ISBN 978-1-960740-04-5

A LABORATORY MANUAL OF MYCOLOGY

Authors
Saba Sana
Iqra Liaqat
Asma Abdul Latif
Kinza Khan
Sikander Ali
Muhammad Nouman Aftab
Muhammad Usman Ahmad
Shamsa Niaz
Nida Sana

Publication Date
February 13, 2024
doi: 10.5281/zenodo.10631334

Scientific Knowledge Publisher (SciKnowPub), USA
info@sciknowpub.com

A Laboratory Manual of Mycology

For Correspondence
Dr. Saba Sana
Assistant Professor
Department of Microbiology
Ikram ul Haq Institute of Industrial Biotechnology
Government College University Lahore, Pakistan
Sabasana22@gmail.com

Authors

Dr. Saba Sana[1]

Miss Iqra Liaqat[2]

Dr. Asma Abdul Latif[3]

Dr. Kinza Khan[4]

Prof. Dr. Sikander Ali[5]

Prof. Dr. Muhammad Nouman Aftab[6]

Dr. Muhammad Usman Ahmad[7]

Miss Shamsa Niaz[8]

Miss Nida Sana[9]

[1,5,7]Department of Microbiology, Ikram ul Haq Institute of Industrial Biotechnology, Government College University, Lahore, Pakistan

[2]Department of Pharmacology & Toxicology, University of Veterinary and Animal Sciences, Lahore, Pakistan

[3]Department of Zoology, Lahore College for Women University, Lahore, Pakistan

[4]Department of Microbiology, Faculty of Veterinary Sciences, The Islamia University of Bahawalpur, Pakistan

[6]Ikram ul Haq Institute of Industrial Biotechnology, Government College University, Lahore, Pakistan

[8]Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan

[9]Institute of Social & Cultural Studies, The University of Punjab, Lahore, Pakistan

DEDICATION

Dedicated
To
My Parents
&
My Family

ACKNOWLEDGEMENTS

I deem it as my utmost pleasure to avail this opportunity to express the heartiest gratitude and deep sense of obligation to my dedicated teacher Prof. Dr. Ali Ahmad Sheikh, Institute of Microbiology, UVAS, Lahore, Pakistan for his valuable suggestions, dexterous guidance and inspiring attitude during preparation of this manual. I have no words to thank to all my colleagues and friends for their interest and advices in my research project; in fact their advices will always serve as a beacon of light throughout the course of my life. Finally, my unreserved love and thanks to my Father (late), Mother, Sisters and Brothers who have always encouraged me and gave all the love and support they could offer.

Saba Sana

Table of Contents

Experiment No.	Title of the Experiment	Page No.
1.	Introduction to laboratory equipment	1-5
2.	Primary culture for isolation of fungi	6-12
3.	Subculture techniques for purification of fungi	13-18
4.	Macroscopic identification: immature and mature colony of fungi (mold)	19-27
5.	Microscopic characters: Cellophane tape and tease mount of fungi	28-33
6.	Microscopic characters: Slide culture technique	34-37
7.	Fungal staining and permanent mount of fungi	38-40
8.	Identification of yeast: negative staining and germ tube test	41-46
9.	Dermatophytes: potassium hydroxide (KOH) mount preparation; hair perforation test	47-50
10.	Inoculum standardization	51-54
11.	Antifungal susceptibility testing (AFST)	55-58
12.	Minimum inhibitory concentration determination: micro-broth dilution method	59-62
13.	Log reduction of fungal spores	63-67
14.	Mycotoxin production and extraction	68-72
15.	Mycotoxin detection by thin layer chromatography (TLC)	73-76
16.	Preservation and revival of fungi	77-81
17.	Screening of soil fungi for antimicrobial production	82-86
18.	Screening of soil fungi for phytase production	87-89
19.	Screening of soil fungi for amylase production	90-92
20.	Effect of physical factors on fungal growth Temperature and pH	93-95

Experiment-1

INTRODUCTION TO LABORATORY EQUIPMENT

Equipment

Any apparatus which operates automatically is known as equipment.
Incubator, Hot air oven and Autoclave

Instrument

Any apparatus which involves handling by personnel is known as instrument.

Petri plate, inoculating loop and glass spreader

Followings are some equipment, instruments used in mycology laboratory.

1. **Microscope**

It is an instrument as well as equipment used to visualize microorganisms ('Micro' means 'small' and 'Scope' means 'to visualize'). Objective lenses of 10x and 40x magnification are used for microscopic visualization a fungus

2. **Hot air oven**

It uses dry heat to sterilize which causes the oxidation of contaminants proteins. It usually operates at 160°C for one hour and at 180°C for half an hour. It is used to sterilize petri plates, glass pipettes and flasks etc.

3. **Autoclave**

It is used to sterilize materials using moist heat which causes the coagulation of proteins. Its operational temperature 121°C at 15 pounds per square inch pressure and exposure time is 15 minutes.

It has following parts:

A pressure gauge to measure the pressure, a steam outlet to release the steam (pressure) and a safety valve to maintain the pressure by upward and downward movement of its piston.

Autoclave can sterilize culture media, thermostable plastic materials (micropipette and tips), material made up of metals.

4. Incubator

This equipment is used to maintenance a particular set temperature for long time in order to provide optimal temperature for microbial growth. An incubator is a sealed unit which controls both the temperatures and the humidity of the internal atmosphere. Thermostat is the component which maintains the temperature and keeps it constant.

Optimum temperature for yeast: 37°C and for filamentous fungi (mold) it is 25°C±3 (22 to 28°C).

5. Weighing balance

It measures the weight of a substance. Sensitive weighing balance can measure the mass up to milligram.

6. pH meter

It measures the pH of a liquid which involves the measurement of the concentration of hydrogen ions. It is used to attain a specific pH. The optimal pH for fungus is 4.5 to 5.6.

7. Centrifuge machine

Centrifuge machine is equipment used to separate different layers by rotation at various speeds. The denser particles move downward and less dense particles remain at top. The molecules are separated on the basis of their size, weight, and density.

It may be used for harvesting of microbial culture (yeast cells/ mycelium from a broth).

8. Biosafety cabinet (BSC)

It is used to achieve a sterile contained environment.

It has following components;

- Blower/Fan to circulate the air
- Visible light lamp to provide proper light for working
- UV light for surface sterilization.
- HEPA (High Efficiency Particulate Air) to sterile the incoming and outgoing air from work area inside the cabinet

It has following classes:

- Biosafety cabinet class I
- Biosafety cabinet class II
- Biosafety cabinet class III

For mostly pathogenic fungi BSC class II is used.

9. Fume hood

Some organic solvents (e.g., acetone nitrile, chloroform and methanol) are volatile in nature and produce fumes which are toxic for the environment and human beings. These solvents are used for mycotoxin extraction. For such chemicals, fume hoods are used. These filter these non-infectious toxic molecules.

10. Neubauer chamber

It is a thick glass slide used to count the number of spores of a fungus. It is used to standardize inoculum in for anti-fungal susceptibility testing.

11. Thin layer chromatographic tank

Thin layer chromatographic (TLC) technique used to detect toxins produced by the fungus. It involves two phases; the mobile phase consists of organic solvents and Silica coated aluminum sheets used as stationary phase. Mobile phase is added to TLC tank.

12. TLC plates

These are silica powder coated plates (plastic or glass sometimes on aluminum sheet). It is used for thin layer chromatographic technique as stationary phase

13. Wooden box/UV box

It is used to detect mycotoxins. In this, we use UV lamp of 360nm wavelength. In it, mycotoxins give fluorescence as some mycotoxins absorb smaller wavelengths of light and transmit light of higher wavelengths.

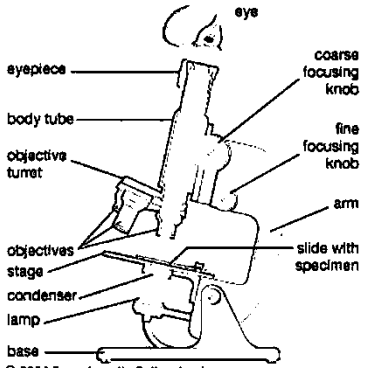
14. Vortex mixer

It is used to mix small of material into liquid mechanically.

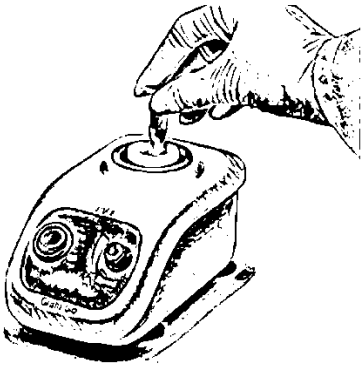
15. Hot plate/magnetic stirrer

It involves the use of a small bar magnet to stir a liquid. It also consists of a heated platform to heat liquids

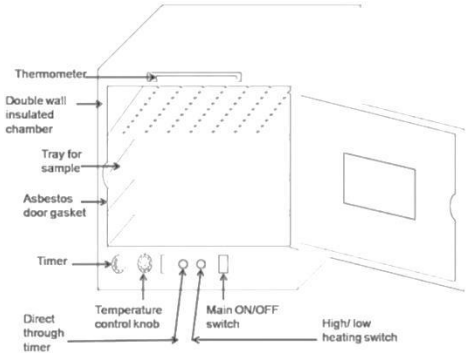
Water bath



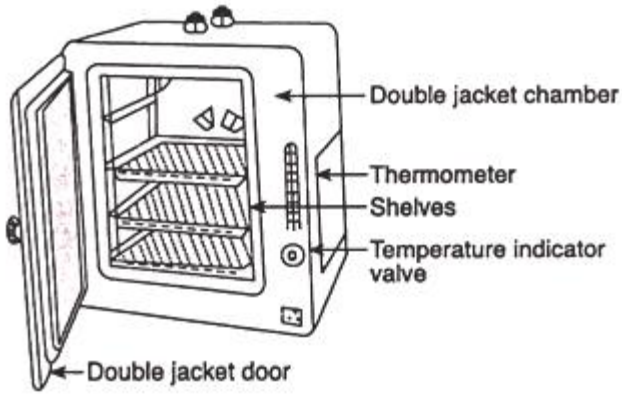
Microscope



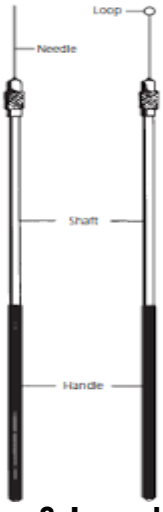
Vortex mixer



Incubator

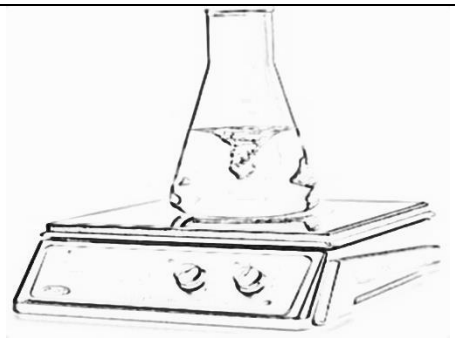


Hot air oven

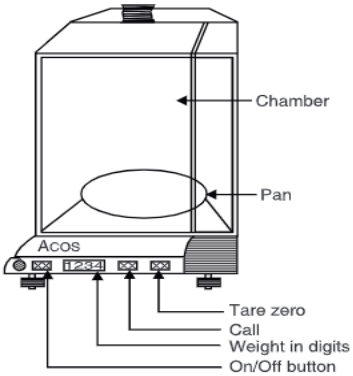


Inoculating loop & Inoculating needle

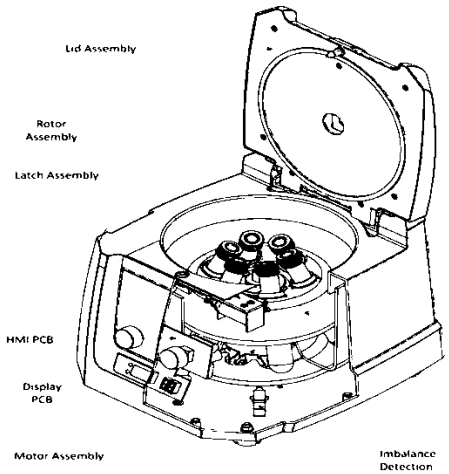
Figure 1: Equipment and instruments commonly used in mycology laboratory



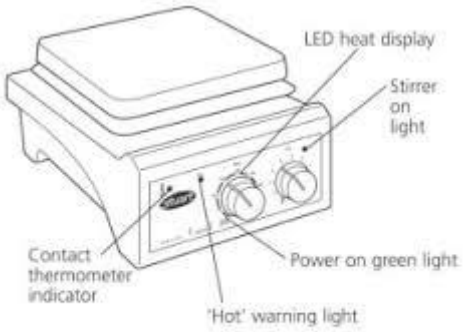
Magnetic stirrer



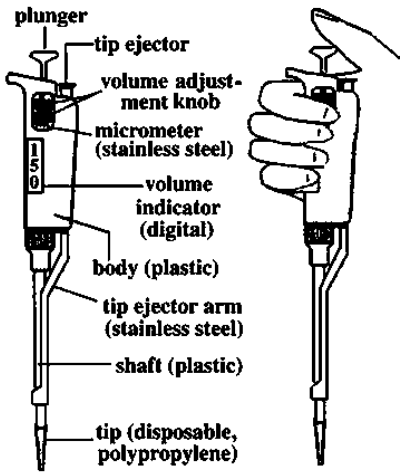
Weighing balance



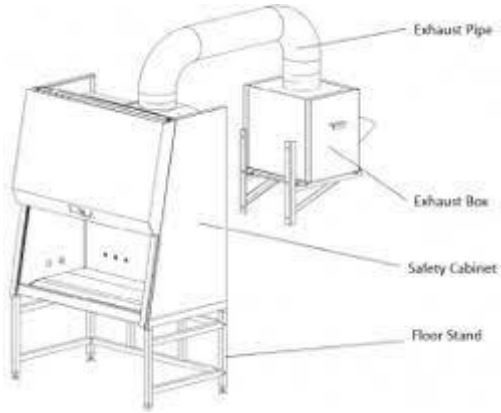
Centrifuge machine



Magnetic stirrer



Micro pipette



Biosafety cabinet

Figure 1: Equipment and instruments commonly used in mycology laboratory

Experiment-2

PRIMARY CULTURE FOR ISOLATION OF FUNGI

Fungi are Eukaryotic micro-organisms. These are either Unicellular or multicellular. Unicellular fungi are called yeast and multicellular fungi are called mold. Yeast optimal conditions on *in-vitro* growth are 37°C/24 hours. For mold optimal conditions are 25±3°C /3-5 days. Some fungi are dimorphic; grow in yeast as well as mold form. This shift in form is temperature dependent. These will be in yeast form at 37°C and in mold form at 25±3°C.

Fungi are ubiquitous (everywhere in the environment, in air soil, water and in everything exposing to environment). Fungi can be isolated from their natural environment and grow *in-vito* by providing required nutrition.

The fungi are beneficial as well as harmful. This course will help to isolate, identify fungi and their metabolites from different sources. The fungal isolation and identification is required for disease diagnosis and for use of beneficial fungi on industrial level.

For isolation of fungi, nutrition must be provided in form of medium. The commonly used medium for fungi is Sabouraud dextrose medium. Both yeast and fungi grow on this medium.

This practical will cover different primary culture techniques for isolation of fungi on Sabouraud dextrose agar (SDA) from samples. We will use soil, air, grains, swab (Buccal cavity).

Isolation Techniques

Isolation techniques (spreading, swabbing, pouring, sprinkling, flooding) of fungi are same as that of bacteria

Purification techniques

Purification technique in yeast is streaking (same as that of bacteria), but in case of molds techniques are different. These techniques will be discussed in next lecture.

Primary culture techniques used for isolation of fungi are;

1. Swabbing

It is a technique which is used for primary culture (mixed growth) of fungi. Different swab samples can be used. For instance:

- Surface swab-from any surface of object to get saprophytic fungi, swab should be moist by dipping it in any diluent like distilled water, normal saline, sterile broth. This is required when wants swabbing from surface.
- Nasal swab
- Throat swab
- Ear swab
- Skin swab

Inoculate these swab samples on Sabouraud dextrose agar by swabbing technique. After this, seal the petri plates with Para-film and incubate these plates for 3 to 5 days at 25°C.

2. Sprinkling

It is technique in which sample is sprinkled on media just like salt on food. Mostly apply on soil (in case of fungi) but can also be apply for liquid samples. For soil sampling, fix the area of 2.5 cm and then remove the litter, collect the soil sample from 10 cm depth in a zipper bag. Sprinkle 15-25mg of soil on SDA. Then incubate the plate for 3-5 days at 25°C.

3. Spreading

In soil some fungi are present in form of spores and some in form of hyphae. For isolation of fungi from soil, soil can be processed in two ways.

- Soil suspension (serial dilutions)
- Soil washing (directly spread)

a. Soil Suspension:

Soil suspension is prepared by adding 10 gram of soil in 90 mL diluent (normal saline). This is ten percent suspension. Place suspension undisturbed for ten minutes at room temperature (RT). All soil will settle down but spores of fungi will remain in upper liquid

phase. Inoculate 100µL of suspension on SDA plate using sterile spreader. Then incubate the media containing suspension for 3-5 days at 25ºC.

However, if our objective is to count the fungi per gram of soil, we can make tenfold-serial dilution from this suspension and inoculate each suspension (100µL) on SDA.

Drawback:

Only spore forming fungi can be isolated as hyphae settle down with soil. Only spores remain in upper liquid phase.

b. Soil washing

In this method we make soil suspension (10%) as mentioned above. This suspension is sieved using MUSCLIN cloth. All liquid and soil particles pass down. The hyphae with some soil residues remain on muslin cloth. Sterile saline is added to remove the soil residues from hyphae. After this process we have hyphae on muslin cloth and spores in filtrate.

- Take 100µL filtrate (containing spores) and inoculate on SDA by spreading with spreader followed by incubation for 3-5 days at 25ºC.
- Take 100µL from hyphal material with help of Pasteur pipette and spread it on SDA and incubate at 25ºC/3-5 days.

4. Settle Plate

This technique is used for isolation of fungi from air. For this prepared sterile SDA plates are directly exposed to air. Just open the plate containing SDA in air and put it in air for 15-60 mints, seal the plate then incubate at 25ºC for 3-5 days.

5. Direct Plating (Embedding)

In this method of primary culture, the fungi infecting the gains (infested fungi) are isolated. Surface contaminating fungi is decontaminated. So that only invaded fungi in maize, rice, wheat grains can be grown on SDA. For decontamination bleach (0.4%) solution is used followed by washing with sterile distilled water to removed bleach residues. Just dip in bleach

for 1-2 minutes and washed with sterile distilled water. Inoculates intact grains on SDA plates with help of sterile forceps

- Small plate (6 cm) = 6 grains
- Medium plate (9cm) =10 grains

Then place the grains on SDA and incubate for 3-5 days at 25^{o}C.

Observations

Mold growth is filamentous, while yeast colonies are just like bacterial colonies but larger in size. Some representative pictures are given below from different samples.

