# LABORATORY PRACTICAL MODULE

**COURSE: MICROBIOLOGY**
**COURSE CODE: MAB 1201**

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## FOREWORD

This Microbiology Practicum Manual is a guidebook for working in the Laboratory for students who take the General Microbiology practical. The main objective is to introduce the basic concepts of microbiology, laboratory techniques, and procedures.

Therefore, mastering these things well will significantly help students in studying microbiology, and they will be able to explore various fields of applied microbiology. The availability of books that are thought of and the readiness of the assistants/lecturers are very supportive of achieving the intended goal. Likewise, laboratory advice and infrastructure must be ready to help achieve goals.

On this occasion, we would like to thank all parties who have helped with the material's content and helped in the technical implementation until the realisation of this book. Suggestions and criticisms from users of this book are highly expected so that subsequent publications can be added/improved.

Jember, 25 July 2023

The Team of Course

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# I. EQUIPMENT AND MEDIA STERILIZATION

Cultivation of microbes in the laboratory requires growth media. This media contains the nutrients needed for microbial growth. The type of media used is adjusted to the type of microbe that will be grown, for example, Nutrient Agar media to grow bacteria and Potato Dextrose Agar media to grow mold. The growth medium can be in the form of solid, semi-solid or liquid media (broth). Solid or semi-solid media is liquid media (material according to the composition of the media) which is added with a gelling agent in the form of agar.

In addition to media, microbial cultivation requires equipment (tools) for microbial growth. Some common equipment used is Erlenmeyer flasks, test tubes, Petri dishes and inoculation loop. Erlenmeyer flasks, test tubes, and Petri dishes are used for microbial growth media, while an inoculation loop transfers microbial cultures.

The equipment and growth media used must be sterile (free from microbes) so that the microbes that grow are actually the expected (target) microbes and not microbes from contamination (unexpected). Sterilization is an effort to liberate the tools or materials from all kinds of life forms, especially microbes. Sterilization can be carried out by physical methods such as heat and filtration or chemical methods (Figure 1). The sterilization method is adjusted to the nature of the materials and tools being sterilized.

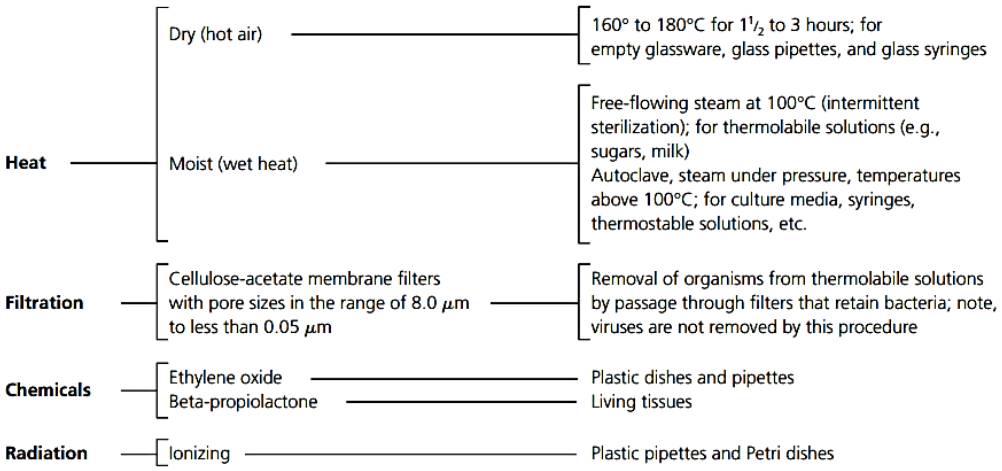



Figure 1. Sterilization Method for Tools and Media (Cappuccino & Welsh, 2019)

**Practical Objectives**

a. Students can sterilize tools.
b. Students can sterilize media.

**Method**

**Tools and materials**

a. Inoculation loop (1)
b. Petri dishes (4 pieces)
c. 100 mL Beaker Glass (1 piece)

d. 20 mL Volumetric flask (1 piece)
e. 1 mL Measuring pipettes (2)
f. Test Tubes (10)
g. Cotton
h. Oven
i. Autoclave
j. Scales
k. Nutrient Broth
l. Bacto agar

**Procedures**

1. Sterilization of tools

1.1 Sterilization of inoculation loop

Heat the inoculation loop until reddish appears in the flame of a Bunsen burner.

