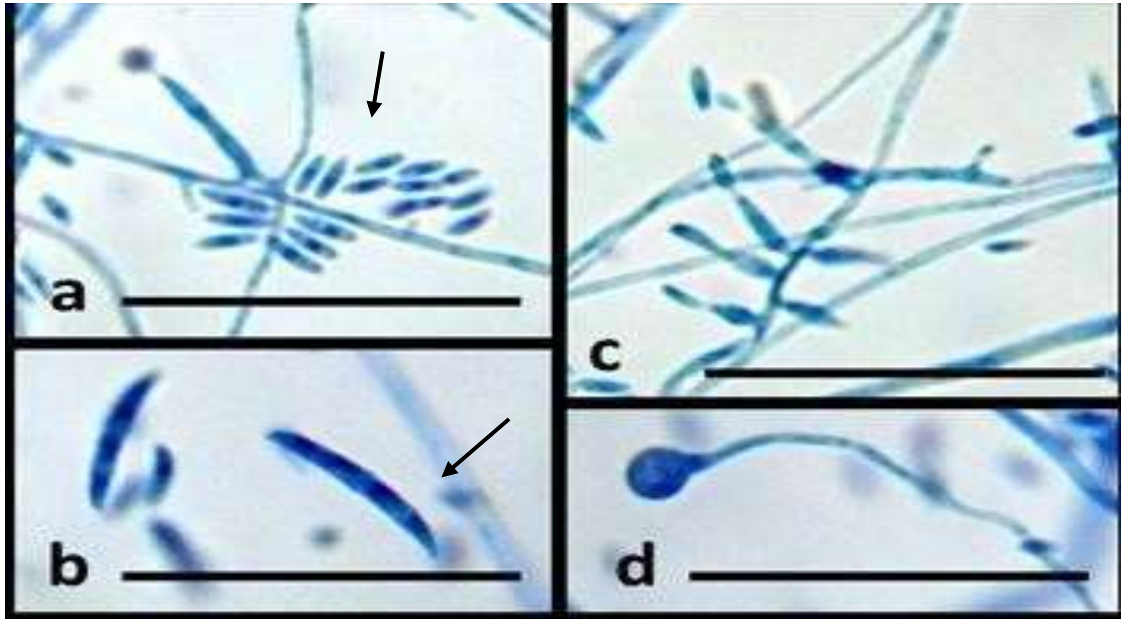




Microspores and Macrospores

- Fusarium sp. grows really fast with varying colony colour depending on isolates.
- Woolly to cottony, flat, spreading colonies. Within few days cover the entire agar plate.
- Conidia are the spores produced by Fusarium.
- They are two types- macro and micro-conidia both of which can be seen under microscope.
- Macroconidia are multicelled while micronidia are single celled

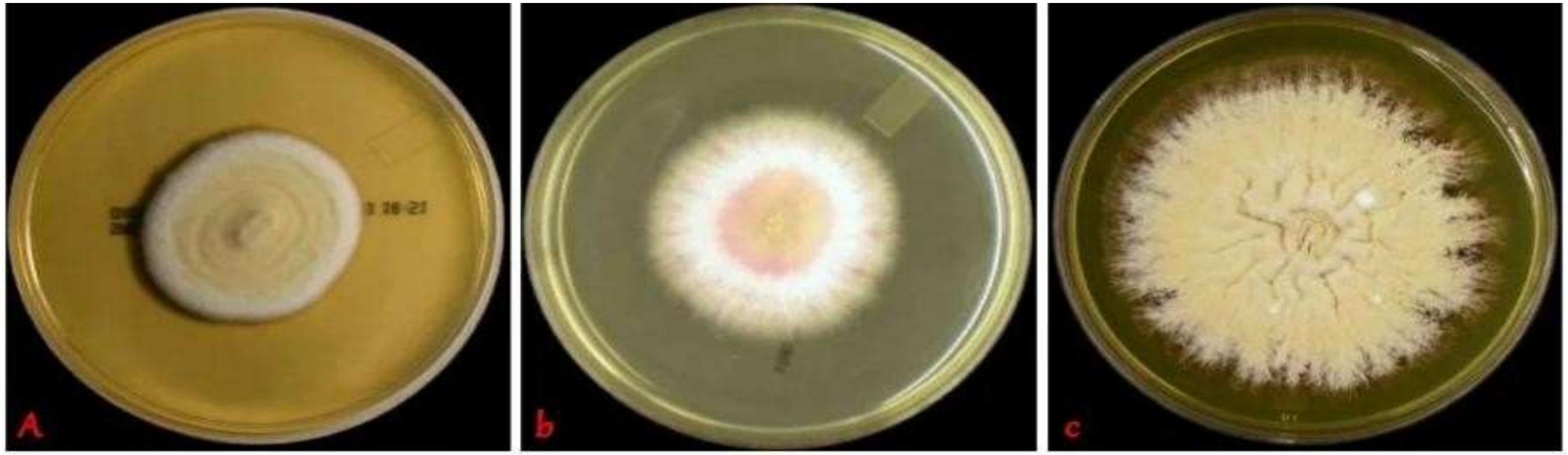




Macroconidia and Micro-conidia

Dermatophytes

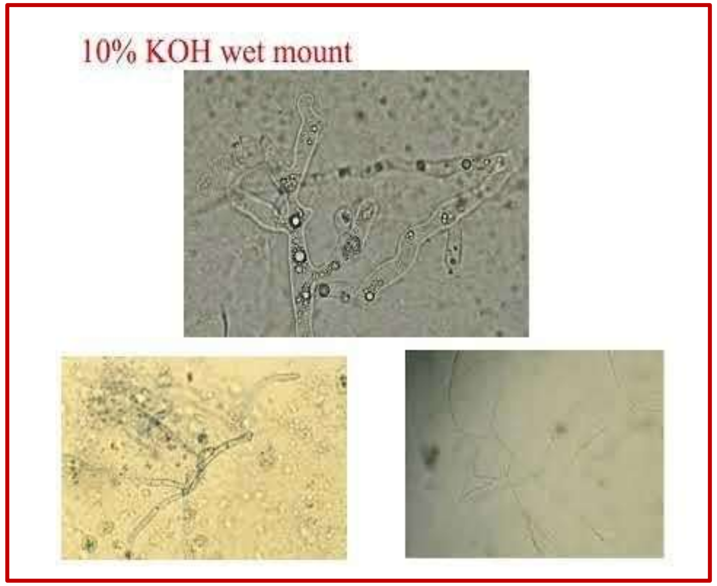
- The dermatophytes are a group of molds that cause superficial mycoses an infection of the hair, skin, and nails.
- They feed on the protein keratin, present in hair, skin, and nails.
- Infections are commonly referred to as ringworm or tinea infections.
- The three common dermatophytes are Microsporum, Trichophyton, and Epidermophyton. These organisms grow well at 25°C.



A. *Trichophytpon Species*, **B.** *Microsporum species*, **C.** *Epidermophyton species*

Macroscopic (colony) appearance of various dermatophytes on SDA.

Identification of dermatophytes



KOH mounts