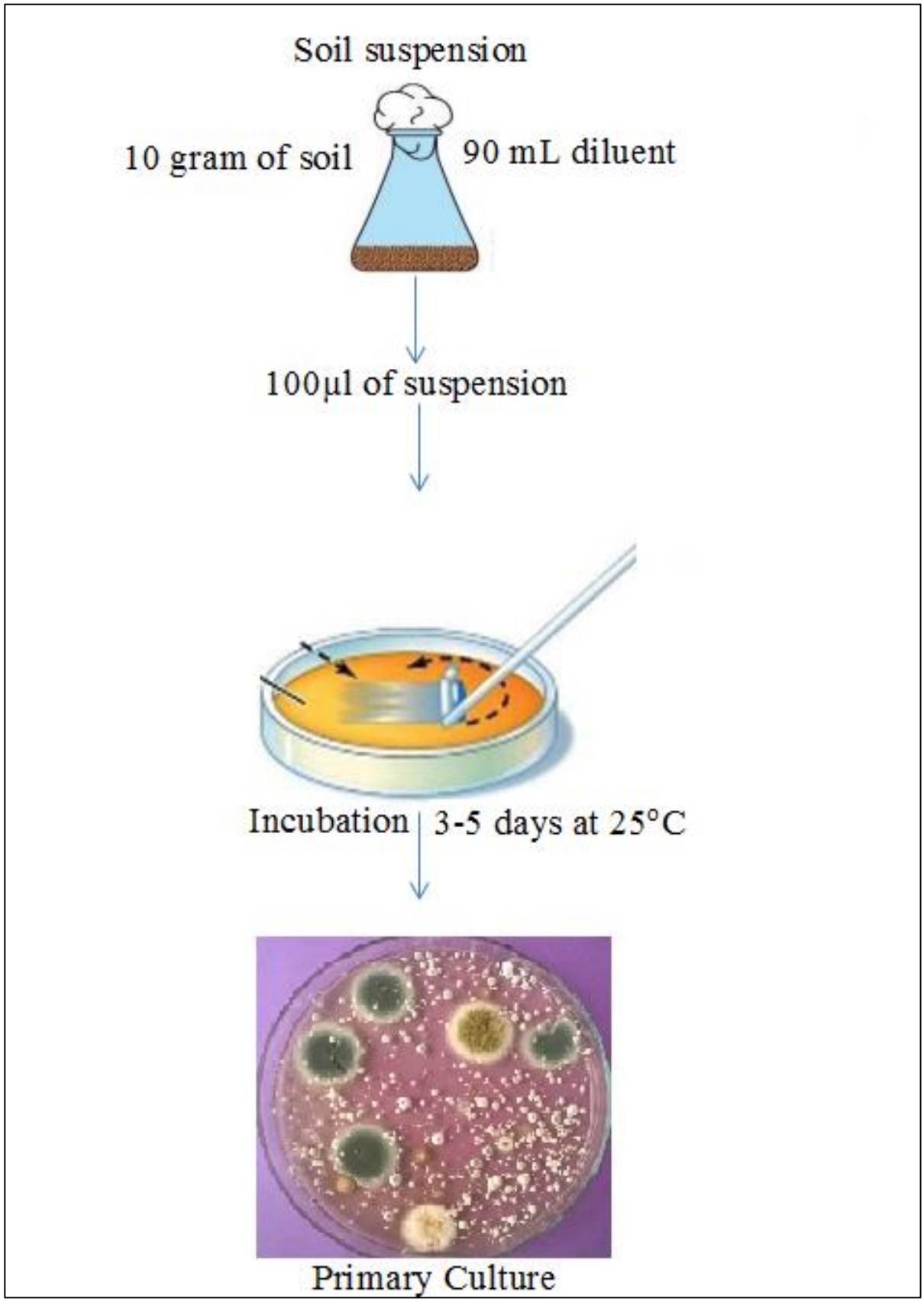


Figure 2.1: Primary culture of fungi by soil suspension

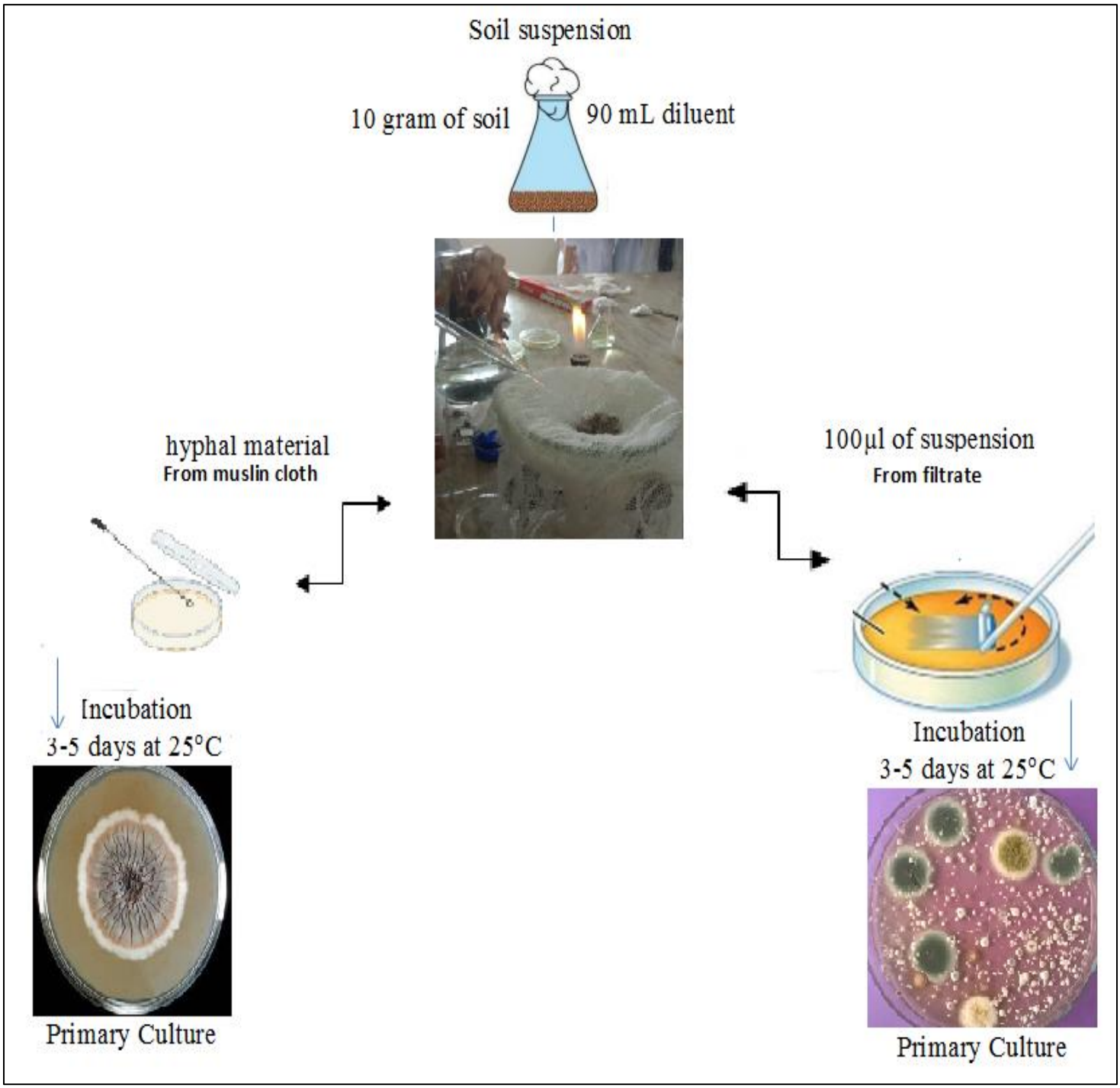


Figure 2.2: Primary culture of fungi by soil washing

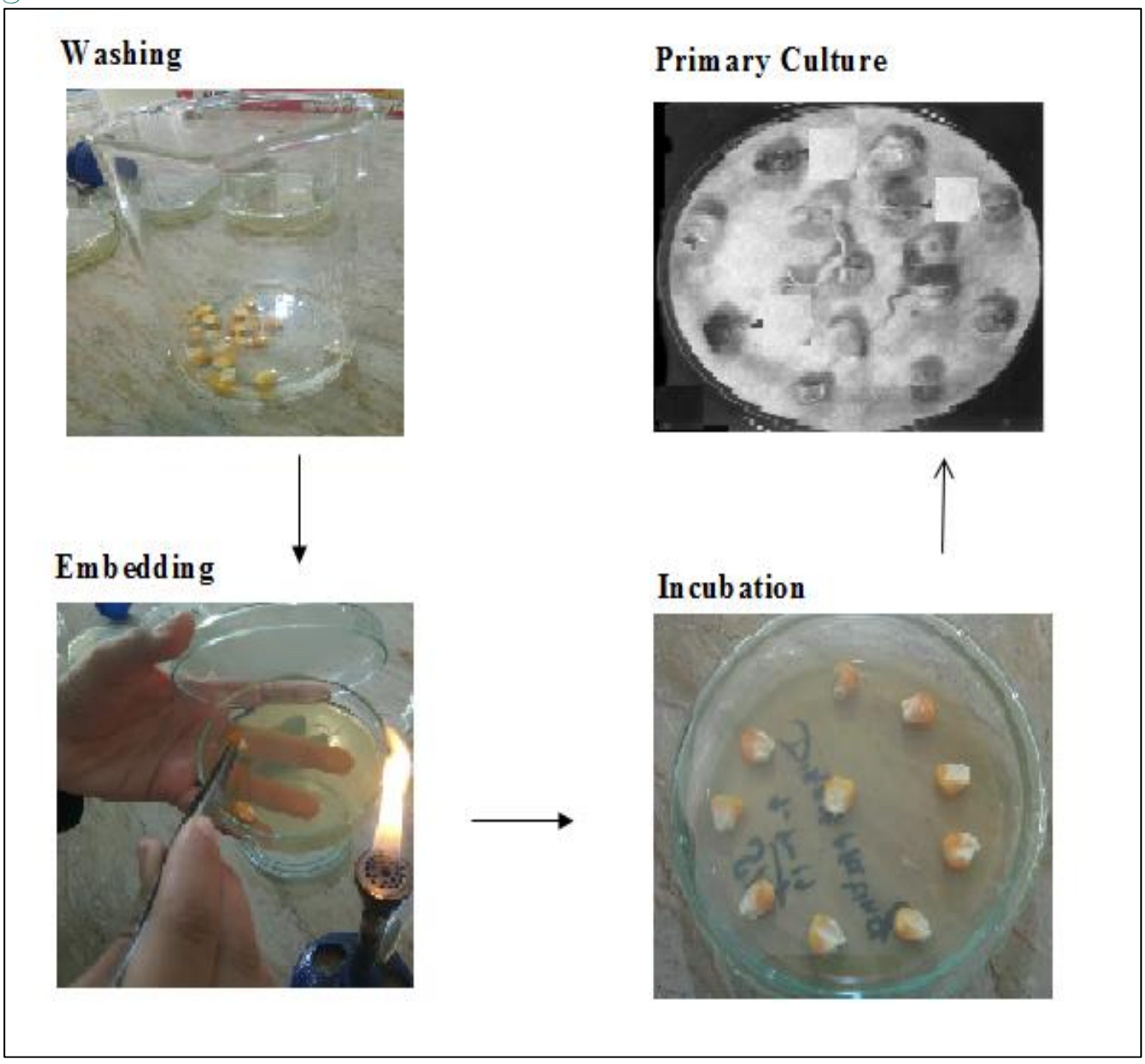


Figure 2.3: Primary culture of fungi by direct plating

Experiment- 3

SUBCULTURE TECHNIQUES FOR PURIFICATION OF FUNGI

As in primary culture, there is mixed growth. For purification sub culturing is performed. The subculture techniques used for fungi are

- Spotting
- Plug inoculation
- Streaking

Requirements

- SDA plates
- Inoculating needle for spotting
- Scalpel
- Well borer
- Inoculating loop for streaking
- Primary culture plate or old growth

A. Spotting

Due to filamentous nature of mold, spotting is performed for subculture of fungi.
Spotting is carried out in two ways

- **Single spot technique;** using an inoculating needle spores rom previous culture/primary culture are transferred to the center of agar just by touching the agar surface.
- **Multi-spot technique;** Using inoculating needle spores from previous culture/primary culture are transferred to three different places in triangle form.

Procedure

1. Sterile the inoculating needle. Take an inoculating needle and sterilize it with 70% ethanol and evaporates ethanol just by passing over flame if it is made up of aluminum. If inoculating needle is made up of platinum then place it on flame and wait until red hot and in case of silver/iron dip it in ethanol and pass through burner.

Single spot Technique

2. Touch sterile needle with a colony from a plate having primary culture/old culture.

3. Place the needle on the center of another plate containing sterile SDA. This is single spotting.
4. Incubate it at 22 to 28ºC for 3 to 5 days upright position.

Multi-spot Technique

1. In case of multiple spotting, take a colony from primary culture.
2. Now touch the needle on the other petri plate containing sterile SDA at three different points in triangular form. Every time while touching the agar surface slightly s rotates the inoculating needle so that spores may be transferred to each point.
3. Incubate it at 22 to 28ºC for 3 to 5 days upright position.

In case of multiple-spot technique the chances of contamination are less as fungi starts growth at three different points on medium and starts using uses nutrition at three points. So, there is less space for contaminants and nutritional deficiency as compared to single spot technique.

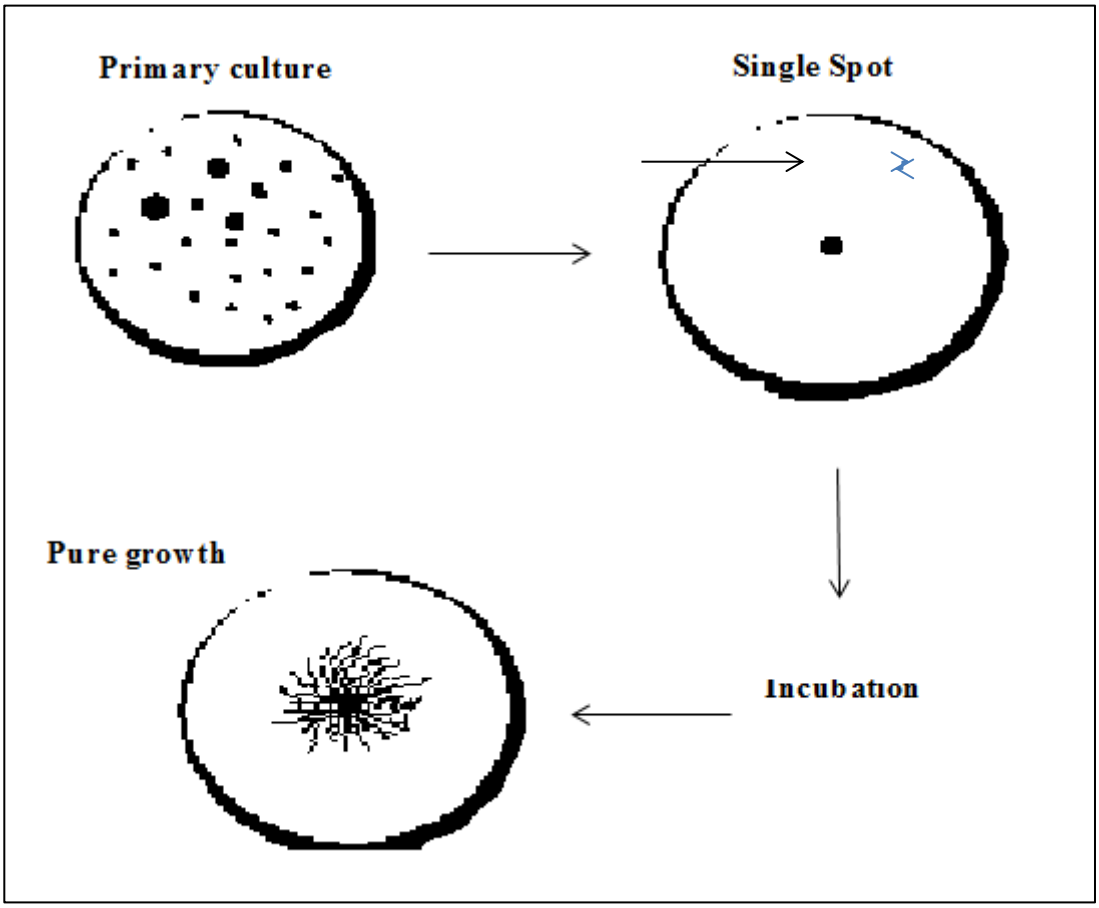


Figure 3.1: Subculture by single spot technique

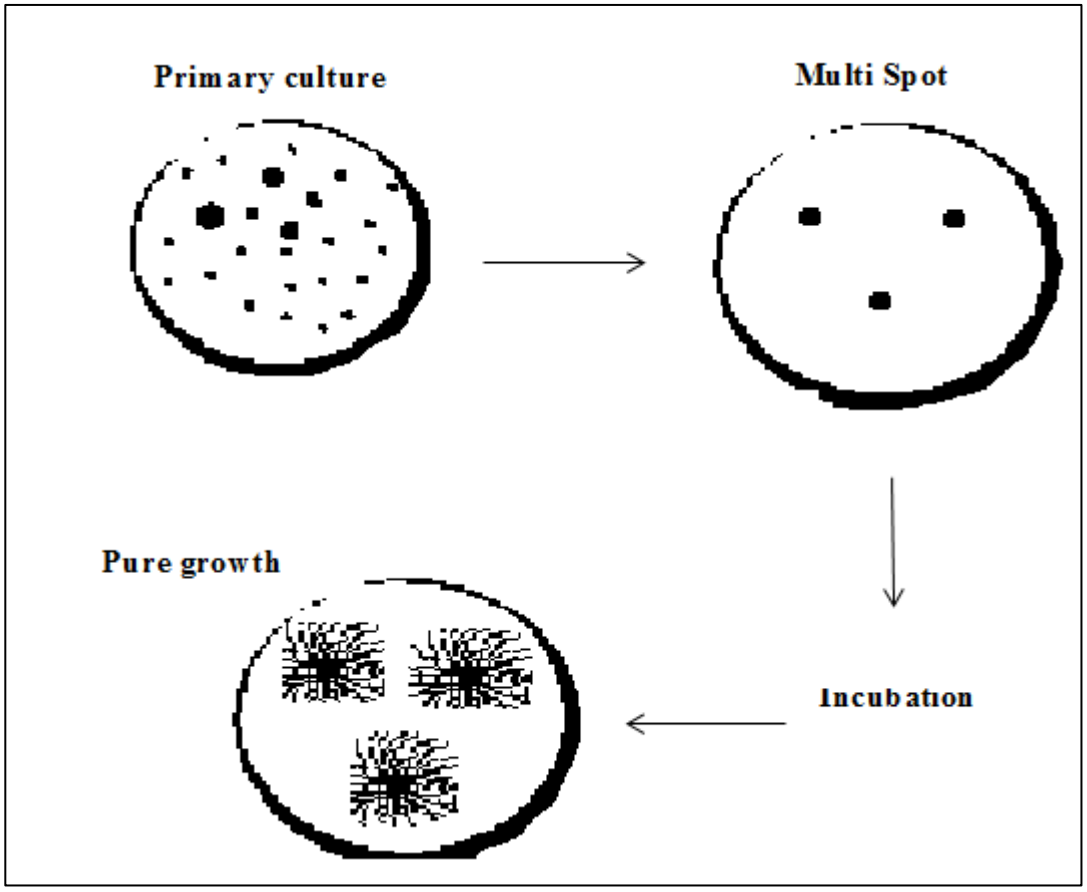


Figure 3.2: Subculture by multi spot technique

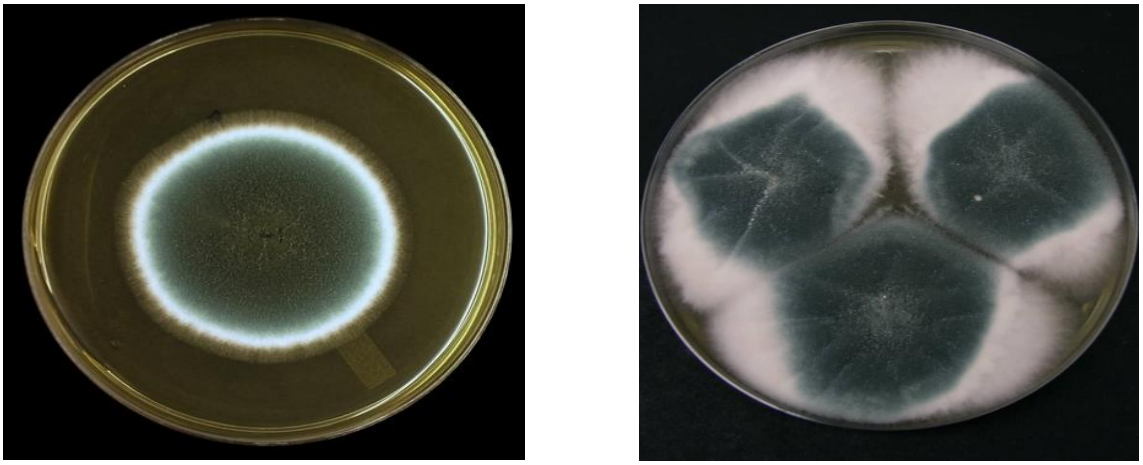


Figure 3.3: Single spot of mold of left side and multispot of mold on right side

B. Plug Inoculation

For sub-culturing of mycelia growth of mold, plug inoculation method is used. For this Purpose well borer is used

Procedure

1. Borer is sterilized by ethanol (70%) followed by evaporation of it over flame.
2. Place borer on primary culture plate or previous growth and gently pressed.
3. A plug of uniform diameter is cut.
4. Transfer this plug in the center of SDA plate with borer and stick
5. Incubate the plate at 25±3°C/3-5 days in upright position.

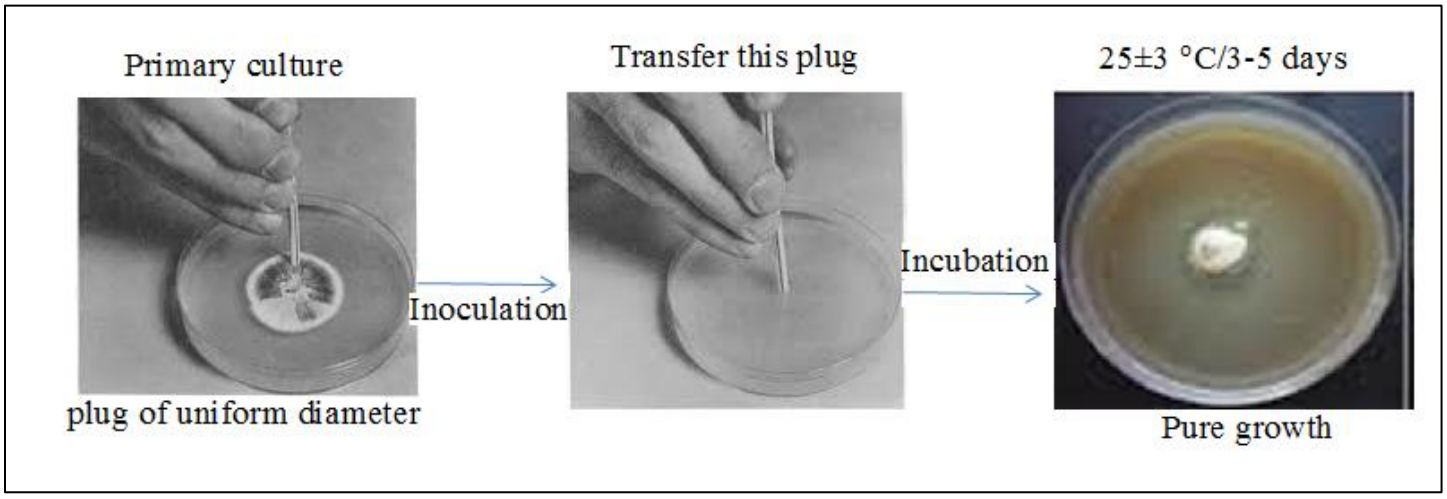


Figure 3.4: Agar plug inoculation for mold

C. Streaking

Streaking is used for sub-culturing of yeast. Inoculating loop is used. Loop is sterilized by flaming.

Streaking may be

- Continuous
- Discontinuous

We will use continuous streaking in different ways;

- One-way streaking
- Two ways streaking
- Three ways streaking
- Four ways streaking

Procedure

1. Sterilize the inoculating loop. Wait until it gets cool.
2. Touch the colony of yeast from primary culture/old culture
3. Do four ways streaking for purification.
4. For yeast incubate streaking plates at 37°C for 24-48 hours in inverted position

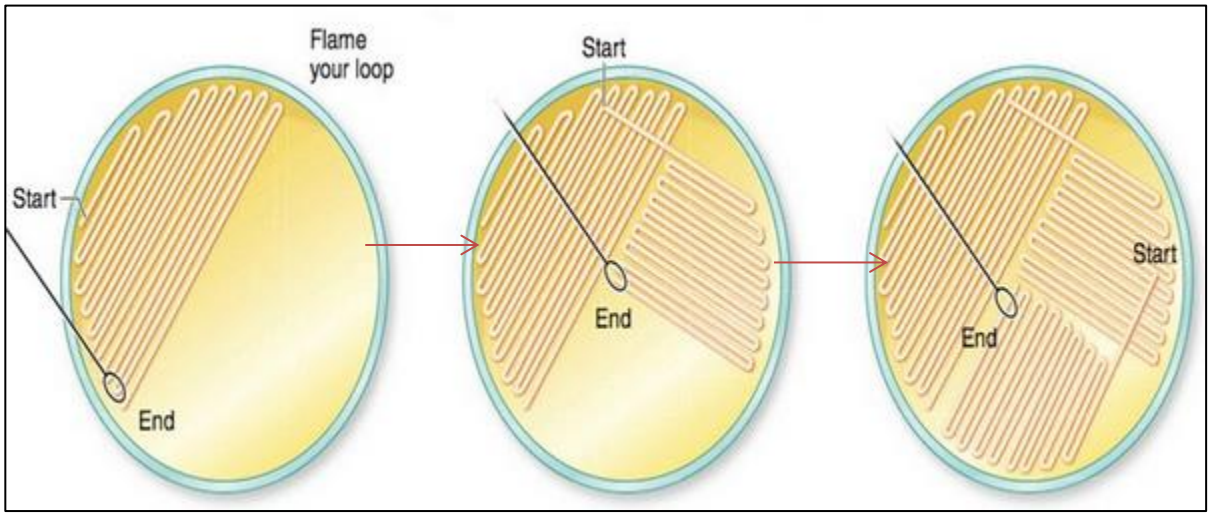


Figure 3.5: Streaking for sub-culture of yeast

Figure 3.6: Yeast growth on SDA

Experiment-4

MACROSCOPIC IDENTIFICATION: IMMATURE AND MATURE COLONY OF FUNGI (Mold)

Fungi are identified once it is purified. As discussed earlier that fungi are either yeast or mold. Both are different cells.

A. Identification of Yeasts

Yeasts are unicellular and their identification is carried out by

Colony morphology on pure culture on SDA

Microscopy; shape, size, budding pattern, budding polarity and presence of special structures

like germination tube in some *Candida* species**,** it is visualized by performing negative staining.

Biochemical tests; these are based on presence or absence of certain enzymes.

Sugar fermentation

Molecular Techniques; DNA is extracted from purified fresh growth and polymerase chain

reaction is carried out using specific primers.

B. Identification of Mold

Mold is multicellular and identification is carried out on two types of characters on immature (early stage) and mature (late stage) of growth. These characters are

a) **Macroscopic characters**

b) Microscopic characters

No Biochemical tests are performed as filamentous fungi have diverse metabolism. Only those characters are targeted for identification which is different in organisms.

In this practical we will discuss **Macroscopic Identification** of purified **Mold** species Macroscopic identification is carried out by observing macroscopic characters from obverse (front side; from lid side of plate) and reverse (Base side of the plate) side of plate.

a. **Macroscopic characters;** are those characters that can be observed with naked out or without aid of any equipment. These include;

- **Color of colony:** It is observed from obverse and reverse side of plate having growth
- **Texture:** It is observed from observe side of the growth. It is the physical appearance of colony. Just like stuff in cloth. The common texture of fungi are fluffy, cottony, granular, dusty and velvety
- **Pigmentation:** Some fungi produced extracellular pigment which diffuses into medium. This pigmentation is observed on reverse side of plate having fungal growth.

Frequently isolated fungi from different samples in previous semesters were;

1. ***Aspergillus***
2. ***Penicillium***
3. ***Rhizopus***
4. ***Mucor***
5. ***Alternaria***

So, we will use above mentioned species for macroscopic identification.

1. ***Aspergillus*;** Among aspergilla the common species were
   - 1.1. *A. niger*
   - 1.2. *A. flavus*
   - 1.3. *A. fumigatus*
   - 1.4. *A. terreus*
   - 1.5. *A. ochraceous*
   - 1.6. *A. parasiticus*

Characters at immature stage of growth:

Obverse side; all *Aspergillus* species are white (color) and cottony (texture just like cotton) at immature stage from obverse side. This is because the hyphae of aspergilli are hyaline.

Reverse side; these are colorless on reverse side of growth. All species of genus *Aspergillus* are not pigment producer.

Characters at mature stage of growth:

Spores are formed on maturation of aspergilla. The spores of genus *Aspergillus* species are pigmented. Every specie produces different colour spores on maturation. Now we will discuss characters of different species on maturation. This is because all species have same characters on immature stage of growth.

1.1. Aspergillus niger

It is industrial organism used for organic acid and enzyme production. It is rarely pathogenic

Immature stage

Obverse side: white and cottony

Reverse side: plain, no pigment

Mature stage

Obverse side: Periphery white (due to mycelia growth if present) at center it is black (due to spores). Texture granular (just like tapal-dany dar tea)

Reverse side: plain, no pigment

1.2. Aspergillus fumigatus

Aspergillus fumigatus is pathogen specie, having small sized spore, and reaches to lower respiratory tract (Lungs) and cause respiratory system disease called aspergillosis. Aspergillosis in chicks called brooder's pneumonia.

Immature stage

Obverse side: white and cottony

Reverse side: plain, no pigment

Mature stage

Obverse side: Bluish green due to spores and texture becomes dusty on maturation.

Once it is old it becomes slate grey in color.

Reverse side: plain, no pigment

1.3. Aspergillus flavus

Flavus (Greek word) meaning yellowish green. It gives yellowish green color on SDA that why its name is *A. flavus*. It is well known for mycotoxin production. Its mycotoxins are called aflatoxins

Immature stage

Obverse side: white and cottony

Reverse side: plain, no pigment

Mature stage

Obverse side: Yellowish green color and mostly cottony to slightly granular texture

Reverse side: plain, no pigment sometimes slightly pale due to dense growth

1.4. Aspergillus terrus

Immature stage

Obverse side: white and cottony

Reverse side: plain, no pigment

Mature stage

Obverse side: Cinnamon brown colour and dusty texture

Reverse side: plain, no pigment

1.5. Aspergillus parasiticus

It is also well known for mycotoxin production. Its mycotoxins are called aflatoxins

Immature stage

Obverse side: white and cottony

Reverse side: plain, no pigment

Mature stage

Obverse side: Dark brownish green (Just like mehndi powder) colour and mostly cottony to slightly dusty texture

Reverse side: plain, no pigment sometimes slightly pale due to dense growth

1.6. Aspergillus ochraceous

It is also well known for mycotoxin production. Its mycotoxins are called Ochratoxins

Immature stage

Obverse side: white and cottony

Reverse side: plain, no pigment

Mature stage

Obverse side: Yellowish slightly brown (Just like camel colour) colour and mostly cottony to slightly powdery texture

Reverse side: plain, no pigment

2. Rhizopus

It is fast growing fungi. Growth appears in 24 hours. It is from zygomycetes

Immature stage

Obverse side: white and fluffy (just like candy ball)

Reverse side: plain, no pigment

Mature stage

Obverse side: Brown on maturation

Reverse side: no pigment. Colorless reverse side

3. Mucor

It is fast growing fungi. Growth appears in 24 hours. Macroscopically it is similar to *Rhizopus.* It is from zygomycetes

Immature stage

Obverse side: white and fluffy (just like candy ball)

Reverse side: plain, no pigment

Mature stage

Obverse side: Smoke like colour, greyish-brown on maturation

Reverse side: no pigment. Colorless reverse side

4. Penicillum

It is antibiotic producing fungi.