1.2 Sterilization of Petri dishes

a. Wrap 4 Petri dishes each with aluminum foil or doorslag paper.

b. Carry out sterilization using two sterilization methods.

c. A total of two Petri dishes were sterilized using an oven at 160°C for 2 hours (Appendix 1. Oven Operational Procedures) and two Petri dishes were sterilized in an autoclave at 121°C 1 atm for 15 minutes (Appendix 2. Autoclave Operational Procedures)

1.3 Sterilization of 1 mL glass pipettes.

a. Wrap two glass pipettes each with aluminum foil or doorslag paper.

b. Perform sterilization in two ways. One pipette is sterilized using an oven at 160°C for 2 hours and one pipette is sterilized using an autoclave at 121°C 1 atm for 15 minutes.

2. Media Sterilization

2.1 Preparation and Sterilization of Nutrient Broth Media (8 g/L)

a. Weigh 0.4 g of nutrient broth using a scale, put it in a 100 mL beaker and add 40 mL of distilled water, then heat until dissolved using a hot plate and add water to a volume of 50 mL.

b. Put 10 mL into 5 test tubes using a measuring/ volumetric flask and cover with cotton.

c. Next, sterilized using an autoclave.

2.2 Preparation and Sterilization of Nutrient Agar Media

a. Weigh 0.5 g of Nutrient Broth and 0.75 g of Bacto Agar using an electric scale, put it in a 100 mL Beaker and add 40 mL of distilled water.

b. Next, heat the solution until it dissolves using a hot plate and add distilled water to a volume of 50 mL.

c. Put 10 mL of media into 5 test tubes using a measuring/ volumetric flask and cover with cotton. Next, sterilized using an autoclave.

## II. ASEPTIC TECHNIQUE

The aseptic technique is a series of routine activities taken to prevent cultures, sterile media and other solutions from contamination by unwanted microbes. Research activities in the field of microbiology have the main aim of growing microbes, both prokaryotes and eukaryotes, that are free from contaminants, so aseptic techniques play an important role. The aseptic technique that is often used in microbiology is the aseptic transfer technique. The aseptic transfer technique is a method or technique for moving or transferring bacterial/ microbial cultures from one place to another aseptically so that the contamination of the culture by other microbes does not occur. This aseptic transfer technique is very essential and the key to the success of microbial procedures that must be known by someone who wants to carry out a microbiological analysis.

**Practical Objectives**

Students can carry out laboratory work aseptically in microbiology.

**Method**

**Tools and materials**

a. Bunsen burner
b. Laminar Air Flow
c. Test tube
d. Petri Dish
e. Cotton
f. Inoculation loop
g. 70% alcohol solution

**Procedures**

1. Some examples of aseptic techniques include the following:
a. Clean the workbench with disinfectant before and after work.
b. Limit the exposure duration of the culture or medium to the air.
c. Keep the Petri dish closed.
d. Effectively sterilizes inoculation loops and other equipment that is being used for culture transfer.
e. Avoid talking while working.

2. Some of the equipment needed to maintain aseptic conditions are as follows:
a. Bunsen burner.
1) The easiest way to create a relatively sterile environment in a laboratory is to use a Bunsen burner.
2) The role of the Bunsen burner in aseptic technique is to reduce airborne contaminants suspended in dust particles.
3) Bunsen burners are best for sterilizing loops (inoculation loop), mouths of test tubes and surfaces of Petri dishes.
b. Laminar Air Flow.
1) Work carried out in laminar air flow will provide a clean workspace.

2) Filtered air eliminates air contaminants entering the work area (Figure 2).

Figure 2. Laminar Air Flow

1. several rules must be known and fulfilled in this aseptic transfer technique, as summarized below:

a. Before Implementation:
1) Remove all unnecessary items from the desk and workspace.
2) Wear clean and hygienic clothing or a laboratory coat before entering the laboratory.
3) It is recommended to wear a clean and hygienic mask.
4) Wear a clean and hygienic hair cover.
5) Never place tubes and other laboratory equipment outside the laboratory.
b. Before and After Implementation:
1) Wash your hands thoroughly and use antiseptic.
2) Sanitize and disinfect the workspace (laboratory and surroundings) with adequate disinfectants, including Laminar Air Flow and incubators.
3) Sterilize all tools and materials before use.
c. When Carrying Out Culture:
1) Don't talk.
2) Work near a fire (Bunsen burner) and in Laminar Air Flow.
3) Open the tube or cup over the fire and keep it away from your nose and mouth.
4) Try not to place the lid (cotton cover) of the test tube on the floor/table base or laminar.
5) Tilt the lid of the Petri dish to be opened as a barrier between the culture and your mouth and nose.
6) Don't open the lid of the Petri dish too wide or for too long. Work quickly.
d. After Culture Implementation:
1) Immediately close all tubes or cups that are still open.
2) Get rid of all equipment or leftover materials that are no longer used.
3) Clean and dry immediately any media spills.
4) Sanitize and re-disinfect the work area (your laboratory).
5) Take off your work clothes and laboratory coat before leaving your work area.