Immature stage

Obverse side: Bluish-green or greyish-green and mostly valvety (just like valvet cloth) or powdery

Reverse side: Diffusible pigment (Yellow/Red).

Mature stage

Obverse side: Bluish green

Reverse side: Diffusible pigment. Colour of pigment may vary in different species. It may be yellow or red.

Its obverse colour is similar to *Aspergillus fumigatus* but texture and reverse side makes *Penicillium* different from *A. fumigatus.*

5. Alternaria

It belongs to the group of fungi called Phaeoid. It is the group which has dematiceous (Pigmented) hyphae.

Immature stage

Obverse side: Olive green or grey green colour, velvety texture

Reverse side: Black-brown

Mature stage

Obverse side: Olive green or grey green colour, velvety texture

Reverse side: Black-brown

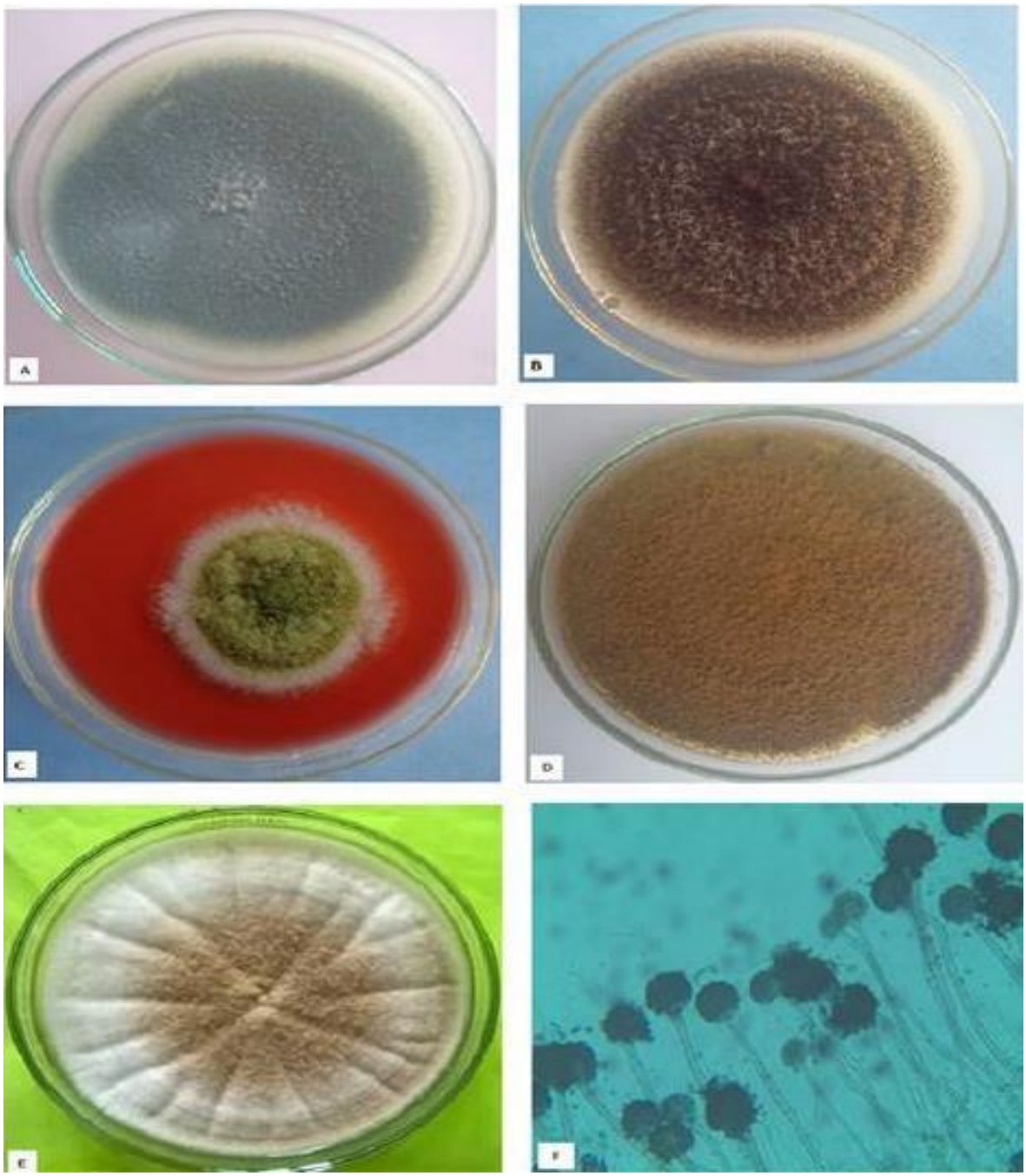


Figure 4.1: Macroscopic and microscopic identification of Aspergillus

species (A): Aspergillus fumigatus: (B) Aspergillus niger: (C) Aspergillus flavus: (D) Aspergillus parasiticusn: (E) Aspergillus terreus: (F) Microscopic view of Aspergillus

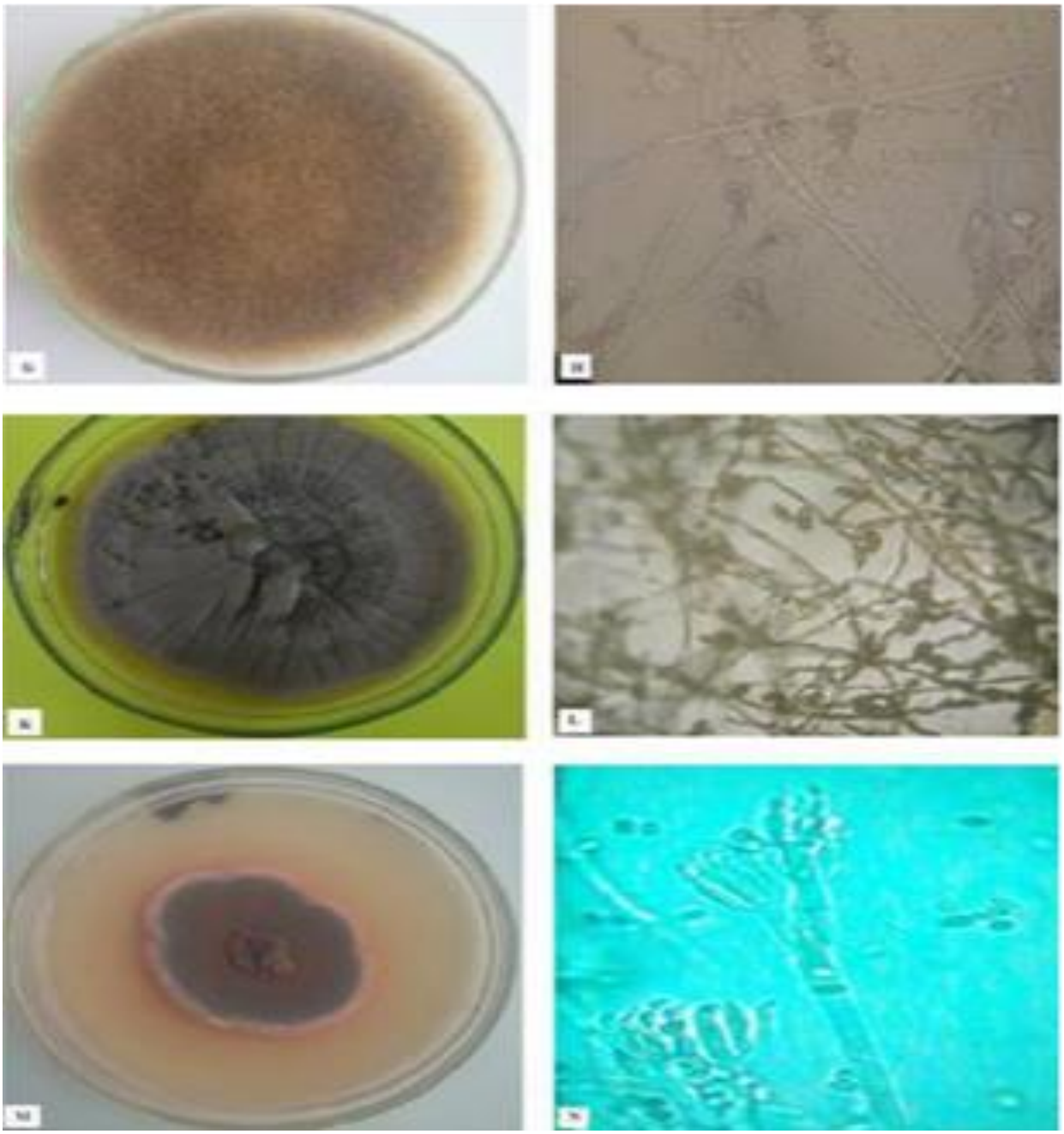
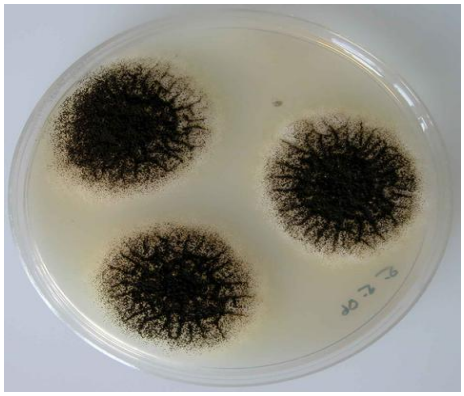


Figure 4.2: ***Macroscopic and microscopic identification of fungal species;***
(G) *Mucor spp.*: (H) Microscopic view of *Mucor spp.*: (I) *Fusarium spp.*: (J) Microscopic view of *Fusarium spp.* (K) *Phaeoid fungi*: (L) Microscopic view of *Phaeoid fungi*: (M) *Penicillium spp.* (N) Microscopic view of *Penicillium spp.*

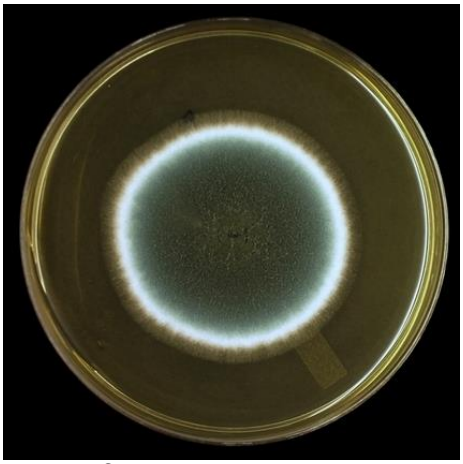
Aspergilus parasiticus

Aspergillus ocharaceous

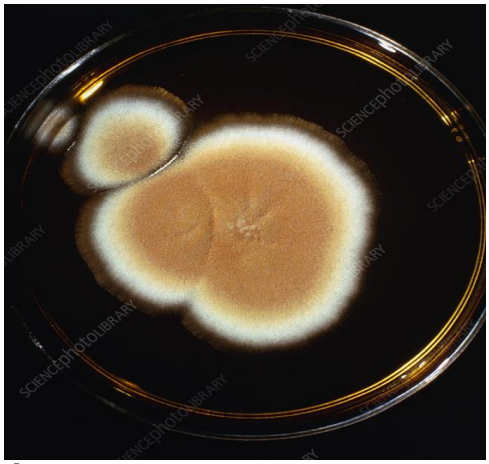
Aspergillus flavus



Aspergillus niger



Aspergillus fumigatus



Aspergillus terrus

Figure 4.3: Macroscopic characters of common fungi

Experiment-5

MICROSCOPIC CHARACTERS CELLOPHANE TAPE AND TEASE MOUNT OF FUNGI

As discussed in previous lecture that mold is identified by macroscopic (observed by unaided eye) and microscopic (observed under microscope) Characters.

In case of filamentous fungi, microscopic characters are

1. Hyphae
2. Spores
3. Special structures like foot cell, vesicle, metulae, sporangium

1. Hyphae

Hyphae are microscopic structure of filamentous fungi and can be classified by several ways, based on septations (cross-walls), pigmentation and role in fungus life cycle. Hyphae help us in identification of filamentous fungi.

Rhizopus and *Mucor*

These have Coenocytic and Hyaline hyphae

Aspergillus and *Penicillium*

Coencytic and hyaline hyphae

Phaeoid Fungi

It is a group of fungi have dematiaceous (pigmented) hyphae

2. Spores

Spores are microscopic structures of fungi produced in asexual and sexual mode of reproduction of fungi. However, asexual spores are helpful in identification. For identification two characters associated with spores are observed. These are shape and arrangement of spores.

Aspergillus* and *Penicillium

Spores of these two genera are circular in shape and are arranged in chains outside the **vesicle** on **phialides** and **metulae**.

Mucor* and *Rhizopus

Have oval shape spores enclosed in vesicle (**Sporangium**).

Alternaria (Phaeoid fungi)

It has septate spores attach with each other through a tube

Fusarium

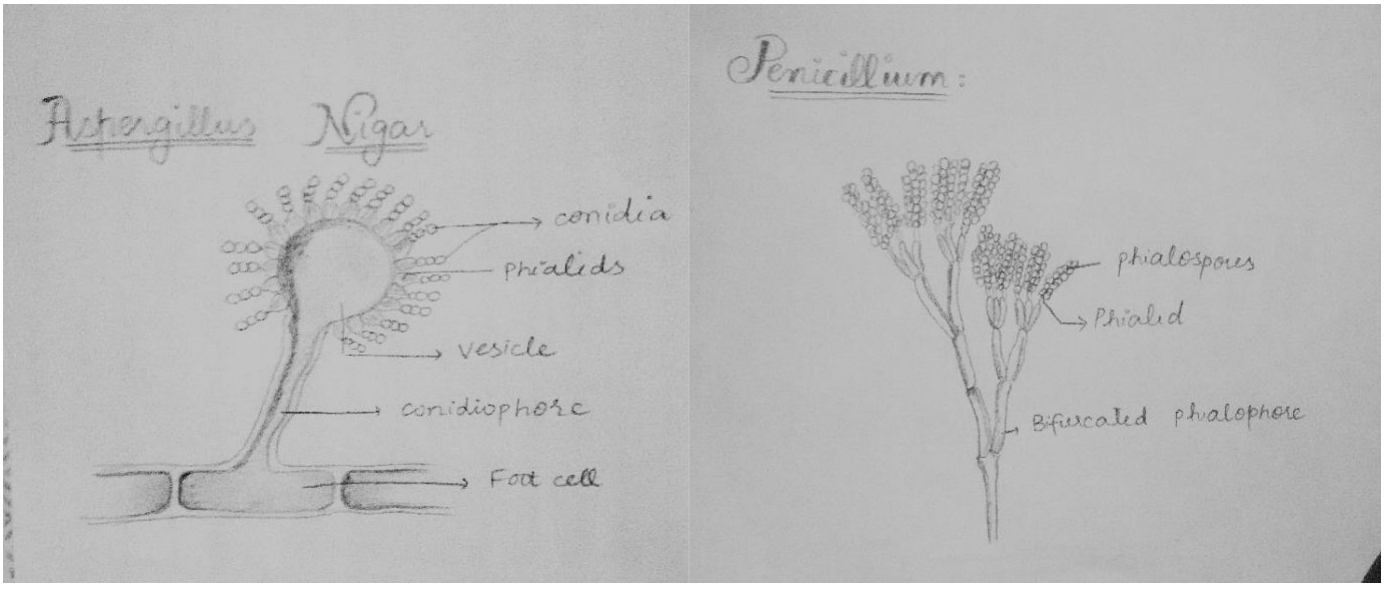
It has fusiform/crescent/sickle shape spores

3. Special Structures

Special structures are not a general character of all fungi. Some fungi have these characters others don't possess.

Vesicle: An oval or round shape vesicle is present at the terminal of reproductive hyphae. It has phialids on its surface. Vesicle is present in all species of genus *Aspergillus*

Foot Cell: An enlarged hyphal cell produces conidiophore. Depending upon position of conidiophore, foot cell are either 'I', 'L' or 'T' shaped.



METHODS FOR OBSERVING MICROSCOPIC CHARACTERS

Microscopic characters can be studied by making slides using following methods;

1. **Tease mount**
2. **Cellophane tape method/Flag method**

   In tease mount and cellophane tape method, disrupted structures are observed. These are quick methods to observe microscopic characters.

3. **Slide Culture**

   This is time taking method. All microscopic characters can be studies in detail and intact structures are seen in slide culture.

In this lecture, we will discuss, first two methods

1. Tease Mount Method

It is the wet mount of fungi. In this a fungal stain is used called Lactophenol cotton blue.

Requirements

- Fungal growth
- Inoculating needle
- Microscopic glass slide
- Cover slips
- Ethyl alcohol (70%)
- Burner

Procedure

- Take a microscopic glass slide and cover slip and clean it by washing with 70% ethyl alcohol. Allow alcohol to evaporate completely
- Place a drop of lactophenol cotton blue on microscopic glass slide
- Pick a fungal growth with inoculating needle
- Mix it with lactophenol cotton blue on glass slide with two sterile needles

- Place cover slip on it
- Observe under microscope at 100x and 400x magnification

Observations Observe at 400x

We can observe the type of hyphae and shapes of spores. Intact structures are rarely observed by it.

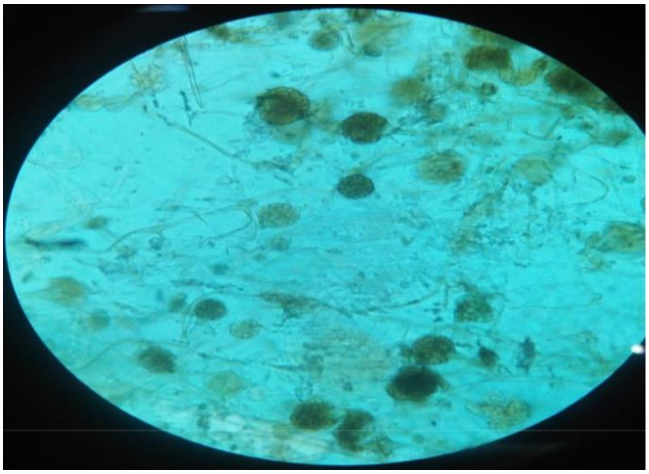


Figure 5.1: Microscopic view of tease mount of fungi

2. Cellophane Tape Method

This method allows studying of microscopic characters. It is modified form of flag method

Requirements

- Fungal growth
- Cellophane tape
- Microscopic glass slide
- Ethyl alcohol (70%)
- Burner
- Scissor

Procedure

- Take a microscopic glass slide and cover slip and clean it by washing with 70% ethyl alcohol. Allow alcohol to evaporate completely
- Cut a small piece of cellophane tape (one sticky side and transparent) approximately equal to length of glass slide
- Hold the cellophane tape with thumb and first finger in such a way that sticky side is outside and non-sticky side towards palm of hand.
- Now join thumb and finger so that cellophane tape appeared as loop.
- Touch the sticky side on fungal growth, fungal growth will stick to tape
- Place the tape on glass slide so that sticky side sticks to slide. Fungal growth is sand-witched between cellophane tape and glass slide
- Observe under microscope at 100x and 400x magnification

When cellophane tape is wrapped by match box stick, it appears just like flag. Then it is touched with fungal growth and attached with glass slide followed by removal of match box stick by cutting. This is cello tape flag method.

Observations

We can observe the type of hyphae and shapes of spores. Intact structures are rarely observed by it.

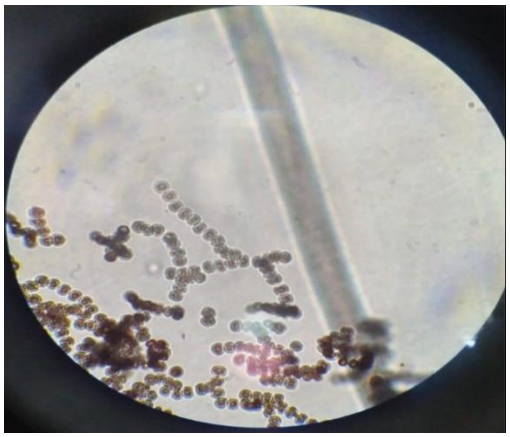


Figure 5.2: Microscopic view of cellophane tape of fungi

Lacto-phenol Cotton Blue: Is commonly used stain for fungi. It is composed of

Cotton blue: A coloring agent imparts blue color to fungal structures

Lactic acid: An organic acid acts as preservative

Phenol Crystals: Inhibit fungal growth

This stain can give color to fungi, kill fungi and preserve the fungal structures for longer time due to lactic acid.

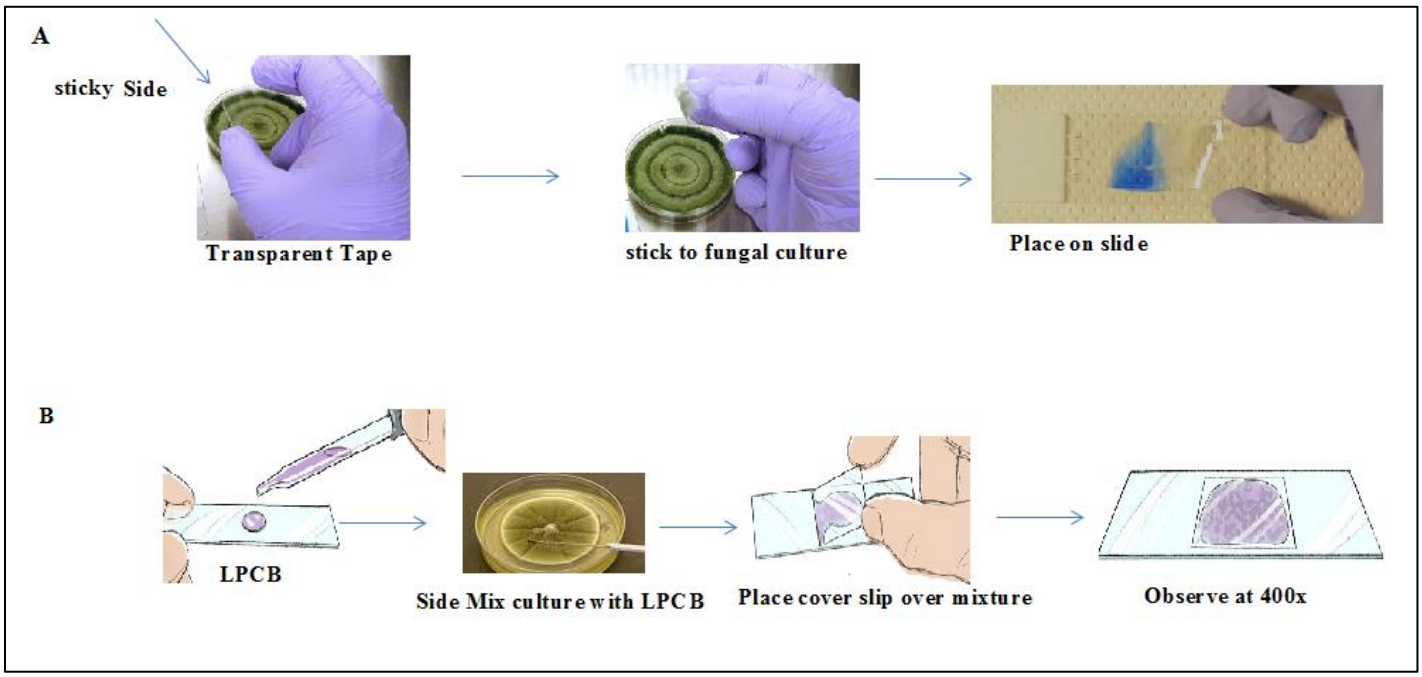


Figure 5.3: Microscopic Observations
A: Cellophane tape, **B:** Tease mount method

Experiment-6

MICROSCOPIC CHARACTERS
SLIDE CULTURE TECHNIQUE

Slide culture means cultivation of fungi on microscopic glass slide. The cultivation is carried out to observe the microscopic characters of fungi.

Complete and intact structures can be observed under microscope. It is used for filamentous fungi; mold.

Slide culture technique can be performed in two-ways;

A. Agar block method

B. Agar drop method

A. Agar Block Method

In this method fungus is grown on a block of agar on microscopic glass slide.

Requirements

- Sabouraud dextrose agar plate
- Glass dropper
- Microscopic glass slide
- Cover slips
- Inoculating needle
- Purified fungus culture
- Sterile petri-plates
- Sterile Cotton /filter paper
- Sterile water
- Incubator

Procedure

1. Take a glass slide and a cover slip, sterile both of these by dipping in ethanol (70%). Allow ethanol to evaporate till slide and cover slips are dried.

2. Cut a block of SDA from media in plate about 1cm^2 size with help of scalpel and place it in the center of the glass slide

3. Inoculate a scanty of fungal spores on in the center of block with help of inoculating needle.
4. Cover the inoculating drop with cover slip
5. Place the prepared slide in sterile petri-plate
6. Also placed a cotton plug/filter paper soaked in sterile water inside petri-plate
7. Incubate the petri-plate at 25°C in incubator
8. Observe the slide on daily basis at 100 and 400x magnification till complete structures develop

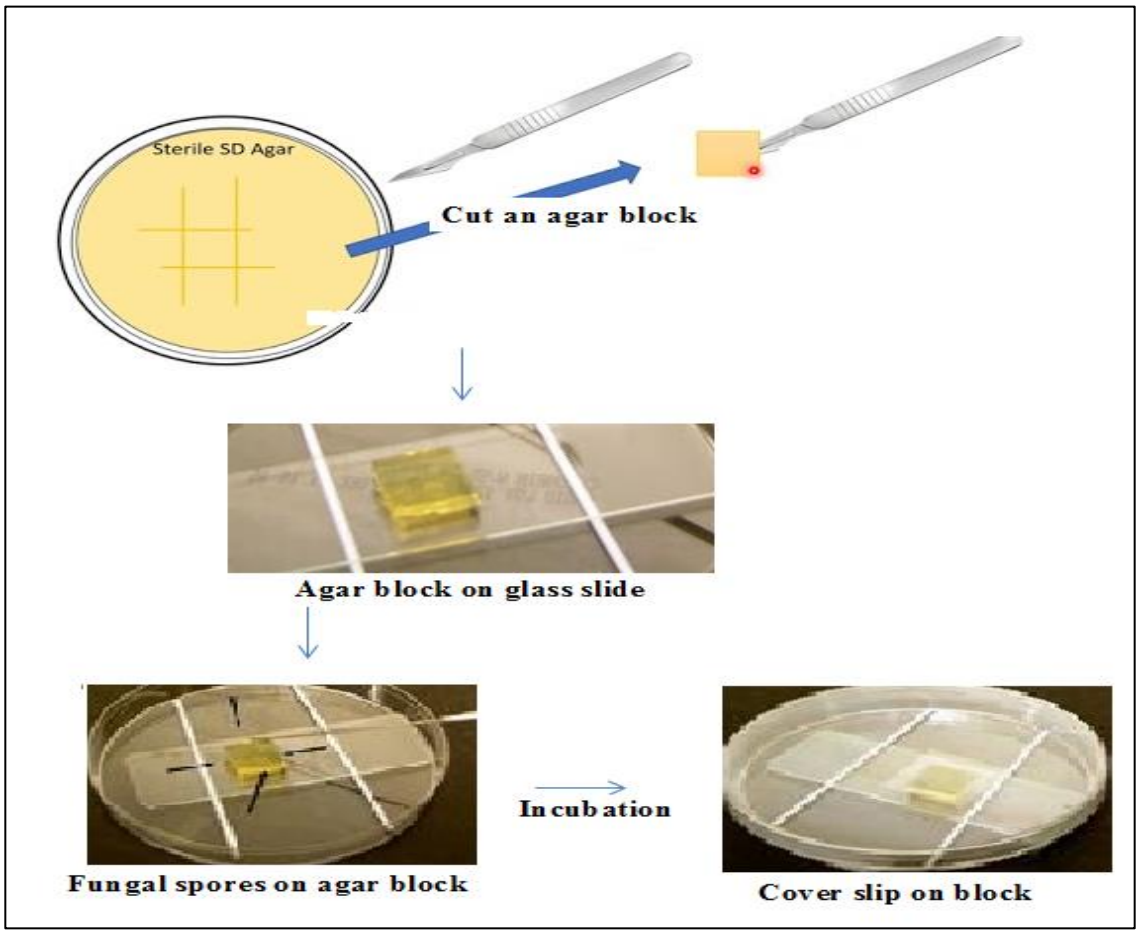


Figure 6.1: Agar block Method for slide culture

B. Agar Drop Method

In this method fungus is grown on a drop of agar on microscopic glass slide.

Requirements

- Sabouraud dextrose agar (45°C-50°C)

- Glass dropper
- Microscopic glass slide
- Cover slips
- Inoculating needle
- Purified fungus culture
- Sterile petri-plates
- Sterile Cotton
- Sterile water
- Incubator

Procedure

1. Take a glass slide and a cover slip, sterile both of these by dipping in ethanol (70%). Allow ethanol to evaporate till slide and cover slips are dried.
2. Place a drop of molten SDA in the center of the slide.
3. Inoculate a scanty of fungal spores on molten agar drop with help of inoculating needle.
4. Cover the inoculating drop with cover slip
5. Place the prepared slide in sterile petri-plate
6. Also placed a cotton plug/filter paper soaked in sterile water inside petri-plate
7. Incubate the petri-plate at 25°C in incubator
8. Observe the slide on daily basis at 100 and 400x magnification till complete structures develop

NOTE: Agar drop method is considered better as compared to agar block method.

- There are less chances of contamination in agar drop method.
- Because the block is thick, this thickness may interfere the focusing of structures under microscope.

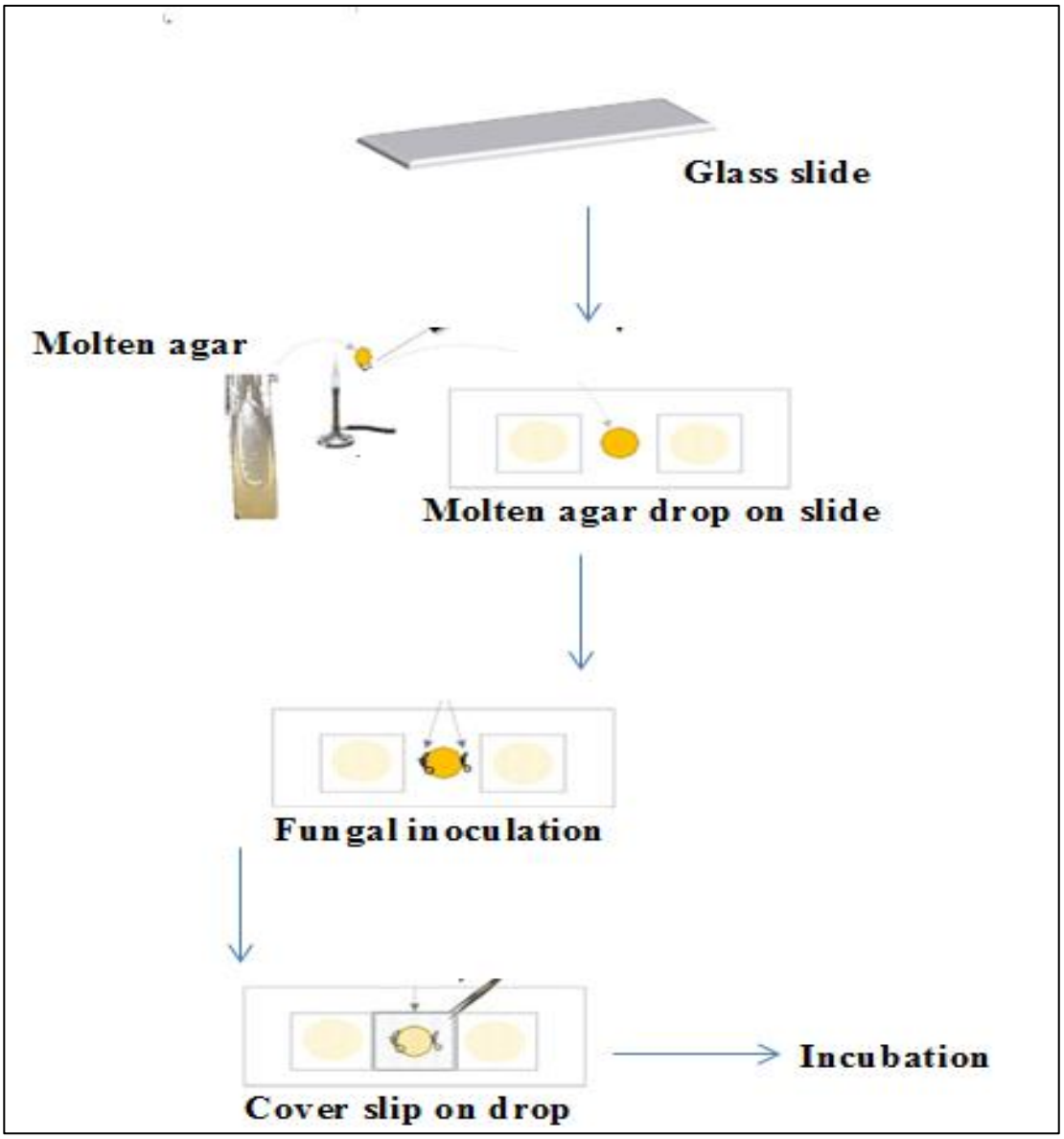


Figure 6.2: Agar drop slide culture

Experiment-07

FUNGAL STAINING AND PERMANENT MOUNT OF FUNGI

One the slide culture is fully matured; this slide can be preserved for further use. Slide is stained to find the colored structures of fungi.

1. **Staining of Slide Culture**

Commonly used stain for fungi is **Lacto-phenol Cotton Blue (LPCB);** Acidic stain for fungi having affinity with positively charged chitin in its cell wall. It is composed of

a. **Cotton blue:** A coloring agent imparts blue color to fungal structures
b. **Lactic acid:** An organic acid acts as preservative
c. **Phenol Crystals:** Inhibit fungal growth

This stain can give color to fungi, kill fungi and preserve the fungal structures for longer time due to lactic acid.

Preparation of Stain

Cotton Blue (Aniline Blue) 0.05 g

Phenol Crystals ($C_6H_5O_4$) 20 g

Glycerol 40 mL

Lactic acid ($CH_3CHOH\ COOH$) 20 mL

Distilled water 20 mL

This stain is prepared over two days.

1. On the first day, dissolve the Cotton Blue in the distilled water. Leave overnight to eliminate insoluble dye.
2. On the second day, wearing gloves add the phenol crystals to the lactic acid in a glass beaker. Place on magnetic stirrer until the phenol is dissolved.
3. Add the glycerol.

4. Filter the Cotton Blue and distilled water solution into the phenol/glycerol/lactic acid solution. Mix and store at room temperature.

Procedure for staining

1. Observe the slide culture on daily basis till all fungal structures developed
2. The drop the stain solution (LPCB) on one side of slide at the edge of cover slip
3. Similarly add stain (LPCB) on all edges of cover slip, till stain penetrates under area covered by cover slip
4. Place the slide at room temperature for overnight, so that stained slide gets dry

Permanent Mount

For permanent mount, mounting solutions are used which fix the slides. One of the mounting solutions is DPX (Dibutylphthalate Polystyrene Xylene). It is in solution form and commercially available in brown glass bottles. When it does come in contact with light, it turns to solid form and sticks to any material. It preserves slides and dry quickly. Mounting fixes, the cover slip. So, chances of cover slip removal are stopped. If cover slip is removed fungal structures will be distorted.

Procedure

1. Once the stain slide culture is dried
2. Take a wooden stick (tooth-pick) and dip into DPX
3. Apply DPX on edges of cover slip.
4. Allow it to dry. For this place the slide at RT for overnight. This will fix the cover-slip to the glass slide.

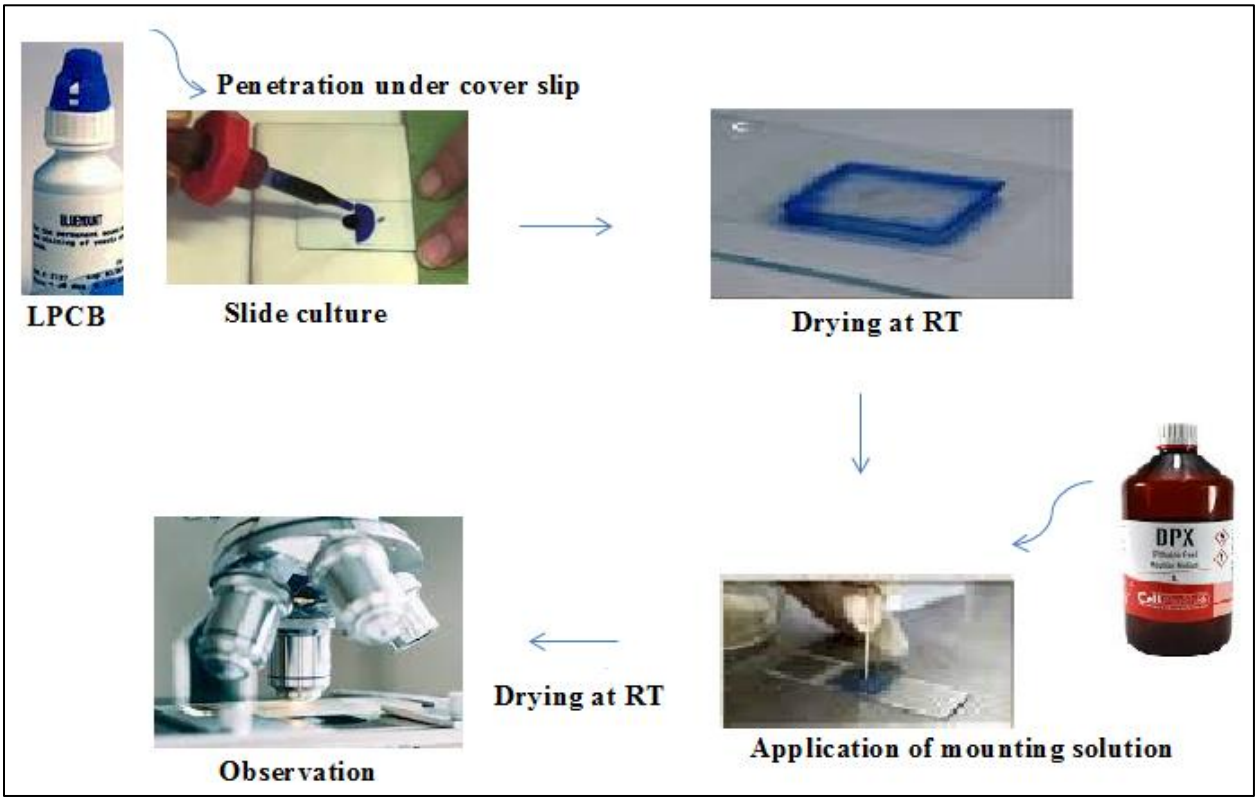


Figure 7.1: Staining and mounting of slide culture of fungi

Experiment-8

IDENTIFICATION OF YEAST: NEGATIVE STAINING AND GERM TUBE TEST

Yeasts are unicellular fungi. Its isolation, purification and identification techniques are similar to bacterial cell (unicellular).

Yeast is identified by;

1. Colony morphology
2. Microscopy
3. Biochemical profile (Particularly sugar fermentation tests)
4. Molecular Techniques

Microscopy helps us to observe;

Shape (spherical/oval)

Size (Larger than bacteria)

Presence of Budding (Mostly yeasts reproduce by budding)

Pattern of budding (Polarity and presence or absence of neck with bud)

Presence of special structures (Germination tube in *Candida albicans)*

Presence of Capsules in *Cryptococcus neoformans*

Techniques for Microscopy

1. Negative staining (For microscopic morphology)
2. Wet mount (For germ tube test)

1. NEGATIVE STAINING

1. Place a very small drop (more than a loop full, less than a free-falling drop from the dropper) of **Nigrosin** near one end of a well-cleaned and flamed slide.

2. Remove a small amount of the culture from the pure growth with an inoculating loop and disperse it in the drop of stain without spreading the drop

3. Use another clean slide (called as spreader slide) to spread the drop of stain containing the organism using the following technique

4. Place one end of the clean slide on the center of the slide with the stain mixed culture. Tilt the clean slide toward the drop forming an angle (45^0) and draw that slide toward the drop until it touches the drop and causes it to spread along the edge of the spreader slide. Maintaining an angle between the slides, push the spreader slide toward the clean end of the slide being stained dragging the drop behind the spreader slide and producing a broad, even, thin smear

5. Allow the smear to dry without heating

6. Observe slide at 1000x magnification using oil immersion lens

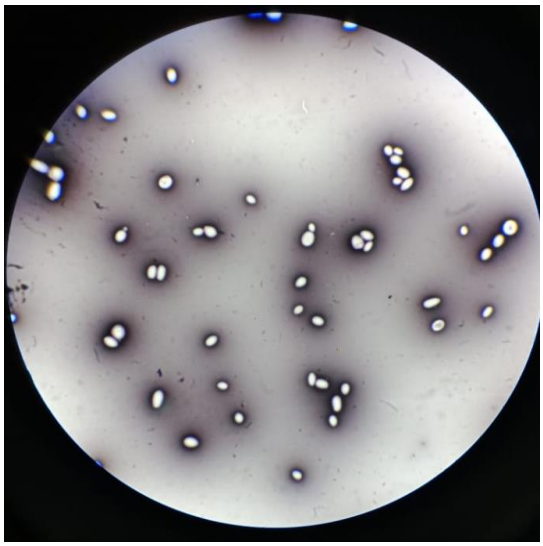


Figure 8.1: Growth of Candida albicans on SDA and microscopic view after negative staining at 100x objective lens.

Microscopy shows that cells are oval shape and larger as compared to bacterial cell, budding without neck is present. One parent cell has one bud at a time (unipolar).

2. GERM TUBE TEST

Germ Tube Test is a screening test which is used to differentiate *Candida albicans* from other yeast. Germ tube (GT) formation was first reported by Reynolds and Braude in 1956. When Candida is grown in human or sheep serum at 37°C for 3 hours, these form a germ tubes, which can be detected with a wet KOH films as filamentous outgrowth extending from yeast cells. It is positive for *Candida albicans* and *Candida dubliniensis*. Approximately 95 – 97% of *Candida albicans* isolated develop germ tubes when incubated in a proteinaceous media.

Principle

Formation of germ tube is associated with increased synthesis of protein and ribonucleic acid. Germ Tube solutions contains tryptic soy broth and fetal bovine serum, essential nutrients for protein synthesis. It is lyophilized for stability. Germ tube is one of the virulence factors of *Candida albicans*. This is a rapid test for the presumptive identification of *C. albicans*.

Procedure

1. Put 0.5 mL of sheep or human serum into a small tube. *Note: Fetal bovine serum can also be used instead of human serum.*
2. Using a Pasteur pipette, touch a colony of yeast and gently emulsify it in the serum. *Note: Too large of an inoculum will inhibit germ tube formation.*
3. Incubated the tube at 37°C for 2 to 4 hours.
4. Transfer a drop of the serum to a slide for examination.
5. Coverslip and examine microscopically under low (10X) and high power (40X) objectives.

Results and Interpretation

Positive Test: A short hyphal (filamentous) extension arising laterally from a yeast cell, with no constriction at the point of origin. Germ tube is half the width and 3 to 4 times the length of the yeast cell and there is no presence of nucleus. *Candida albicans* and *Candida dubliniensis are positive for this test.*

Negative Test: No hyphal (filamentous) extension arising from a yeast cell or a short hyphal extension constricted at the point of origin. *C. tropicalis*, *C. glabrata* and other yeasts are negative for this test.

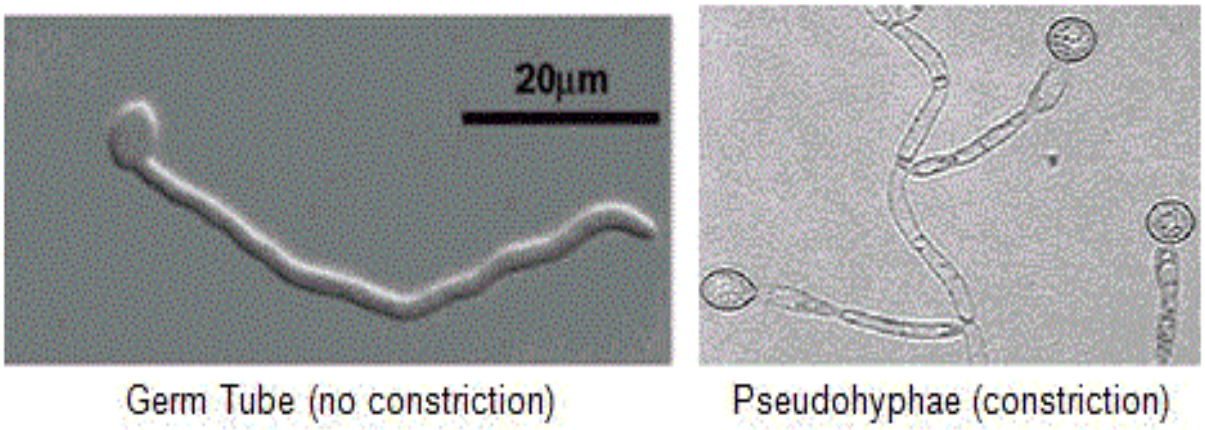


Figure 8.2: Germination tube test under microscope

Limitations

1. *C. tropicalis* may form early pseudohyphae which may be falsely interpreted as germ tubes.
2. The yeast formerly named *Candida stellatoidea* also produces germ tubes; however, it has been combined with *C. albicans* and no longer exists as separate species.
3. This test is only part of the overall scheme for identification of yeasts. Further testing is required for definite identification.