## III. CULTURE TRANSFER TECHNIQUES

The process of transferring microbes from one medium to another is called subculture. This technique is carried out routinely for the preparation and maintenance of microbial culture stocks as well as for microbiological analysis needs.

Microbes are always present in the environment, both in the air, equipment and where we work in the laboratory. The presence of these microbes can potentially be a contaminant, so proper aseptic technique is very necessary during the subculture process. Several important steps need to be considered in the subculture process (Figure 3), as follows:

1. Label the tube or Petri dish that you will inoculate with the name of the organism, date and your initials before you subculture.
2. Hold the culture stock tube and the tube you are going to inoculate in the palm of your hand, use your thumb to hold and separate the two tubes so that they form a "V" shape in your hand.
3. Sterilize the inoculation loop by holding it in a micro incinerator or the hottest part of a Bunsen burner until the wire turns red hot. Once the inoculation loop is sterilized, hold it in the circle of your hand and let it cool for 10-20 seconds. Never place it on your work desk.
4. Open the cap of each tube by holding the first cap on your little finger and the second cap on your next finger. Hold the cap on the hand holding the inoculation needle. Never place the tube cap on the work table as this will compromise the aseptic procedure.
5. After removing the tube cap, sterilize the neck and mouth of the tube by passing it through an incinerator or Bunsen burner flame 2-3 times quickly. Cool the inoculation needle by touching the inside wall of the tube before removing a small amount of the inoculum sample. You can test whether the inoculation needle is hot or not on the empty part of the media.
6. Depending on the culture medium, an inoculation loop or needle can be used to obtain inoculum from the agar culture slant, but be careful when touching the surface of the solid medium in areas showing growth so as not to gouge the agar.

a. Transfer of culture from slant media to liquid media: take the culture from slant media then gently shake the inoculation loop or needle into the new medium to release the microbes.

b. Transferring liquid culture to a slant medium: obtain a full circle of liquid culture and place it on the bottom of the slant medium. Lightly drag the circle onto the surface of the hardened agar in a straight line or zigzag direction from the bottom of the medium to the top.

c. Transfer the culture from the slant media to the agar medium using the puncture method: take the inoculum from the slant medium, insert the inoculation needle into the medium in the new tube in a straight line and quickly pull along the insertion line.

7. After the inoculation process, re-sterilize all equipment including the inoculation loop, test tube necks and Petri dishes.
8. Replace the test tube lid according to its original position.
9. Re-sterilization of the inoculation loop or needle is carried out to remove remaining microbes.