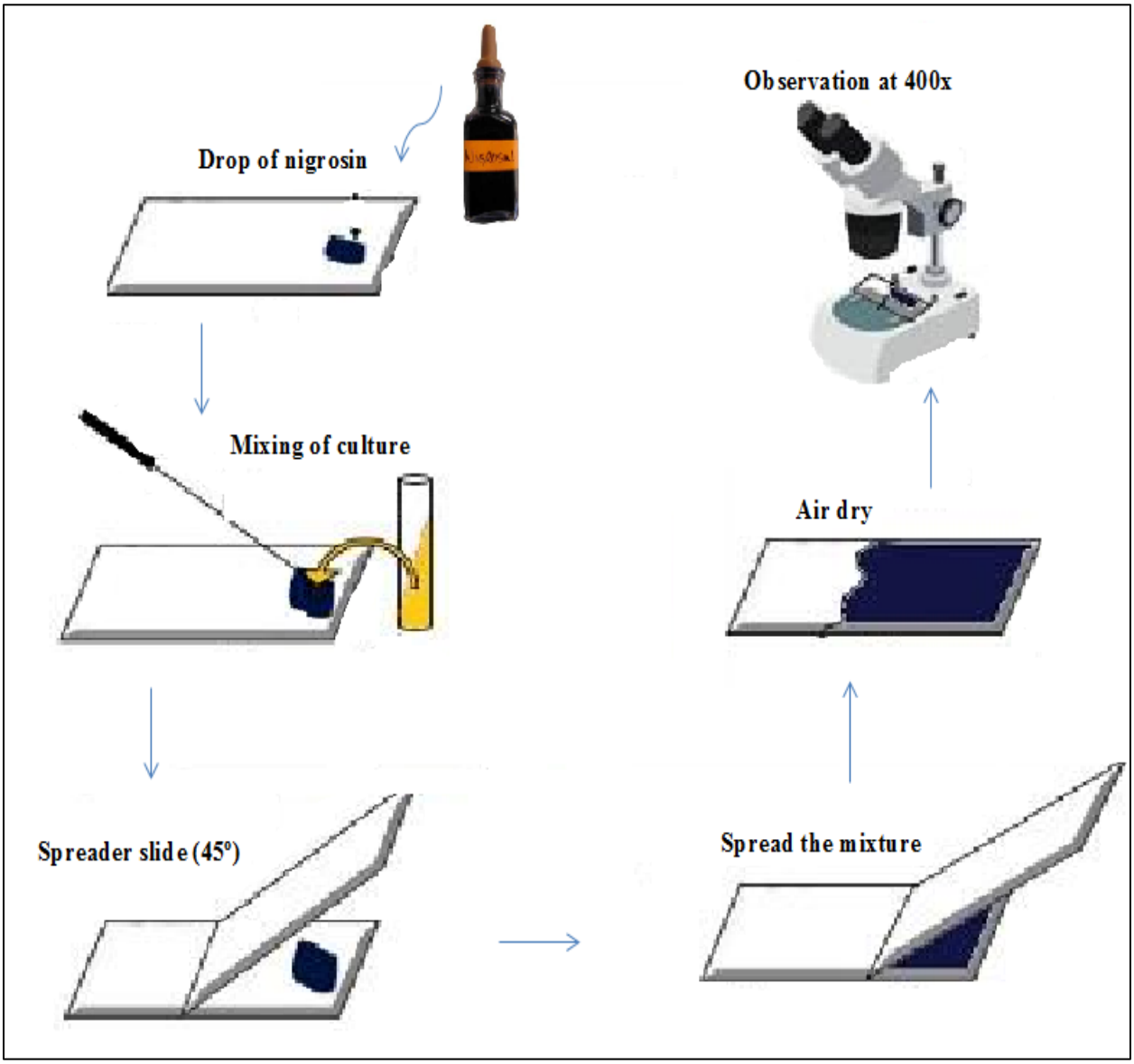


Figure 8.3: Negative staining technique

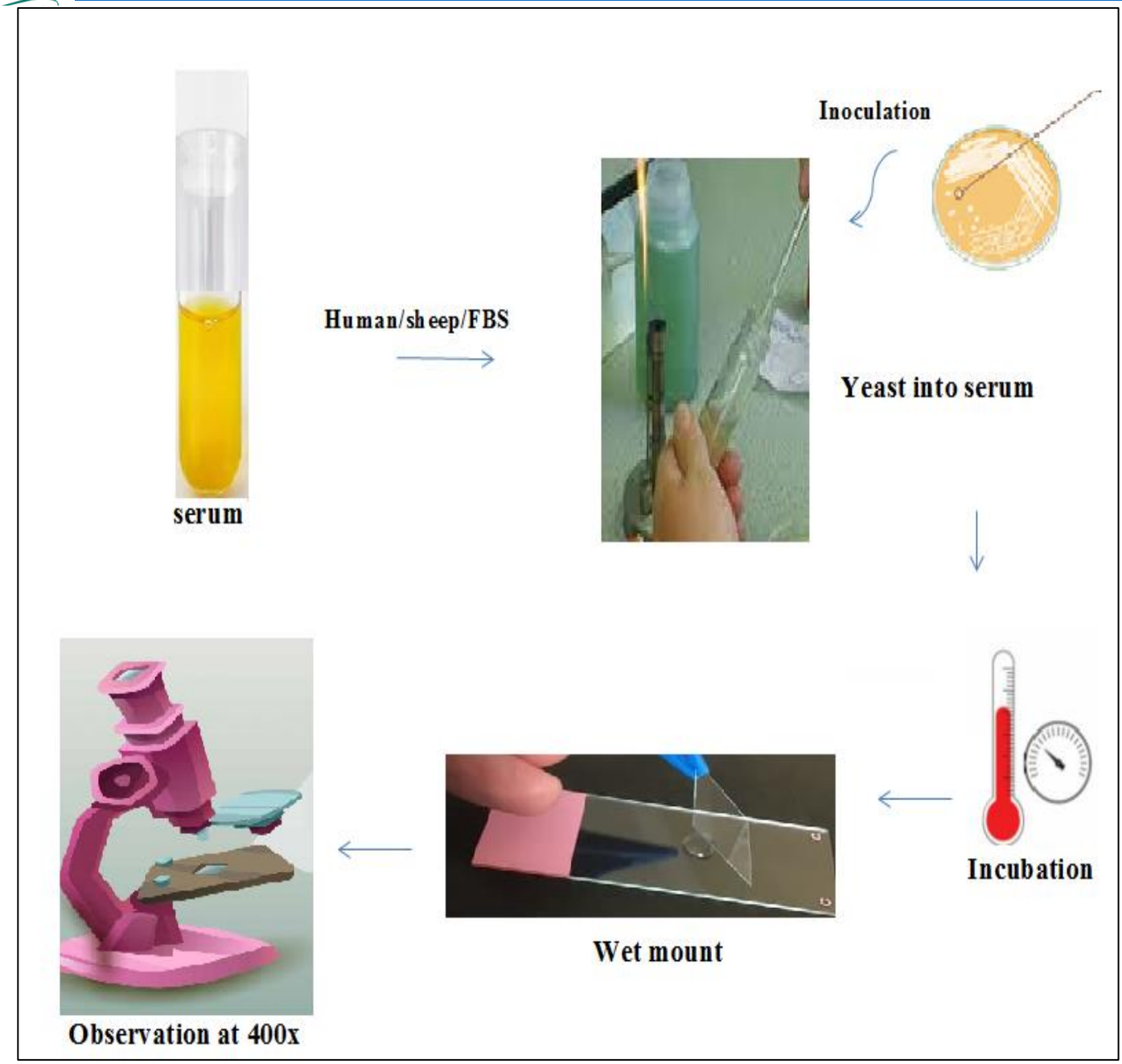


Figure 8.4: Germination tube test

Experiment-9

DERMATOPHYTES: POTASSIUM HYDROXIDE (KOH) MOUNT PREPARATION; HAIR PERFORATION TEST

1. POTASSIUM HYDROXIDE (KOH) MOUNT

This test is performed to detect dermatophytes in specimen (skin scrapings). When specimen such as skin, hair, nails or sputum is mixed with KOH (A strong alkali), it softens, digests and clears the tissues (e.g., keratin present in skins) surrounding the fungi so that the hyphae and conidia (spores) of fungi can be seen under microscope

KOH available in form of pellets and 10-40% solution is recommended for this test. Solution (10%) is prepared by dissolving 10 grams of pellets into distilled water.

Procedure

1. Place a drop of KOH solution on a slide.
2. Transfer the specimen (small pieces) to the drop of KOH, and cover with glass. Place the slide in a petri dish, or other container with a lid, together with a damp piece of filter paper or cotton wool to prevent the preparation from drying out.

   Note: To assist clearing, hairs should not be more than 5 mm long, and skin scales, crusts and nail snips should not be more than 2 mm across.
3. As soon as the specimen has cleared, examine it microscopically using the 10X and 40X objective lenses

Modified Method with Fluorescent Dye

Solution A

- Potassium hydroxide 10 g
- Glycerin 10 ml
- Distilled water 80 mL

Dissolve the KOH in water and add glycerol.

Solution B

- Calcofluor white 0.5 g

- Evans blue 0.02 g
- Distilled water 50 mL

Dissolve Calcofluor white powder in the distilled water by gentle heating.

Procedure

1. Mix one drop of each solution on the Centre of a clean microscope slide.
2. Place the specimen in the solution and cover with a coverslip,
3. Gently heat the slide and examine microscopically for the presence of fungal elements that fluoresce a chalk-white or brilliant apple green color, depending on the filters use

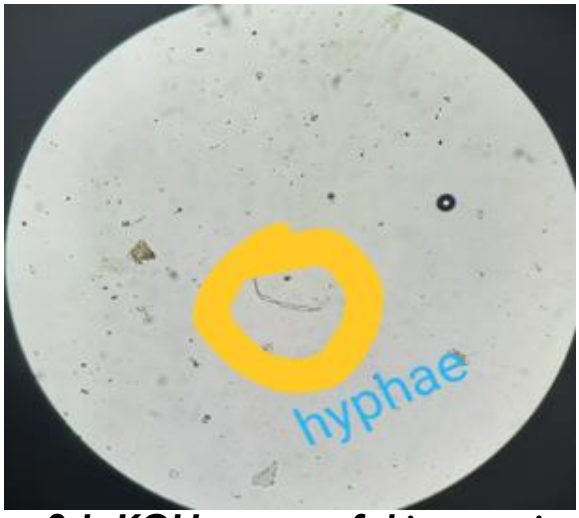


Figure 9.1: KOH mount of skin scrapings

2. HAIR PERFORATION TEST

This test is performed to distinguish between isolates of dermatophytes, particularly *Trichophyton mentagrophytes* and its variants.

Requirements

- Autoclaved hair cut into short pieces (1cm)
- Sterile distilled water 5 mL in a suitable vial.

Procedure

1. Place hair in water in vial.
2. Inoculate with small fragments of the test fungus.

3. Incubate at room temperature.
4. Individual hairs are removed at intervals up to 4 weeks and examined microscopically in lactophenol cotton blue.

   Place a drop of lectophenol cotton blue on a microscopic glass slide. Transfer a piece of hair from vial in it. Put a cover slip over it.
5. Observe at 40x objective lens
6. Isolates of *T. mentagrophytes* produce marked localised areas of pitting (Wedge shaped lesion) and marked erosion whereas those of *T. rubrum* do not.

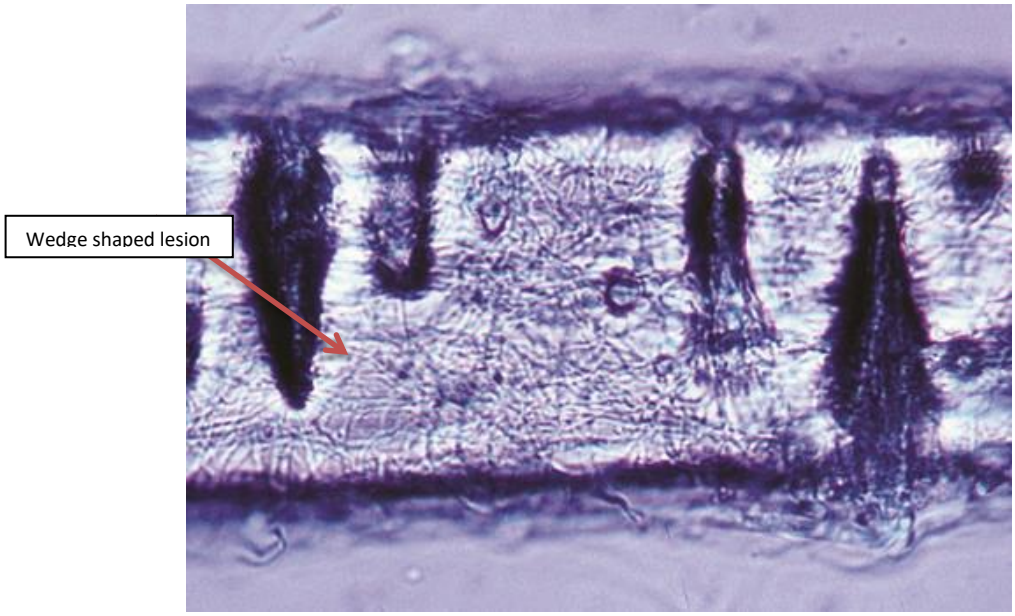


Figure 9.2: Hair under microscope having wedge shaped lesions

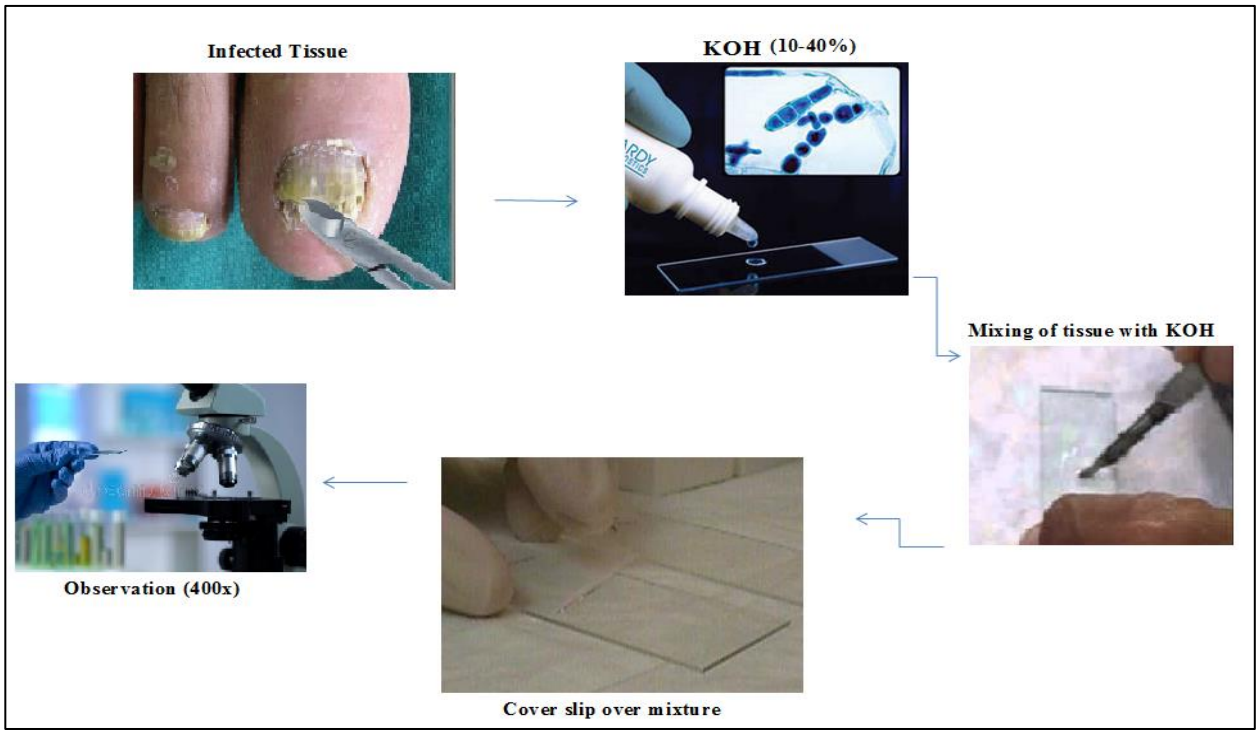


Figure 9.3: KOH mount preparation for dermatophytes

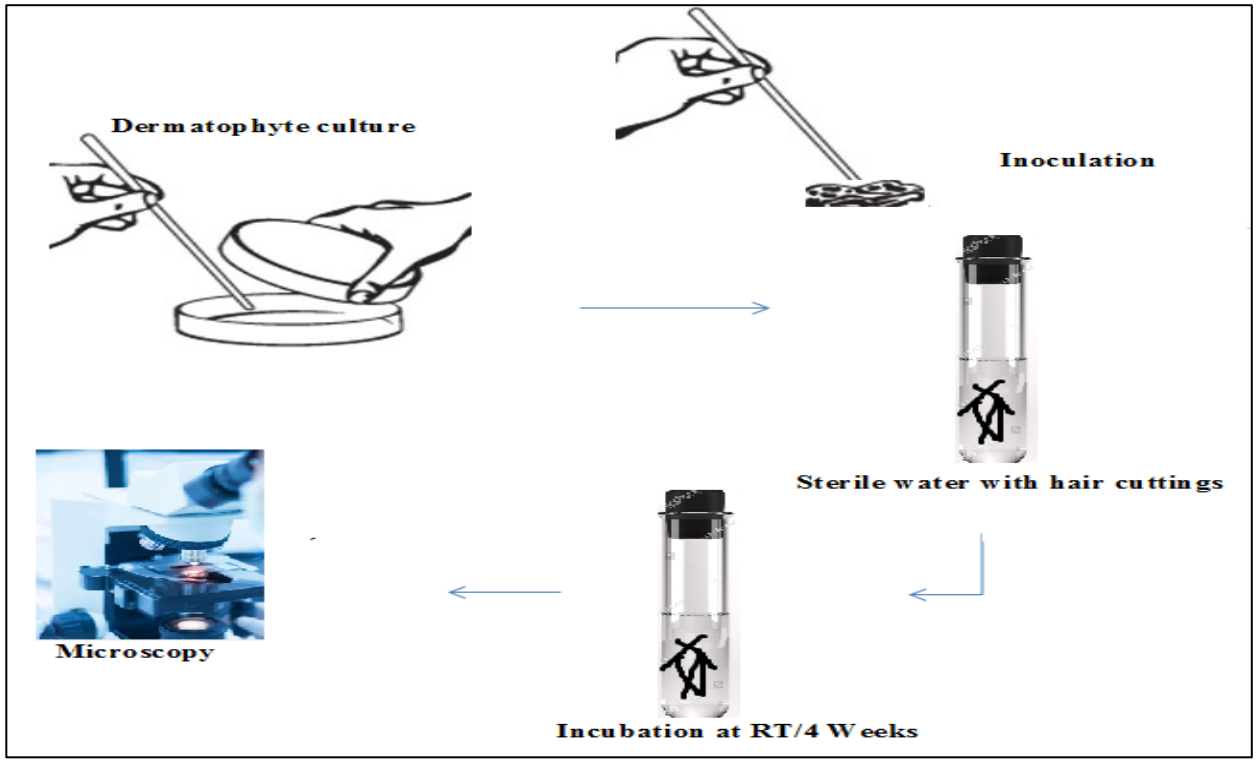


Figure 9.4: Hair perforation test

Experiment-10

INOCULUM STANDARDIZATION

To adjust a particular no. of cells/spores to inoculate a medium is called standardization of inoculum. This standardization of inoculum is required in

- Fermentation experiments
- Antimicrobial susceptibility testing

1. Standardization of Yeast

This method is same for yeast as bacteria. In this method, cell suspension is prepared from purified fresh growth (24 hours) of yeast in sterile normal saline. This suspension is compared with a standard solution (0.5 MacFarland solution). Turbidity is matched, if yeast suspension is less turbid then more yeast cells are transferred to cell suspension and vice versa until turbidity of standard solution and cell suspension exactly becomes the same.

In second method we can take the optical density of yeast cell suspension at 600nm wavelength using spectrophotometer or ELISA reader. The OD value must be between 0.085-0.1 at 600 nm.

We require $1x10^6$ yeast cells /mL. The turbidity of 0.5 MacFarland solution is equal to $1x\ 10^6$ yeast cells /mL and 0.085-0.1 O.D value.

2. Standardization of Mold

For mold we count the spores by Nubauer chamber as described in previous lecture.

a. Counting of Fungal Spores Using Nubauer Chamber

The Chamber is a glass slide consisting of two large central squares called counting areas (which can be seen in its entirety with the 10X objective). Each counting area consists of **9 large boxes**. Each large box is divided into 16 small boxes each with 16 small squares

inside. The central large box has 25 medium boxes, each medium box further divided into 16 small boxes. We will use large boxes located at four corners for counting of spores

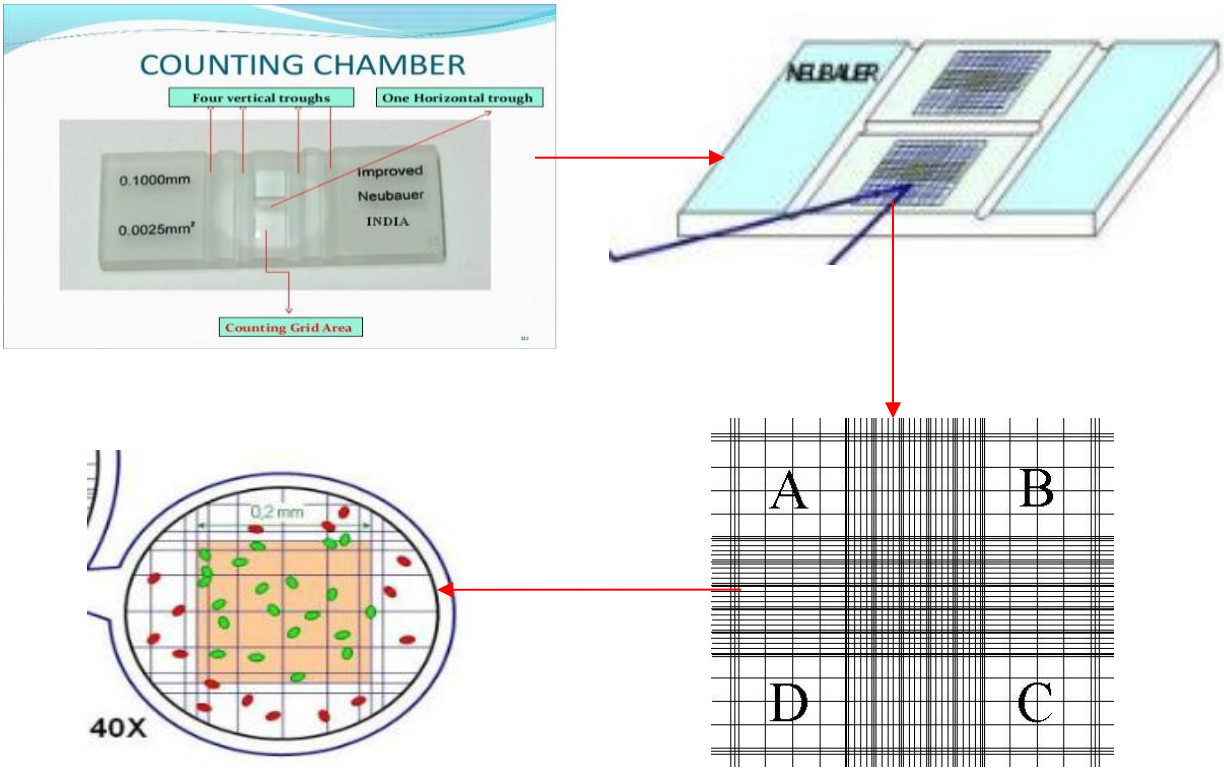


Figure 10.1: Small and large boxes in counting chamber

Two information are written on one side of slide;

0.0025mm^2

0.01mm

0.0025mm^2 is the area of one small box, while 0.01mm is the depth throughout chamber counting area.

Using this information, we can calculate the volume accommodated by ONE LARGE BOX (We have to count spores in large boxes, it means large box is a box of our interest that's why we must know how much volume it can accommodate).

Let's calculate the volume accommodated by one large box;

Volume/large box= Area of large box X Depth **(A)**

= ? X 0.01mm

Area of one large box: ?

We can determine the area of one large box by multiplying area on one small box with 16.

As each large box has 16 small boxes of area 0.0025mm^2 each.

Area of one large box= 0.0025mm^2 X 16

=0.04mm^2

Now put the information in above formula **(A)**;

Volume/large box= Area of large box X Depth

= 0.04mm^2 X 0.01mm

= 0.004mm^3/ 0.004µL

mm^3 = µL

So, each large box 0.004µL volume capacity

Procedure

1. Prepare a spore suspension sterile normal saline from a pure fungal culture.
2. Prepare a chamber for use.

   Carefully clean all surfaces of the counting chamber and cover-slip.

   Take care to ensure that all surfaces are completely dry using non-linting tissue.

   Center the coverslip on the counting chamber.
3. Pipet 20 microliters of the spore suspension into one of the two counting chambers.

   Use a clean pipet tip.

   Be sure that the suspension is thoroughly, but gently, mixed before drawing the samples.

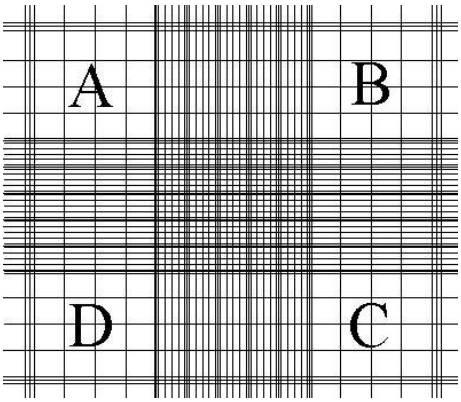
   Fill the chambers slowly and steadily.

   Avoid injecting bubbles into the chambers.

Do not overfill or under-fill the chambers.

4. Count the spores.

   Count all of the spores in each of the four corner squares labeled A, B, C and D in Figure below the page under 40X objective lens.



For counting the spores follow the rule; leave lower and left

- DO NOT, count the spores touching the bottom or left borders.
- DO count the spores touching the upper or right borders.

Determine the Spore Count

- Calculate the total spores counted in the four corner squares and determine average no. of spores/large box.

Average spores= A+B+C+D/4

- Average no. of spores /large box means are equal to average no. of spores/0.004µL
- Calculate spores/mL by unit method;

EXAMPLE: If the calculated average (n) of spores in the four corners squares of the chamber is 30:

0.004µL volume contains spores=30

1µL volume contains spores= (30/0.004) *1

1000µL (1mL) contains spores= (30/0.004) *1*1000

Cells/mL = 30 x 10,000 = 300,000 spores/mL or $3X10^5$spores/mL

Experiment-11

ANTIFUNGAL SUSCEPTIBILITY TESTING (AFST)

Antifungal susceptibility testing is performed to evaluate the effect of antifungals on pathogenic fungi.

Antifungal

The agents that kill or inhibit the growth of fungi are called antifungals. The major groups of antifungal agents used clinically are:

(1) Polyenes (amphotericin B and lipid formulations of amphotericin B)

(2) Azoles (fluconazole, itraconazole, voriconazole, and posaconazole)

(3) Echinocandins (caspofungin, micafungin, and anidulafungin)

(4) Fluorinated pyrimidines (flucytosine)

(5) Allylamines (terbinafine)

Methods for AFST

There are several methods for antimicrobial testing. The methods included in our course are;

- Disc diffusion method
- Well diffusion method
- Micro-Broth dilution method

1. Disc diffusion method

Antifungal discs are commercially available. These discs have a particular concentration of antifungal absorbed on it. In dis diffusion these discs are used. This is called disc diffusion because antifungal starts diffusion from disc into the agar. If it is effective against fungi, it inhibits the growth around itself to where effective concentration is diffused. This area appears as zone of inhibition. To perform Disc diffusion followings are the steps;

1. Standardization of inoculum (10^6 cells / mL for yeast and 10^6 spores/ mL)
2. Make a uniform fungal lawn on Muller Hinton agar by swabbing
3. Apply discs with forceps or disc dispenser
4. Incubate at 37°C for 24 hours in case of yeast. Incubate at 28°C for 3 days in case of molds

Observation

Observe the plates for the presence or absence of zone of inhibition (Area around the disc where no growth is present). If present, diameter of zone of inhibition is measured in mm and compared with CLSI (clinical laboratory standards institute) available standards to find out whether our test fungi is sensitive or resistant to that particular antifungal.

Sensitive: it means fungus is killed by that drug

Resistant: Fungus is not killed by that drug

2. **Well Diffusion method**

Principle is the same as for disc diffusion. Drug diffuses into medium. But instead of disc, antifungal solutions of required concentrations are poured into wells made in Muller Hinton agar plate. This test is carried out as;

1. Standardization of inoculum (10^6 cells / mL for yeast and 10^6 spores/ mL)
2. Make wells with the help of well borer. Three wells on 6cm diameter plate and 5 wells may be prepared on 9cm diameter Muller hinton agar plate. Well base is sealed by molten agar so that when we pour antifungal solution, it does not seep under the agar. It must be diffused into medium that's why we seal the base of agar with minute (10µL) of molten agar.
3. Make a uniform fungal lawn on Muller Hinton agar by swabbing
4. Pour antifungal solution into wells so that well is completely fills.

5. Incubate at 37°C for 24 hours in case of yeast. Incubate at 28°C for 3 days in case of molds. Plates are incubated in upright position either yeast or mold.

Observation

Observe the plates for the presence or absence of zone of inhibition (Area around the well where no growth is present). If present, diameter of zone of inhibition (ZOI) is measured in mm and compared with CLSI (clinical laboratory standards institute) available standards to find out whether our test fungi is sensitive or resistant to that particular antifungal.

If are testing a natural product/herbal product then presence of zone of inhibition indicates that the tested product has antifungal activity.

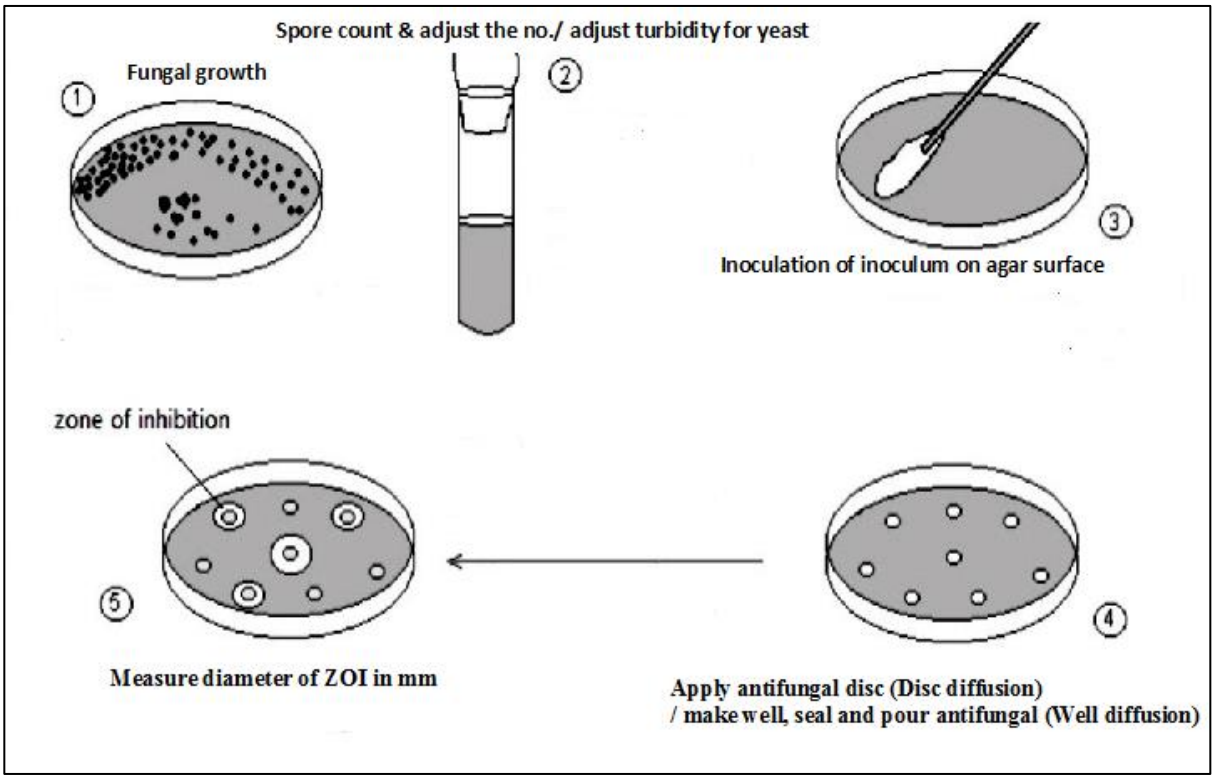


Figure 11.1: Antifungal susceptibility testing

Figure 11.2: zone of inhibition

Disc diffusion (left side); Well diffusion (right side)

Experiment-12

MINIMUM INHIBITORY CONCENTRATION DETERMINATION MICRO-BROTH DILUTION METHOD

This test is performed using broth (Muller hinton broth) instead of agar. It is performed using small quantity (µL). In this method, Minimum inhibitory concentration (MIC) of drug is determined. MIC value just like Zone of Inhibition (ZOI) helps to determine either fungus is sensitive or resistant to a particular antifungal. CLSI also provided standards based on MIC along with ZOI.

Micro-titration plate: 96 wells micro-titration plate flat bottom is used in this method. This plate has rows and columns. There are 12 columns and 8 rows. Rows are designated by letters (A-H), while columns are designated by number (1-12). This plate has 96 small pockets having capacity to accommodate 250µL volume. These pockets are called wells. So, this is 96 wells micro-titration plate.

This test is performed as mentioned below;

1. Standardization of inoculum (10^6 cells / mL for yeast and 10^6 spores/ mL)
2. Take 96 wells flat bottom micro-titration plate
3. Add 100µL sterilized Muller hinton broth in each well (A1-A12) as a diluent
4. Add100µL antifungal agent in first well (A1) and mix properly
5. Now prepare two-fold serial dilutions till 10th well (A10). Two-fold dilutions are prepared by transferring 100µL from 1st well to second well. Then mix it in second well and transfer 100µL to 3rd well and mix. Similarly transfer 100µL from previous well to next well (have 100µL diluent. already added in first step) till 10th well. Now discard 100µL from 10th well to keep the uniform volume in all wells. Now all wells

have 100µL volume after this step. But first 10 wells have antifungal in them. The highest concentration of antifungal is in 1st well and the lowest in the 10th well of plate.

If we know the concentration of antifungal, we are using then we can determine the concentration of antifungal in each well. In above figure, we used antifungal stock having concentration of 100mg/mL. It was two-fold diluted serially up-to 10th well. So, in first well its concentration remain half (50mg), in second well it again diluted two times, so concentration become 25 and so on.

6. Now add 100µL of standard inoculum in each well up-to 11th well.
7. Incubate this plate at 25ºC for 3-5 days for mold and at 37ºC for 24 hours if test fungi are yeast.

Observations

Observations can be read either by micro-plate reader by taking optical density (OD value) at 490nm for mold or 600nm for yeast or visually by presence of mycelial growth in wells

According to performed procedure;

A12: Negative control; must be sterile after incubation, there must be no growth. It will remain transparent

A11: Growth control; add broth and inoculum. No antifungal. So, after incubation there must be growth, which is represented by turbidity. It must be turbid

A1-A10: are test wells having broth + antifungal (varying concentration in each well) + inoculum.

We will check test wells for presence or absence of growth.

MIC: Highest dilution (Lowest concentration) of drug that inhibits the fungus growth is MIC.

EXAMPLE: Suppose the drug we tested have antifungal activity proven by well diffusion. Now we want to determine its minimum concentration that inhibits fungus (MIC). We will perform micro-broth dilution as mentioned.

Suppose that there is no growth from A1-A7. From A8 growth starts and in A9 turbidity is also present. This result indicates that this antifungal is able to stop fungus growth up-to concentrations present in A1-A7. Among all these concentrations the lowest or minimum concentration is in 7th well. So, the concentration in 7th well is MIC of that particular drug.

MIC is written as;

$$MIC \geq \text{Concentration in } 7^{th} \text{ well (example only)}$$

$$MIC \geq 0.78mg$$

It means 0.78mg concentration of antifungal and all concentrations higher than that are able to inhibit fungus growth. But minimum concentration is 0.78mg.

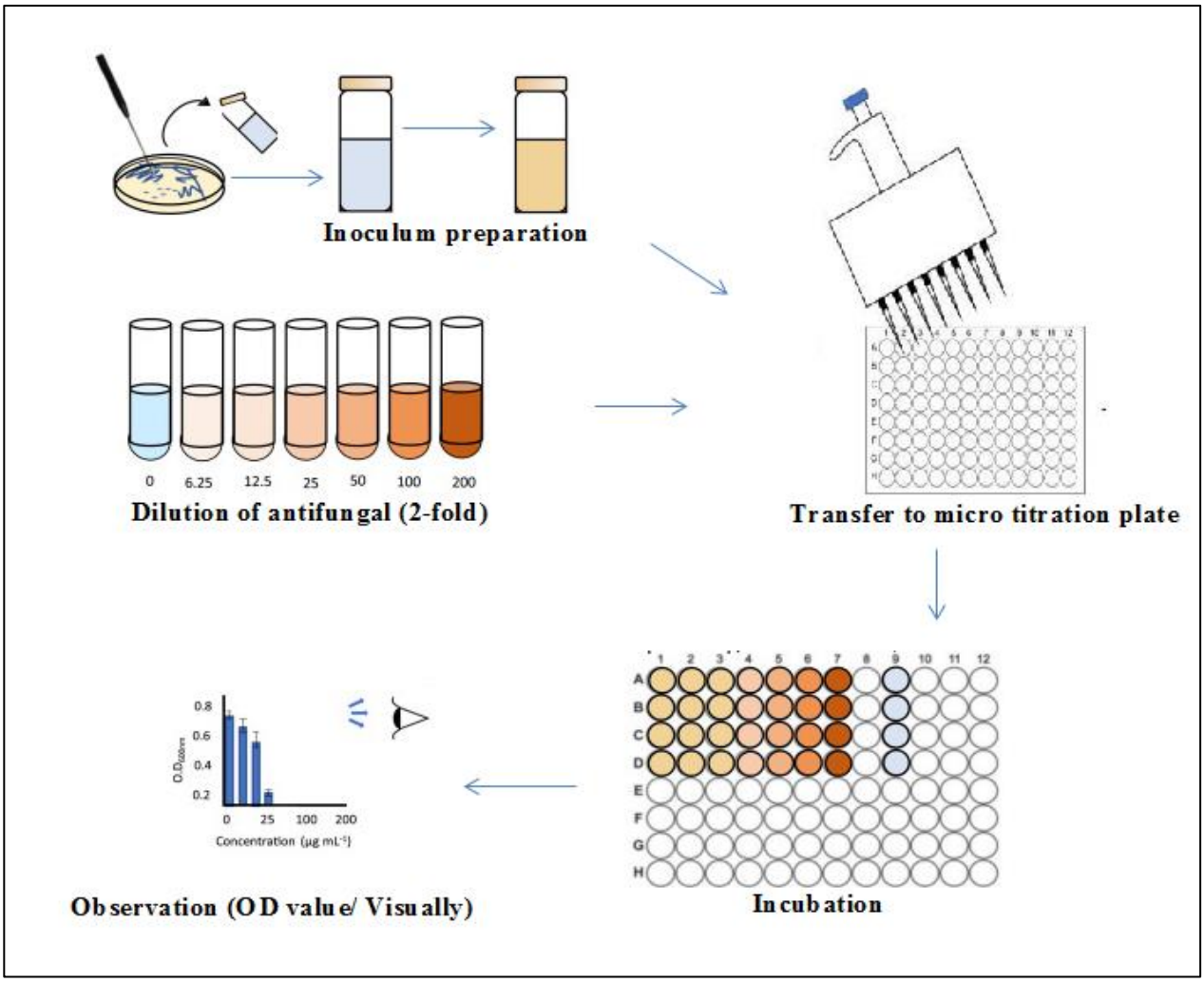


Figure 12.1: Minimum Inhibitory Concentration (MIC) for fungus

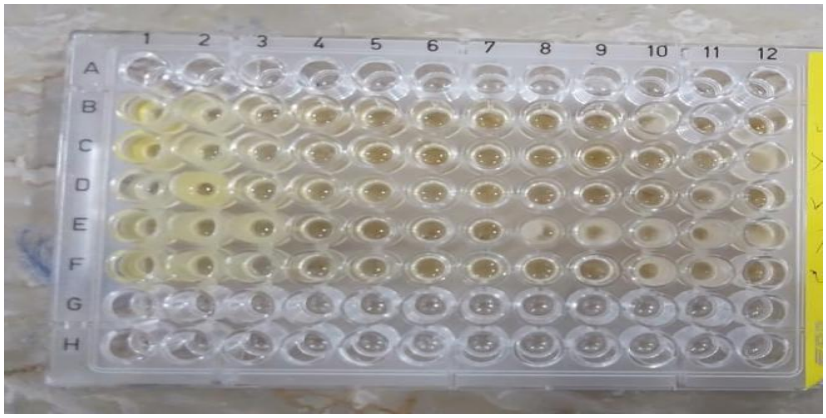


Figure 12.2: 96 well micro-titration plate

Experiment-13

LOG REDUCTION OF FUNGAL SPORES

Molds play an important role in various ecosystems and are used for the production of different types of foods and food supplements, beverages, enzymes, antibiotics, and other pharmaceuticals. However, molds are also responsible for a vast amount of global food spoilage and cause damage to a wide range of building materials and household surfaces. Especially dangerous are food related fungal species, which are capable of producing toxic metabolites known as mycotoxins, many of which are known human carcinogens, neurotoxins, immune-suppressants, and endocrine disruptors.

The occurrence of unwanted mold causes economic loss on a global scale; a challenge made even more pressing by the lack of a truly effective response measure to prevent it. Recently, several studies have indicated that the problems caused by fungal contamination are rising at an alarming rate due to improper agricultural practices and global climate change. Moreover, globalized economies demand the long-distance transport of food from producer to consumer, meaning frequent changes in local climate and long storage times, further exacerbating the issue. Current approaches to avoid mold contamination usually involve the use of groups of chemicals such as fungicides and disinfectants, where disinfectants are typically alcohol, peracetic acid, iodophors, aldehydes, chlorine, peroxygen-based, or a mixture of these chemicals. The other method to lower the burden of fungal spores is heat treatment.

Procedure

In current practical, we will use heat (50, 60 and 100°C/10 minutes) and disinfectant (10% bleach (Sodium hypochlorite), interaction time 0, 5, 10 and 15 minutes). We will evaluate the effect of disinfectant and heat on spores of *Aspergillus flavus* by log reduction methods. *Aspergillus flavus* is a common contaminant of food, feed and ingredients. Its contamination leads to mycotoxin production under inappropriate storage conditions.

Preparation of spore suspension

Prepare spore suspension from purified growth of mold (*Aspergillus flavus*) in normal saline. Adjust a spore suspension at $1x10^6$ spores/mL of saline. For this purpose, use Neubaer chamber to count the spores as mentioned in previous experiment of spore counting.

1. Inactivation by Heat

- Prepare three test tubes of spore suspension (each tube has $1x10^6$ spores /mL). Label these tubes as 1 (for 50°C), 2 (for 70°C) and 3 (FOR 100°C) and 4 (Room temperature, no heat treatment).
- Incubate Tube 1 in water bath, showing adjusted temperature of 50°C for 10 minutes.
- After incubation take out of tube from incubator and allow it to cool.
- Now prepare ten-fold serial dilutions of treated spore suspension (50°C).

  For ten-fold serial dilution take five test tubes. Each tube having 9 mL sterile normal saline in it, label these tubes as (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5})

  Now add one mL of treated spore suspension in first tube (10^{-1}), mix it well by pipetting and transfer one mL from 1st tube to second tube labeled as 10^{-2}. Similarly, from second tube after mixing transfer one mL to 3rd tube (10^{-3}), from 3rd to fourth tube (10^{-4}) and from fourth tube to 5th tube (10^{-5}). From 5th tube discard one mL so that each tube has equal volume of diluted spores. These are ten-fold serial dilutions.

Take five sterile SDA plates and label these as (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5})

- From each dilution spread 100µL on respectively labeled sterile SDA plates.
- Incubate the plates at 25±3°C for 3 days
- Count the colonies on plate having countable colonies.
- Calculate CFU/mL using following formula.

CFU/mL= No. of colonies counted x dilution factor x corrective factor

Corrective factor is 10 if we inoculate 100µL from each dilution.

Dilution factor is the reciprocal of dilution. If you are choosing a plate on which you spread 10^{-2} dilution. You select that plate and count colonies in that plate. Suppose you count 20 colonies. The no. of colonies will be 20 to put in formula and dilution factor will be 10^{+2}.

So;

CFU/mL= 20 x 10^{+2} x 10

CFU/mL = 20000 or in scientific notation $2x10^4$

Similarly Tube 2 and 3 will be incubated at 70ºC and 3 100ºC respectively followed by serial dilutions and inoculation 100µL from each dilution, incubation at 25±3 ºC for 3 days. Count colonies per mL as mentioned above. Similar procedure will be applied on spores incubated at room temperature.

Interpretation

At the end of experiment, we will have four CFU/mL values for each treatment.

CFU/mL in tube incubated at room temperature/ 10 mins

CFU/mL in tube incubated at 50ºC temperature/10 mins

CFU/mL in tube incubated at 70ºC temperature/10 mins

CFU/mL in tube incubated at 100ºC temperature/ 10 mins

We will compare CFU/ mL of each tube and find which tube has the lowest value. The tube has the lowest value, that temperature is effective in the inactivation of spores. That why inactivated or killed spores remained unable to grow on SDA plate

2. Inactivation by Disinfectant

We will use 10 percent bleach solution and evaluate its effect on inactivation of *A. flavus* at three different interaction times (0, 5, 10 and 15 minutes)

- Prepare spore suspension in normal saline (10^6 spores/mL). Prepare 9 mL spore suspension and add one mL of bleach solution into it. This will reduce the pure bleach solution to 10 percent (desired concentration).
- Incubate tube at room temperature (RT).
- Just after adding (0-minute interaction time) take one mL from this mixture and make ten-fold serial dilutions as mentioned above.
- From each dilution spread 100µL on sterile SDA plates
- Incubate the plates at 25±3°C for 3 days
- Count CFU /mL as mentioned above.

Repeat this experiment on mixture of spore suspension and bleach solution (10%) after 5, 10 and 15 minutes of exposure time and count CFU/mL.

Interpretation

At the end of experiment, we will have four CFU/mL, at 0 time, at 5 minutes, at 10 minutes and 15 minutes. We will compare and find out lowest CFU/mL. The time at which the lowest CFU/mL is obtained will be the most effective interaction time for 10 percent solution of bleach (sodium hypochlorite) to inactivate or kill the spores of *A. flavus*

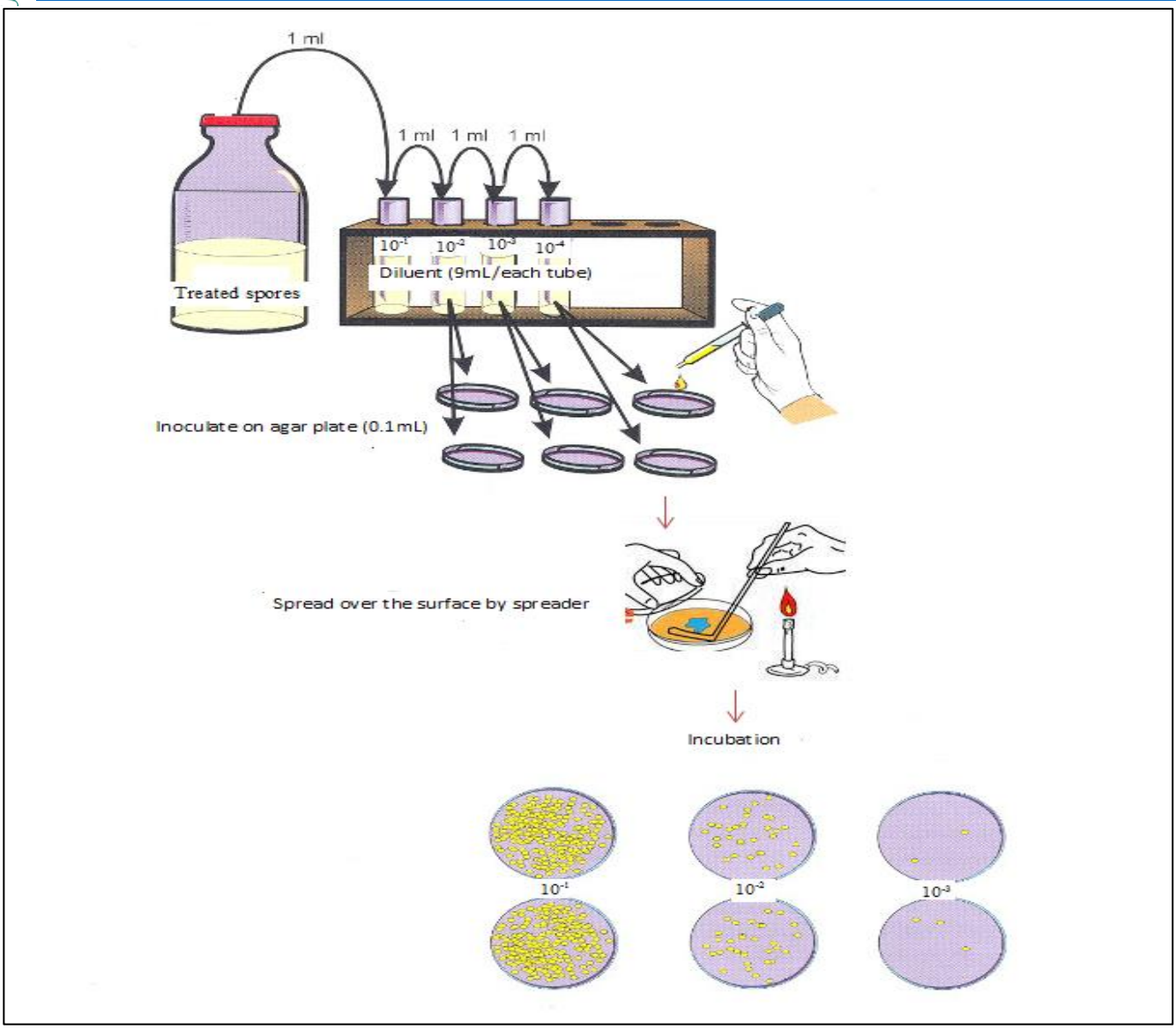


Figure 13.1: Effect of treatment by log reduction method

Experiment-14

MYCOTOXIN PRODUCTION AND EXTRACTION

Mycotoxins are toxic secondary metabolites produced by certain fungi. Their toxicity is associated with animal, human, birds and microbial cells. Species from following fungal genera famous for mycotoxin production are;

- *Aspergillus*
- *Penicillium*
- *Fusarium*

There are more than 300 recognized mycotoxins. However, important mycotoxins are;

1. **Aflatoxins (AF)**; Produced by *Aspergillus flavus* and *Aspergillus parasiticus.* The four naturally occurring aflatoxins are;

   AF B_1 (The most potent mycotoxin)

   AF B_2

   AF G_1

   AF G_2

2. **Ochratoxins (OT);** Produced by *Aspergillus ochraceous*
3. **Zeralenone;** Produced by *Fusarium spp.*
4. **Fumonisins;** Produced by *Fusarium spp.* and some *Penicillium spp.*

These fungi contaminate various types of feedstuff, and under inappropriate conditions, the produce mycotoxins. Mycotoxins may build up to the injurious levels (Above the permissible limit provided by WHO and European Union; like 20ppb for **AF B_1**); it may leads to toxicity of consumer (animals, birds or human). The toxic effects caused by consumption of preformed mycotoxins along-with feed/food are called mycotoxicosis.

Procedure

In current experiment, we will proceed to determine either a fungi is toxigenic or non-toxigenic.

1. Inoculum Preparation

Spore suspension from pure cultures of *fungi* will be prepared in normal saline. Briefly, normal saline will be added to screw capped glass test tubes (10mL each) and sterilized by autoclaving. Fungal spores from pure cultures of *fungal* species will be picked with inoculation loop under sterilized conditions. Spores will be transferred to normal saline tube and evenly mixed.

2. Mycotoxin production

Inoculum (Fungal spores) will be inoculated in SD broth.

- Broth (100mL each) will be prepared in conical glass flasks (250mL) following the instructions of manufacturer, pH (4.5) adjusted using electrical pH meter (PHS-3C) and autoclaved.
- Fungal spore suspension (01mL) will be inoculated using micropipette (200-1000µL) into respective labeled broth flasks under sterile conditions.
- Flasks will be properly wrapped using brown paper and placed in incubator (25±3°C) for 45 days.

3. Mycotoxin Extraction

- Representative fungal culture samples at 45-days post incubation will autoclaved at 121°C for 15 minutes under pressure of 15 psi and used for extraction of mycotoxins.
- Samples will be homogenized using a tissue homogenizer (JANKE & KUNKEL IKA-WERR/DS3PI-45).
- Autoclaved and homogenized broth culture (12.5 grams) will be poured in a blue capped bottle (100mL).
- Extraction mixture will be prepared by adding,

Chloroform	(45mL)
Methanol	(05mL)
NaCl	(2.5g)
Distilled water	(5mL)

Chloroform and **Methanol** are organic solvents. They are used because mycotoxins are organic in nature. Most of the mycotoxins are solubilize in organic solvents. These are sparingly soluble in water

NaCl breaks the contact between mycotoxin and mycelium and helps mycotoxins to dissolve in organic solvent.

- All components will be mixed and placed in shaking incubator at 37°C for 30 minutes.
- Mixture will be passed through whatman filter paper 42 (2.5µm pore size).
- The filtrates will be evaporated to dry, crystals crushed to fine powder and shifted to glass vials.

- To separate mycotoxins from crushed powder, chloroform (1mL) will be added to it. It will be mixed and allowed to stand for one minute to settle down crushed powder. Liquid phase will be separated using sterilized glass pipette and added to separate tube.

(The washing of crushed powder with chloroform will be repeated three times in order to obtain all the mycotoxins)

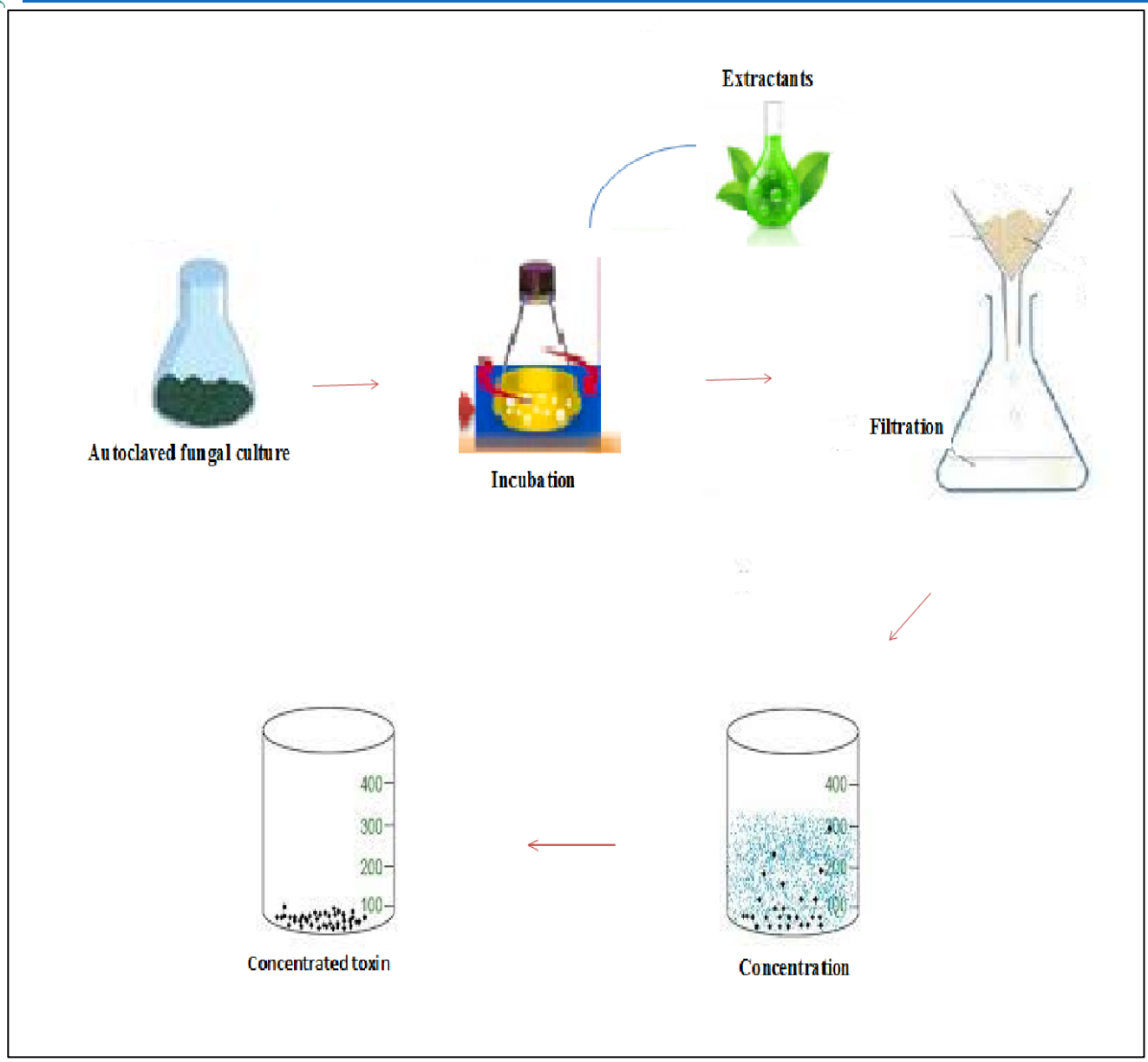


Figure 14.1: Mycotoxin production and extraction

Experiment-15

MYCOTOXIN DETECTION BY THIN LAYER CHROMATOGRAPHY (TLC)

Mycotoxins are detected and quantified by several methods;

1. **Culture based techniques**

In this we grow fungus on media to see if it is producing toxins. These methods are not very accurate and only use for qualitative detection.

2. **Immunological techniques**

In this case we use ELISA (Enzyme linked immune-sorbent assay) for mycotoxin detection and quantification.

3. **Chromatographic techniques**

This technique is used for separation, detection and quantification of mycotoxins.

3.1. Thin layer chromatography (TLC):

- Semi-quantitative
- Less expensive
- Less Time consuming

3.2. High performance Liquid chromatography (HPLC) is used. It is used for quantification

Every chromatographic technique uses two phases

- mobile phase
- Stationary phase

THIN LAYER CHROMATOGRAPHY

We will use thin layer chromatography to detect the mycotoxins produced by fungi in broth or in feed. For this purpose, we have to extract mycotoxins. In previous hand out provided detailed procedure for mycotoxin extraction after their production in broth culture. In this method we can identify either a fungus is mycotoxin producing or Not.

Principle

The principle of thin layer chromatography is the movement and separation of molecules on the basis of affinity with the mobile or stationary phase. If the molecules have higher affinity with the mobile phase they will go faster with the movement of mobile phase and reach closer to the top of the plate. If they have more affinity with the stationary phase, these will stay closer to the bottom and move slowly with the mobile phase.

Stationary phase in TLC

- It is silica coated on supporting material.
- Material could be of glass, plastic plates or aluminum sheets.

Mobile phase

- Mobile and stationary phase change with the nature of the test substance.
- Mobile phase is liquid and I is Chloroform and acetone in 95:5 respectively**.** Chloroform is used because mycotoxins are more soluble in it.
- We need standard to compare our test substance. Mycotoxin standards are also available.

This mobile phase is prepared in a glass tank known as TLC Tank. Tank must be washed and dried before preparation of mobile phase.

Procedure

- Take a silica coated sheet and draw a line of 1cm away from bottom with led pencil. Draw Line gentle so that it does not disturb the silica coating.
- Then draw another line 15cm away from that line on top of plate.
- Apply the standard (2µL) and sample (20µL) on this sheet with help of sample charging syringe or pipette. Samples must be in placed on TLC plate in form of spots. All spots must be an appropriate distance (1cm apart from the other) to avoid cross contamination.

It must be noted that all samples volume (20µL) must not be spotted at once. Spot 5 microliter at a time let it dry and applies whole samples in fraction of 5µL. That means you will pour four times to complete 20 microliters.

- Put this TLC sheet into tank where mobile phase is present but make sure that sheet doesn't touch with walls of tank.

Also make sure that mobile phase doesn't touch your sample (spots). Samples must be above the level of mobile phase. It means spots must Not dip in mobile phase.

- After doing this close the tank and note the movement of mobile phase. Mobile phase will move upwards by capillary action and will separate the mycotoxins on silica according to their chromatographic mobility.
- As the mobile phase moves up to a distance of 15cm (Touches the line drawn at top). Then remove the TLC plate.
- Let the plate to dry the plate and observe it under UV lamp at 360nm wavelength of UV light.

Observations

Aflatoxins produced blue and green fluorescent in UV light. We compare bands with the standards bands (mycotoxins). Mycotoxins showing Blue fluorescence are Aflatoxin B_1 and B_2. The former is the most common toxin found in food and has the lightest molecular weight thus moves farthest on the TLC plate with the mobile phase. The Green fluorescence substances are G_1 and G_2. G_2 is the heaviest mycotoxins of all four thus have a green band closest to the bottom of the plate.

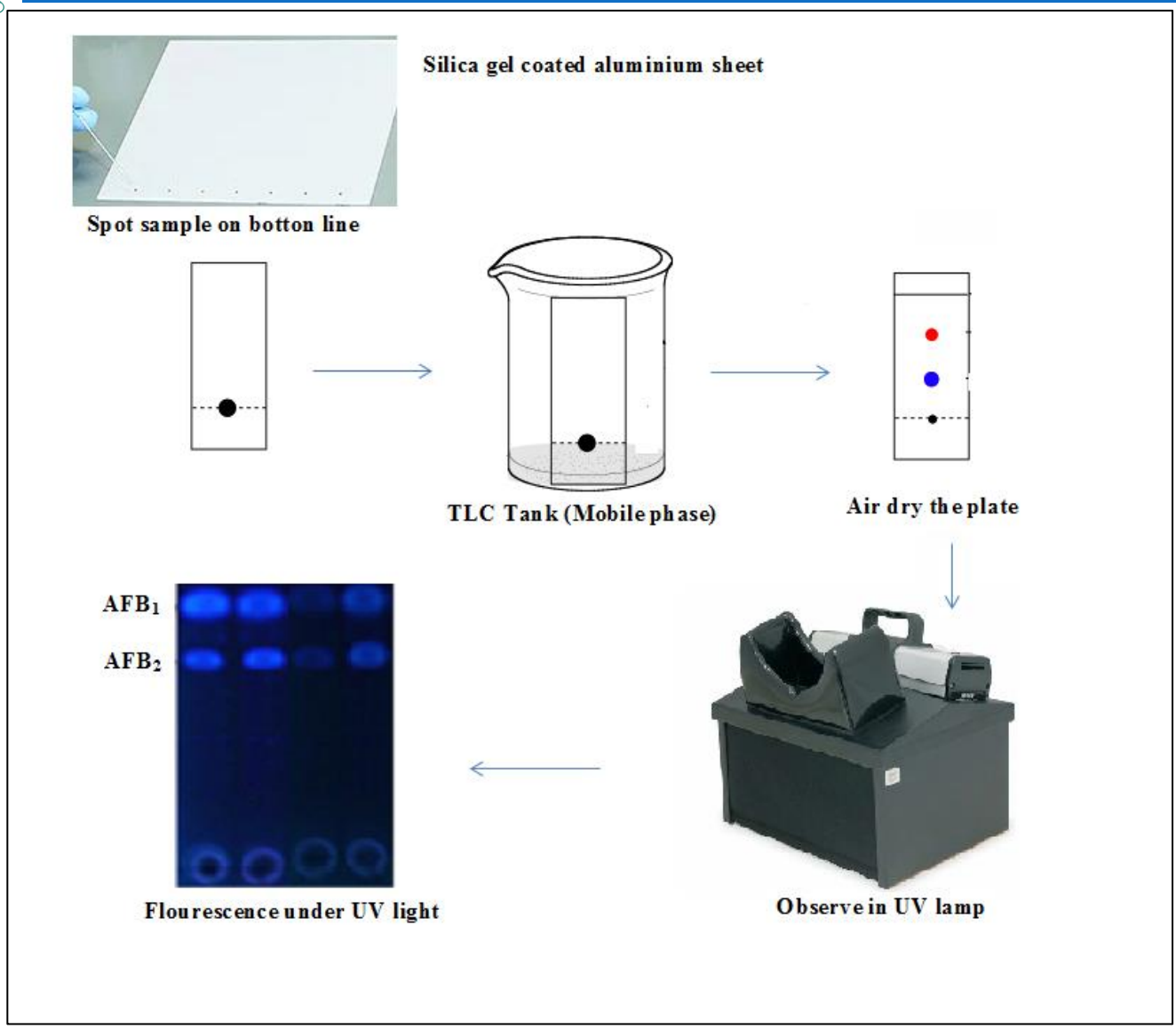


Figure 15.3: Mycotoxin Detection by Thin Layer Chromatography

Experiment-16

PRESERVATION AND REVIVAL OF FUNGI

Once fungal cultures are identified, these are preserved for future use. So that, time consuming activity of isolation, purification and identification of fungi may not be repeated. There are different methods used for preservation of fungi. The method is considered the best which does not change the character of microorganisms. For preservation culture must be purified and preferably in log phase of growth. It increases the chances of revival.

1. Sub-culturing

Sub culturing is the method use for the preservation of microorganisms but characters of microbes lost during this method. Some genes present on plasmid but by repeated *in-vitro* cultivation of bacterial cells under different physico-chemical conditions may lead to the plasmid lose.

Method: In this method slants of Potato dextrose agar or Sabouraud dextrose agar are prepared. Fungi are inoculated and incubated at recommended temperature (25°C for mold/7 days; yeast 37°C/48 hours). Once growth appeared m it is placed at low temperature (4°C)

Revival: Growth is sub-cultured on new slants after 15 days.

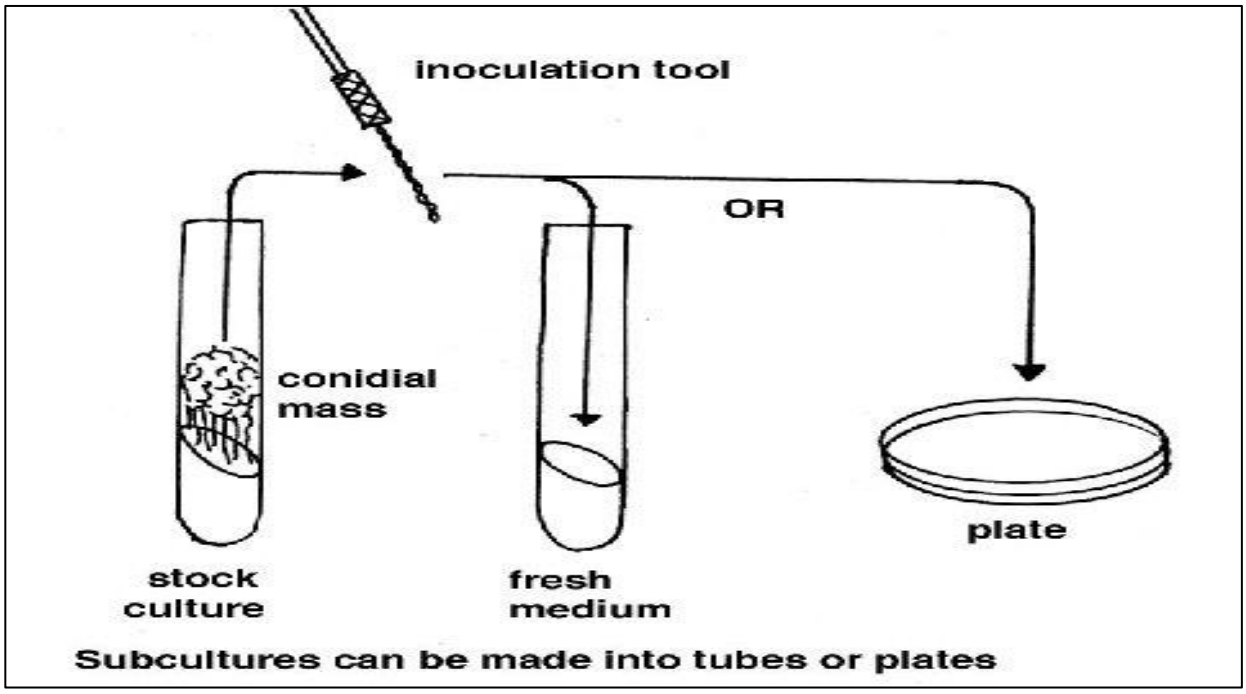


Figure 16.1: Subculturing for revival of cultures

2. Lyophilization

It is method for long term preservation and convert microbes into dry form.

Methods: Yeast can be preserved in this method. Yeast cells are placed in Lyophilizer which converts growth of yeast into powder form.

Revival: Lyophilized powder is inoculated into broth followed by incubation at recommended conditions for microorganisms.

3. Cryopreservation

In cryopreservation microorganisms are stored at low temperature and metabolism rate of microbes become low. Cryo-protective agents are used. Without use of cryoprotectant, water in fungal cell become crystalized and damage cell membrane. Cryoprotective used agents are glycerol (10-30%) and 10% dimethyl sulph oxide (DMSO). Cultures remain preserved for months and years. It is mostly performed for yeast and mycelia of fungi.

Method: In this method overnight broth culture is used and 15% glycerol is added to it. In one mL broth culture, 150µL sterile glycerol is added. It is gently mixed and placed at low temperature (-20ºC or -80ºC).

Revival: Bring the preserved culture to room temperature. It is centrifuged at 6000 rpm for 10 minutes. Discard the supernatant and pellet is washed by adding sterile normal saline followed by centrifugation. Supernatant is discarded and the washed pellet again suspend in small quantity of saline. This suspension is inoculated on SDA by spreading followed by incubation at recommended temperature.

4. Preserve in Soil

Mostly fungal spores are preserved in sterile soil. Spores can be preserved for several years. It is long term and commonly used method for preservation of fungal spores. It is only used for mold not for yeast.

Method: In this method soil added in bottle and autoclaved and incubated at room temperature. It is autoclaved at second day. After two times of autoclaving spores suspension in normal saline from a pure culture is transferred into it followed by incubation at 25ºC for 10 days. Then it is placed either at low temperature or room temperature.

Revival: For revival the soil is sprinkle on sterile SDA plate followed by incubation at 25±3ºC for 5 days. Or some soil is suspended in one mL sterile normal saline and mixed by vortex, place undisturbed for 5 minutes. Soil settles down and upper liquid phase is spread on SDA followed by incubation.

Silica powder can be used as storage material instead of soil using same method.

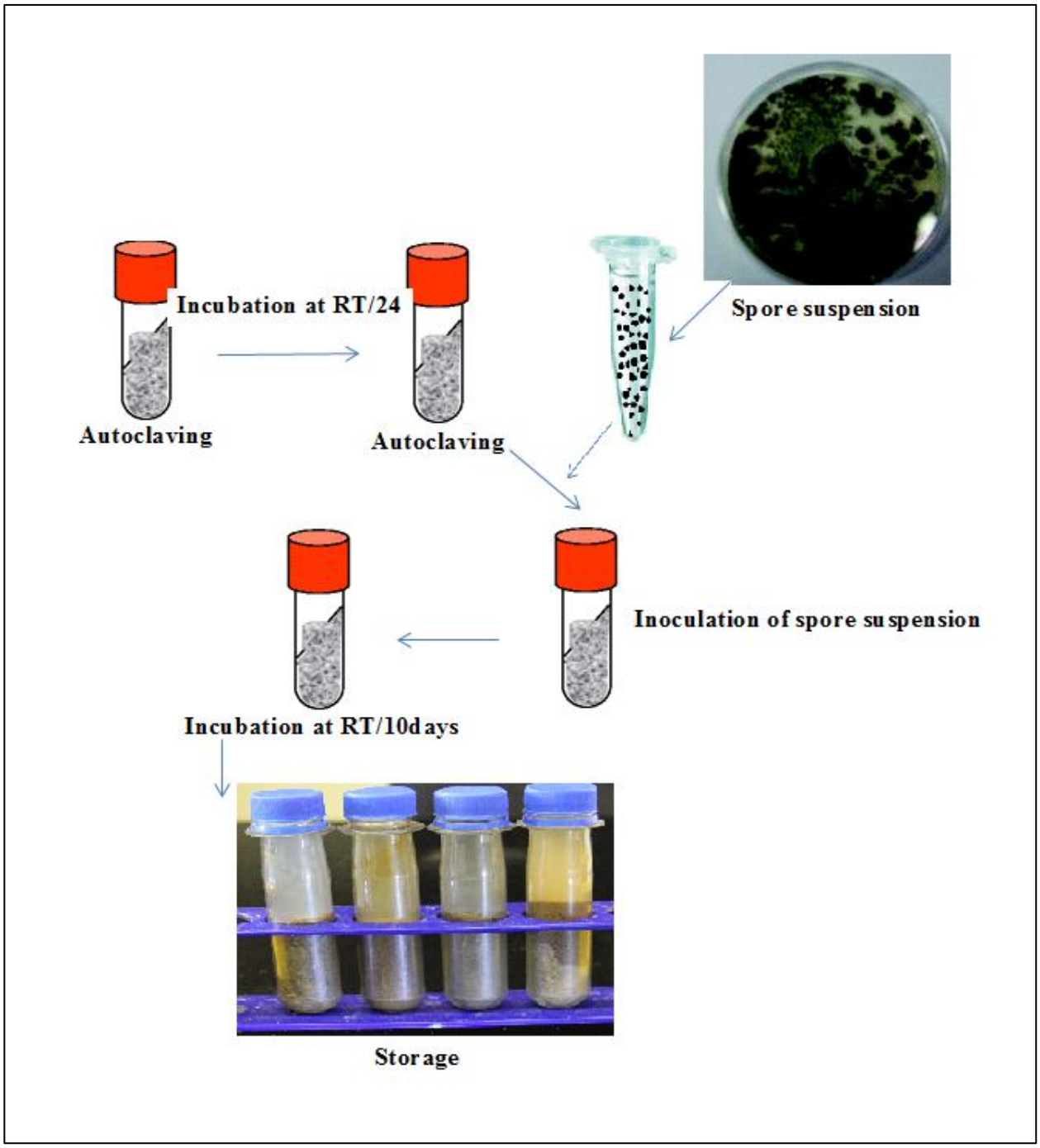


Figure 16.2: Preservation of filamentous fungi in soil

5. Micro Bank

Micro-bank is commercially available. It contains cryo-vials for the storage of microbial strains. These cryovials of 1.5mL contains micro-beads suspended in a buffer. These are charged polystyrene beads.

Methods: Pure culture is transferred into cryovial and placed at room temperature for 2 minutes. After that, buffer is completely removed. During this period microbial cells become adhere to beads. Vials are stored at -20 or -80°C. It is long term preservation methods and microbes are preserved for several years.

Revival: For revival, one bead in removed from a vial and rub agar plate followed by incubation at recommended temperature.

Figure 16.3: Cryovial with microbeads (left side) and microbank (Rightside)

Experiment-17

SCREENING OF SOIL FUNGI FOR ANTIMICROBIAL PRODUCTION

Antibiotic is a drug used to treat infections caused by bacteria that can cause illness to humans and animals. To date, over 100 different antibiotics are available to cure minor and life-threatening infections. Antibiotic resistance occurs when the effectiveness of drugs and chemicals designated to cure diseases are reduced. Scientists are continuously searching for novel antibiotic producing microbes because drug resistant strains of pathogen emerge more quickly than the rate of discovery of new drugs and antibiotics. Consequently, a number of antibiotics that can fight against pathogenic bacteria had been discovered. It is important to discover new antibiotics as the emergence of new diseases and reemergence of multiple-antibiotic resistant pathogens have caused current antibiotics ineffective. There are many sources where antibiotics can be discovered, however, soil is the most important source for the discovery of novel antibiotic producing microorganisms. Soil microorganisms have continually been screened for their useful biological active metabolites, such as antibiotics since long ago.

In current experiment we will screen the soil borne fungi for antimicrobial production. We will evaluate the antibacterial activity of soil fungi against test bacteria;

- *Staphylococcus aureus*
- *Bacillus cereus*
- *Pseudomonas aeruginosa*
- *Escherichia coli*

Soil sampling, preparation and plating

1. Soil samples will be collected
2. Soil (1g) samples will be dissolved in 9 mL of sterile Phosphate Buffer Saline (PBS) or normal saline to make 10% soil suspension.

3. Soil suspension will be allowed to stand undisturbed for five minutes at room temperature
4. The upper liquid phase from soil suspension will be spread over four SDA (Sabouraud dextrose agar) plates. The plates will be incubated at 28°C for 5 days
5. The soil fungi will appear on SDA plates. It will be a mixed growth with different types of fungi. This **master plate** will be used in next step.

Preliminary screening for antimicrobial producing fungi

1. Preparation of test bacterial culture. We will grow the test bacteria in nutrient broth. Fresh growth (log phase) of bacterial culture will be used.
2. This bacterial culture (1mL) will be added into 10mL soft nutrient agar (Nutrient agar containing 0.75% agar). Mix it well
3. This mixture of bacteria and soft agar will be overlaid on master plate
4. Plate will be incubated at 37°C for 24 hours.
5. After incubation formation of inhibition of zone (ZOI) will be observed. The soil fungi that inhibited the growth of test bacteria by producing inhibition zone will be purified (sub-culturing) by plug inoculation on SDA. This fungus will be identified as per standard procedure (Macroscopic, Microscopic characters and by Molecular technique such as PCR).

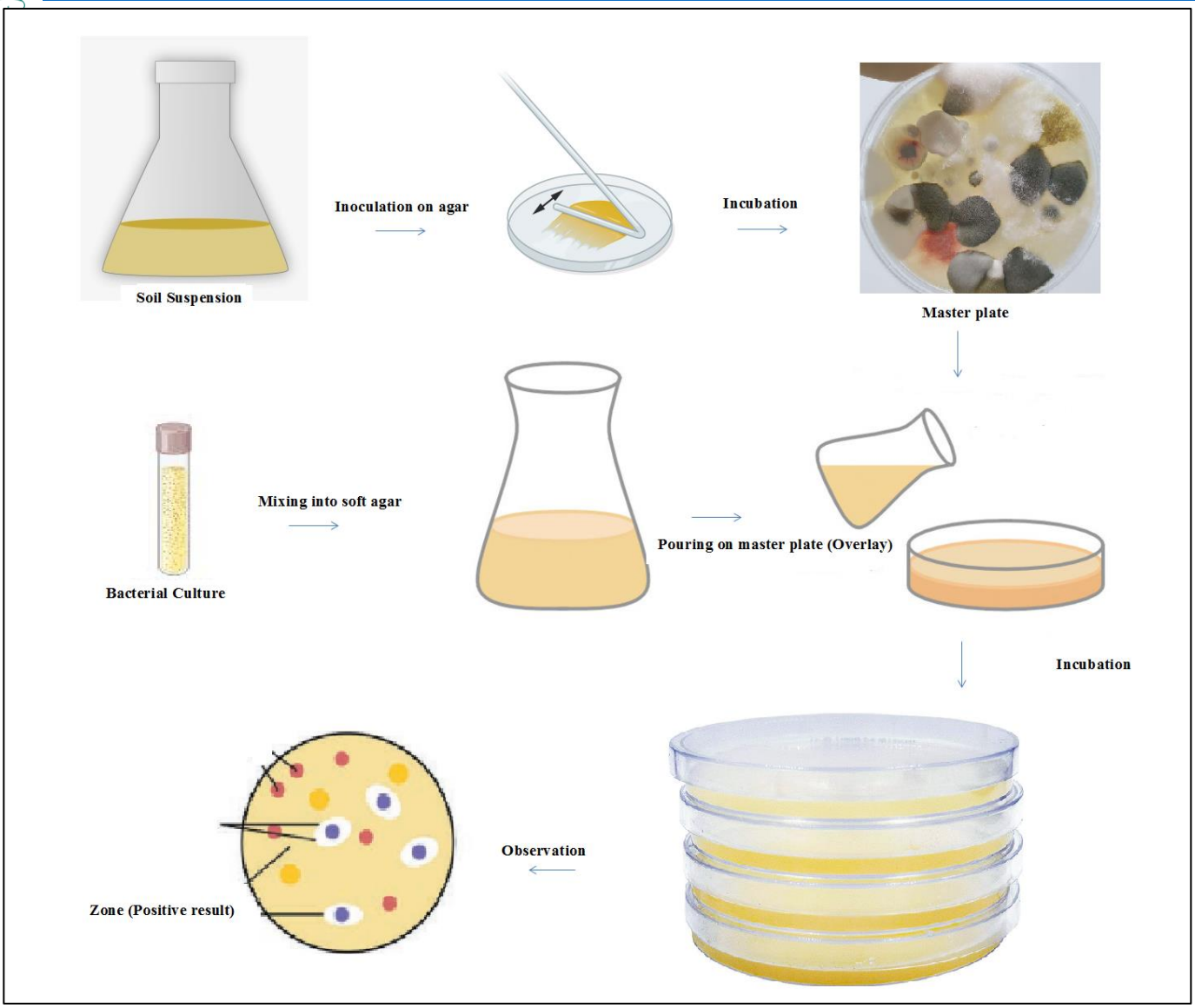


Figure 17.1: Screening for antibiotic producing fungi

Secondary screening for antimicrobial producing fungi

Secondary screening is used for confirmation of antibacterial activity.

1. The purified fungal spores will be inoculated into 100mL Sabouraud dextrose broth (SDB) in 250mL Erlenmeyer flask.
2. The flask will be inoculated at 28ºC for 7 days.
3. After incubation, the broth culture will be centrifuged at 10,000rpm for 15 minutes. The supernatant will be collected
4. The supernatant will be filtrated via 0.25um membrane filter (Syringe filter)

5. This filtrate will be used as crude antimicrobial in further testing
6. The presence of antimicrobial activity will be tested by **well diffusion** of **disc diffusion** method as mentioned in previous classes.

a. Disc Diffusion Method

Standardization of bacterial inoculum

In this method, cell suspension of tested bacteria is prepared from purified fresh growth (24 hours) in sterile normal saline. This suspension is compared with a standard solution (0.5 MacFarland solution). Turbidity is matched, if bacterial suspension is less turbid then more bacterial colony is transferred to cell suspension and vice versa until turbidity of standard solution and bacterial cell suspension exactly becomes the same.

In second method we can take the optical density of bacterial cell suspension at 600nm wavelength using spectrophotometer or ELISA reader. The OD value must be between 0.085-0.1 at 600nm.

Preparation of discs

If we want to choose disc diffusion method for secondary screening, we have to prepare discs of cell free fungal supernatant (CFS). A disc of 7mm will be cut from whatman filter paper with punching machine. These discs will be sterilized by placing in petri-plates using hot air oven (Dry heat sterilization 180ºC/30minutes).

5. Make a uniform bacterial lawn on Muller Hinton agar by swabbing
6. Apply discs with forceps or disc dispenser
7. Incubate at 37°C for 24 hours

Observation

Observe the plates for the presence or absence of zone of inhibition (Area around the disc where no growth is present. Presence of zone of inhibition indicates that the tested fungal cell free supernatant (CFS) have antibacterial activity.

b. Well Diffusion Method

Principle is the same as for disc diffusion. Drug diffuses into medium. But instead of disc, CFS (cell free supernatant) of fungi is poured into wells made in Muller hinton agar plate. This test is carried out as;

1. Standardization of bacterial inoculum (mentioned in disc diffusion)
2. Make wells with the help of well borer. Three wells on 6cm diameter plate and 5 wells may be prepared on 9cm diameter Muller hinton agar plate. Well base is sealed by molten agar so that when we pour CFS solution, it does not seep under the agar. It must be diffused into medium that's why we seal the base of agar with minute (10µL) of molten agar.
3. Make a uniform bacterial lawn on Muller Hinton agar by swabbing
6. Pour CFS into wells so that well is completely fills.
7. Incubate at 37°C for 24 hours

Observation

Observe the plates for the presence or absence of zone of inhibition (Area around the well where no growth is present). Presence of zone of inhibition indicates that the tested fungal cell free supernatant (CFS) have antibacterial activity.

The CFS having antibacterial activity in secondary testing can further is used for MIC determination. The active components in cell free supernatant can be detected by Fourier transmission Infra-red spectrometry (FTIR)

Experiment-18

SCREENING OF SOIL FUNGI FOR PHYTASE PRODUCTION

The major portion of animal feed contains (0.83-9.15%) of phosphorus in form of phytate or Phytic acid. Phytic acid is considered as anti-nutritional, due to its ability to chelate with divalent cations such as calcium, magnesium, iron and zinc. It forms insoluble phytate+metal complexes and inhibits the action of other enzymes (amylase, cellulose and phosphatase) in GIT system and reduces the digestibility of nutrition.

Phytase (myo-inositol-hexa kis phosphatephos phohydrolase EC 3.1.3.8) are the enzymes that hydrolize phytic acid, liberating phosphorous. Phytase enzyme can be found in animals, plants, bacteria, yeast and filamentous fungi. However, among microorganisms, phytase activity has been frequently detected in fungi, particularly in *Aspergillus* species.

In current experiment, we will screen the fungi isolated from soil for phytase production. For phytase enzyme screening we required a substrate and detection system. So that enzyme action on substrate can be detected or measured.

Requirements

Substrate (Phytase or phytic acid): We will use phytase screening medium which have phytase in form of sodium phytate. Phytase screening medium contains (PSM) was prepared by mixing: 15g glucose, 2g NH_4NO_3, 0.5g KCl, 0.5g $MgSO_4.7H_2O$, 0.3g $MnSO_4.7H_2O$, 0.001g $FeSO_4.7H_2O$ and 20g agar in 1liter of distilled water (pH 5.6). Medium will be autoclaved at 121°C and 15lbs pressure for 15mins. Filter sterilized sodium phytate (2%) was added in PSM as substrate. After that pour the medium into sterile petri-plates

Staining solution: Two solutions are used for confirmation of phytate hydrosis by phytase enzyme. These solutions are used separately one after other.

First solution: First staining solution is cobalt chloride (2%).

Second Solution: Second solution is a mixture of two solutions in equal volumes (1:1). These two solutions are ammonium molybdate (6.25%) and ammonium vanadate (0.42%).

Procedure

Isolation of Phytase producing Fungi from Soil

1. Phytase screening agar will be prepared in petri-plates
2. Soil suspension (10%) will be prepared in normal saline. It will be allowed to stand at room temperature (RT) for 5-10 minutes.
3. Upper liquid phase from soil suspension (100µL) will be inoculated on PSM by spreading
4. Plates will be incubated at 28°C for 3 days.
5. Colonies showing zone of hydrolysis will be selected.
6. These colonies will be purified on phytase screening medium. These colonies (growth) on PSM will be used for confirmation of phytase production. Again, zone of hydrolysis will appear around colonies. This zone of hydrolysis may be due to two reasons:
    a. Fungi have ability to produce phytase enzyme
    b. Acid producing fungi have the ability to solubilize sodium phytate causing the formation of clear zone

So, to rule out that zone of hydrolysis is due acid production, we will use a double staining technique.

Confirmation of Phytase producing fungi

a. The 3 days old purified growth on PSM
b. Pour cobalt chloride (2%) solution on plate with purified fungal growth on PSM
c. Incubate the plate for 5mins at RT
d. Decanted the cobalt chloride solution
e. Pour the second solution (mixture of (6.25%) ammonium molybdate and (0.42%) ammonium vanadate (1:1) was flooded on culture plates
f. Re-incubated the plates for 5 minutes at RT

Observations

Isolates will be confirmed as positive for phytase if the zone of hydrolysis retains after double staining. These salts of staining solutions re-precipitates the acid solubilize phytate and rule out false positive results.

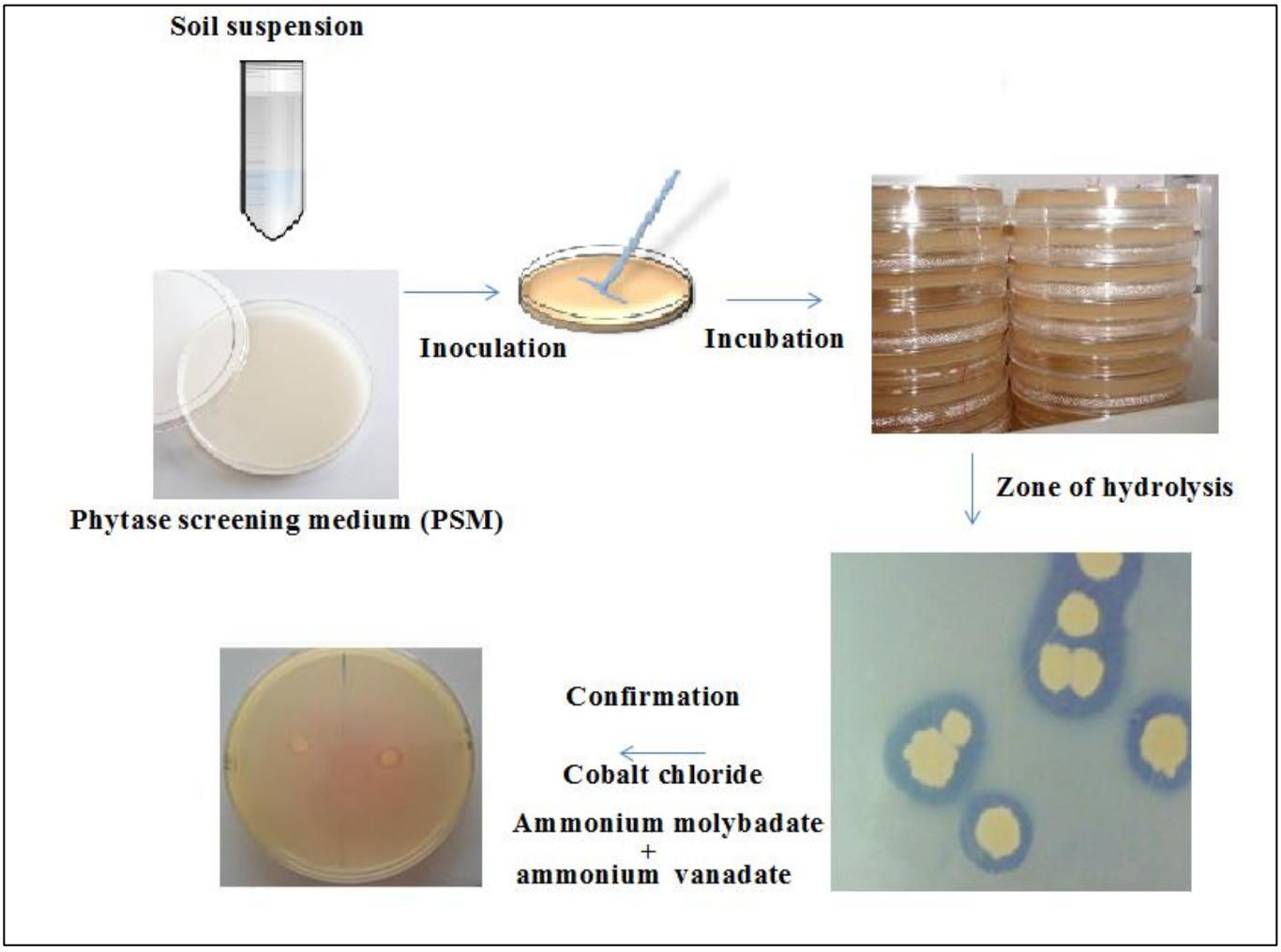


Figure 18.1: Phytase producing organism on phytase screening medium (PSM)

Experiment-19

SCREENING OF SOIL FUNGI FOR AMYLASE PRODUCTION

Amylases are among the most important enzymes which hydrolyze starch and convert it into simple sugar like glucose. These are used in food industry, textile industry, paper industry biofuel production and as food aid.

Amylases can be obtained from several sources, such as plants, animals and microorganisms. Major advantage of using microorganisms for the production of amylases is the economical bulk production. Amylase can be obtained from several fungi, yeast, bacteria and actinomycetes; however, especially fungi, have gained much attention because of the availability and high productivity of fungi,

In current experiment, we will screen the fungi isolated from soil for amylase production. For amylase enzyme screening we required a substrate and detection system. So that enzyme action on substrate can be detected or measured.

Requirements

Substrate (Starch)

Starch agar contains Ingredients (g / Litre); Meat Extract 3.000, Peptic digest of animal tissue 5.000, Starch (soluble) 2.000, Agar 15.000 Final pH (5.6). Medium will be prepared according to recommendations. Medium will be autoclaved at 121°C and 15lbs pressure for 15mins. After that pour the medium into sterile petri-plates

Iodine solution

It will be used for presence or absence of amylase enzyme. It reacts with starch and gives blue/dark purple (blackish) color product. So, if amylase hydrolyzes the starch, there will be no such reaction and no color will appear (positive result for amylase production). On other hand if amylase is not present or produced by fungi, starch will not be hydrolyzed and blue color will form (negative result for amylase production).

Procedure

Isolation of amylase producing Fungi from Soil

1. Starch agar will be prepared in petri-plates
2. Soil suspension (10%) will be prepared in normal saline. It will be allowed to stand at room temperature (RT) for 5-10 minutes.
3. Upper liquid phase from soil suspension (100µL) will be inoculated on starch agar by spreading
4. Plates will be incubated at 28°C for 3 days.
5. Pour iodine solution on starch agar plate with fungal growth
6. Decant the excessive iodine

Observations

Colonies showing zone of hydrolysis will be selected as these are positive for amylase production.

These colonies will be purified and identified as per standard procedures.

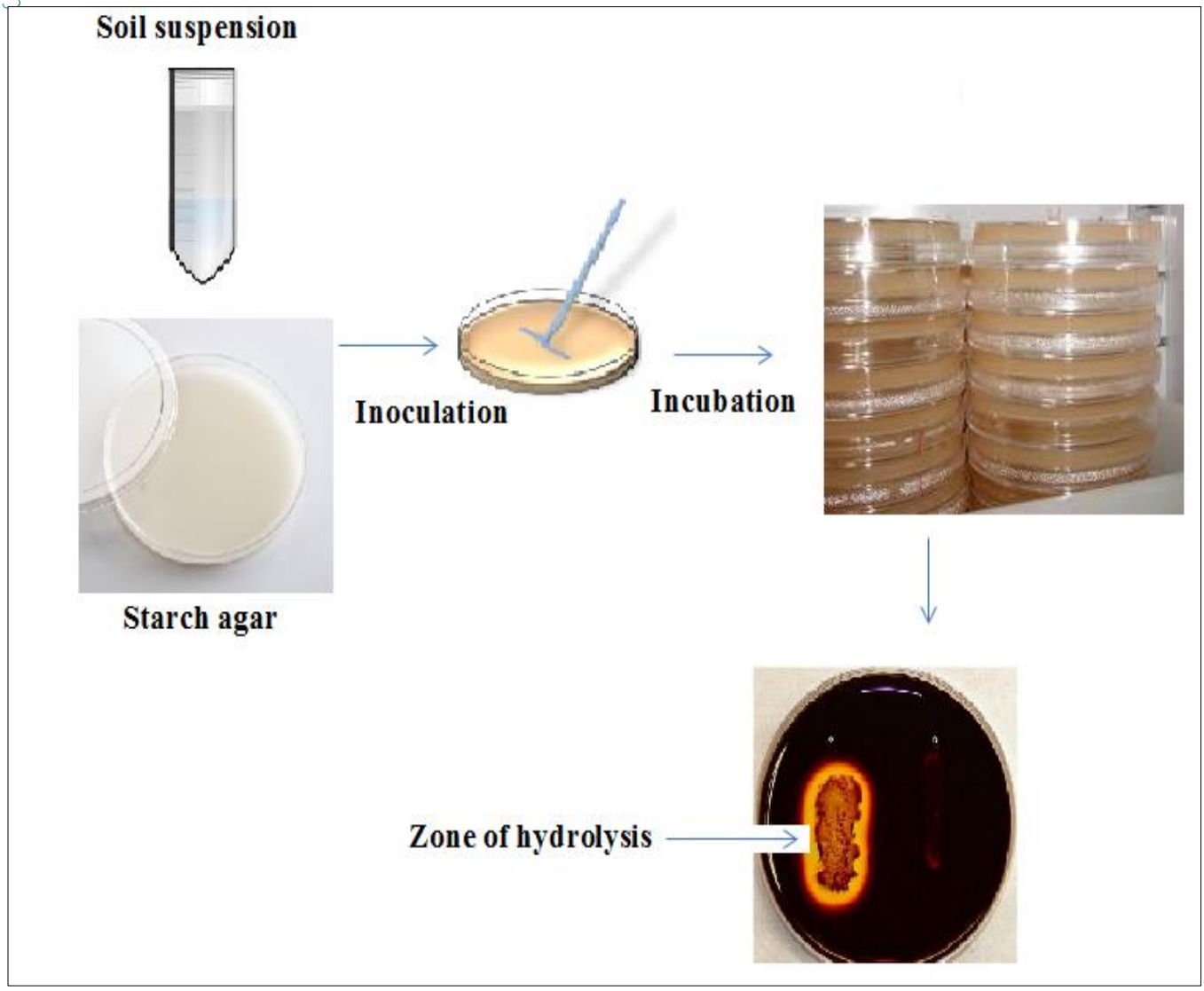


Figure 19.1: Amylase producing fungi on starch agar

Experiment-20

EFFECT OF PHYSICAL FACTORS ON FUNGAL GROWTH TEMPERATURE AND pH

Fungal growth is dependent upon chemical and physical factors. Chemical factors are actually the nutrients and physical factors include temperature, pH and moisture. Every fungus has optimal conditions at which metabolic activity of fungi is highest. In current experiment, we will evaluate the effect of temperature, pH on growth of *Aspergillus flavus*. *A. flavus* is a saprophytic and pathogenic fungus. It has cosmopolitan distribution. The name flavus means yellow in Latin language. Filamentous fungi are able to grow on all types of food especially on a wide variety of agricultural products worldwide. It produces significant quantities of mycotoxins called as aflatoxins. In immune-compromised individuals it can cause aspergillosis.

Requirements

- Sabouraud dextrose broth (SDB)
- Purified culture of *A. flavus*
- Neubar chamber
- Microscope
- Normal saline
- Micropipette and tips
- pH meter
- Incubator

For growth optimization we will use three different pH values (5.6, 7.2 and 9.0) and three different temperatures (25, 37 and 42°C). Prepare the standard (10^6 spores/mL) spore suspension in normal saline using Neubauer chamber as we discussed in previous experiments.

A. Procedure

1. Prepare SDB in a three conical flask of 250mL. Each flask has 100mL SDB.
2. Label these flasks with pH-5.6, pH-7.2 and pH-9.0.

3. Adjust pH of the SDB that is mentioned on flask
4. Sterilize medium by autoclaving (121°C, 15lbs for 15 minutes)
5. Inoculate each flask with one mL spore suspension of *A. flavus* (10^6 spores)
6. Incubate inoculated flasks at 25°C for 5 days with constant shaking at 150 RPM (revolutions per minute)

Use the same step 1-5 for temperature 37 and 42°C. Just change incubation temperature for three pH values.

B. Biomass Determination

1. Biomass is the mycelial growth of *A. flavus* in flask. After incubation determine the biomass of produced by *Aspergillus flavus* in each flask.
2. Take Whatman filter paper and dry in in oven
3. Place the filter paper on weighing balance to determine its weight in grams (W_0)
4. Place the filter paper on funnel and prepare setup for filtration
5. Now mix the fungal growth in flask and pour it onto filter paper
6. Allow the filtration to run. During this the whole medium flow down and mycelia will remain on filter paper
7. Once filtration is completed, take out filter paper with mycelia and properly squeeze it
8. Weight the filter paper with mycelia. This will be final weight in grams (W_f)
9. Growth or biomass can be determined by subtracting the weight of filter paper (W_0) from weight of filter paper with mycelia (W_f)

$$\text{Biomass (g)} = (W_f) - (W_0)$$

We will compare the biomass of all flasks; flask showing highest biomass indicates the optimal conditions of pH and temperature for *A. flavus*

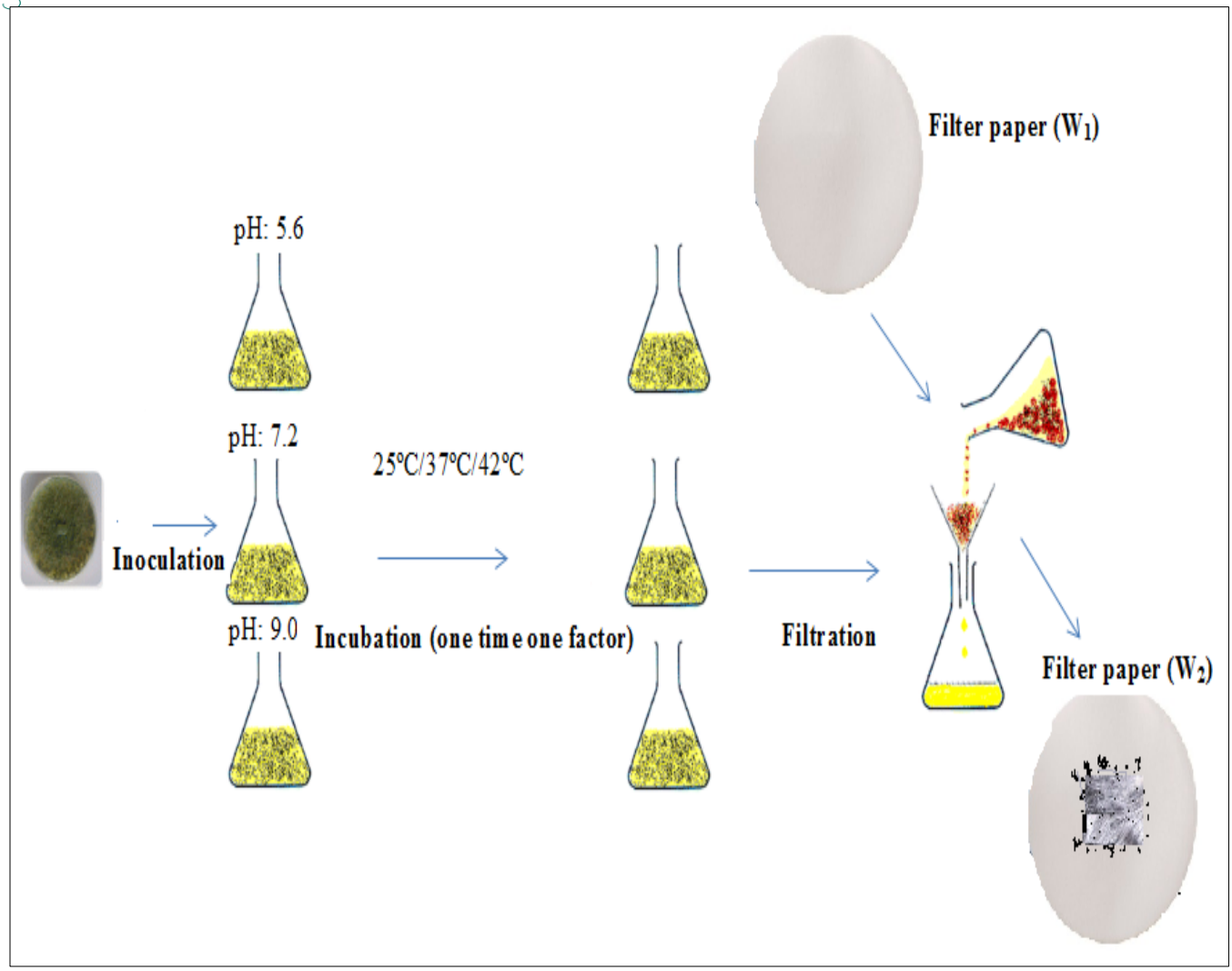


Figure 20.1: Effect of factors on fungal biomass

Author Contributions

Conceptualization; N.S; I.L; S.S validation; K.K; A.A.L, writing—original draft preparation S.S; N.S, I.L, K.K; A.A.L, writing-review and editing S.S; I.L; N.S; S.N and visualization: S.A; M.N.A; U.A; S.N; N.S

Conflicts of Interest

The authors declare no conflict of interest.