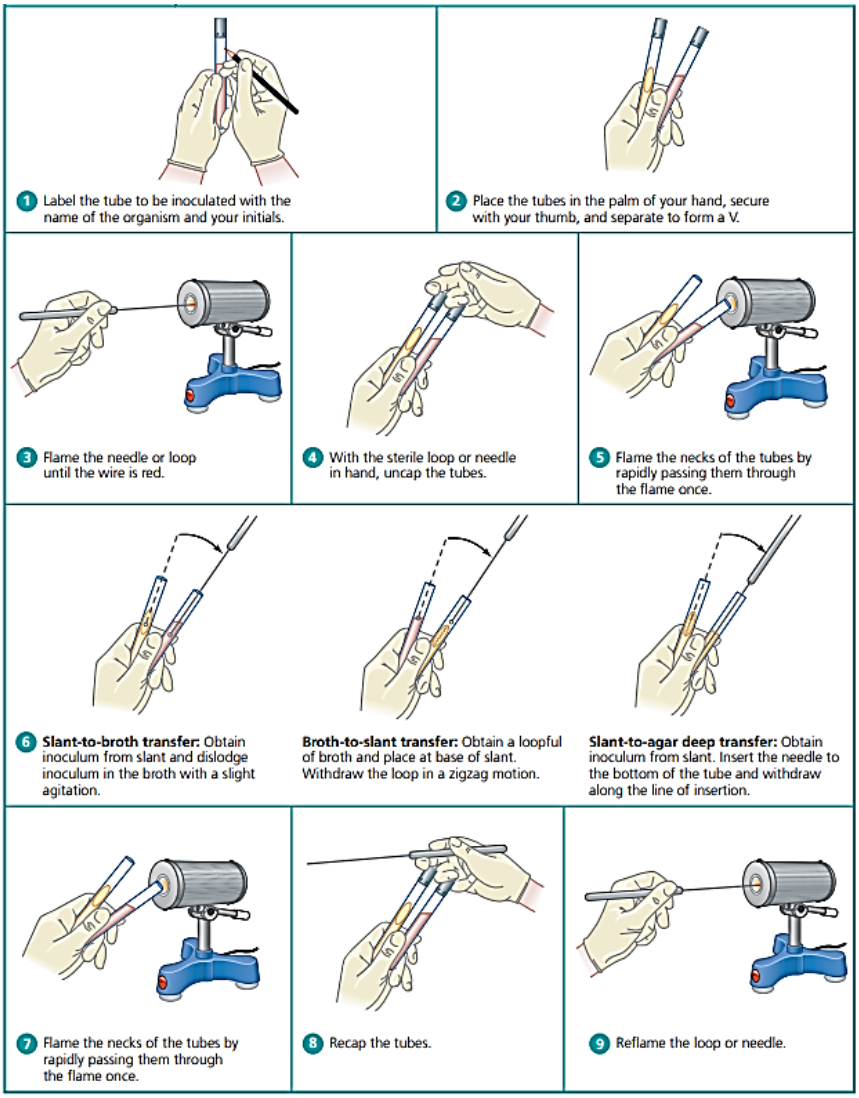



Figure 3. Culture transfer technique (subculture) (Cappuccino & Welsh, 2019)

**Practical Objectives**

Students can carry out culture transfers by applying aseptic microbiology techniques.

**Method**

**Tools and materials**

a. Laminar air flow
b. Alcohol 70%
c. Rolled tissue
d. Bunsen burner
e. Inoculation loop
f. Volumetric pipette
g. Nutrient Agar (NA) media, slant in a test tube

h. Nutrient Agar (NA) media in Petri dishes
i. Nutrient Broth (NB) media in a test tube
j. Bacillus sp culture. on the slant agar media
k. Bacillus sp culture. on NB media

**Procedures**

1. Transferring the culture from liquid media to liquid media
a. Label the new media that you will inoculate.
b. Take 100 µL culture of Bacillus sp. in NB media and inoculate into new NB media in a test tube, homogenize.
c. Carry out microbiological aseptic activities.
2. Transferring the culture from liquid media to solid media.
a. Label the new media that you will inoculate.
b. Take the liquid culture (100 µL) of Bacillus sp. and inoculate it into NA media in a Petri dish using the spread plate method.
c. Carry out microbiological aseptic activities.
3. Transferring the culture from solid media to solid media.
a. Label the new media that you will inoculate.
b. Take a culture of Bacillus sp. on slanted NA media and inoculate on new slanted NA media using the zig-zag streak plate method.
c. Carry out microbiological aseptic activities.

## IV. PURE CULTURE ISOLATION TECHNIQUES

Microbial populations in nature consist of various species that live together. We can separate the population into pure cultures in the laboratory. This pure culture only contains one type of organism and is not mixed with others. Pure cultures are prepared to study the morphology and biochemical properties of these microbes.

The method used to obtain pure culture is by isolation. The isolation technique determines the success of obtaining a pure culture that is separate from other cultures. There are two ways of isolation to obtain pure culture, namely:

1. Streak plate method

In principle, this method is to scratch a suspension of material containing microbes on the surface of the medium. The technique can use the 4 quadrant technique. After incubation, the scratch marks will grow into separate colonies (Figure 4).

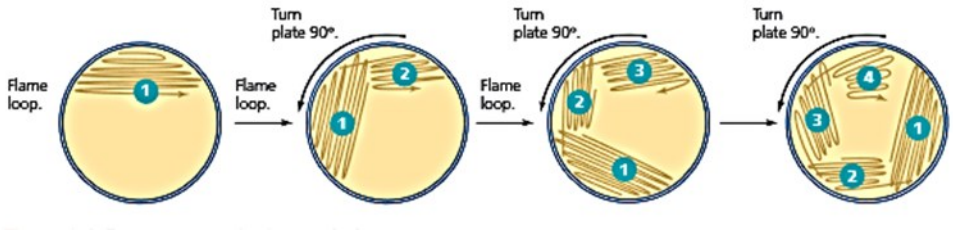



Figure 4. Four quadrant technique in the scratch method (Cappuccino & Welsh, 2019)

2. Spread Method

The spread method consists of two techniques, i.e. spread plate and pour plate.

a. Spread plate is a spreading method by inoculating isolates on solid agar media in Petri dishes and spreading using a sterile L-shaped glass spreader.

b. Pour plate is a spreading method by inoculating the isolate on solid agar media which melts at a temperature of around 40°C-50°C, and then is poured into a sterile Petri dish.

The difference between these two is that the microbes that grow using the spread plate technique are aerobic, whereas with the pour plate method, the growth can be aerobic (on the surface of the agar) and facultative anaerobic/anaerobic (below the surface of the agar).

**Practical Objectives**

Students can practice pure culture isolation techniques.

**Method**

**Tools and materials**

a. Sterile Petri dishes
b. L-shaped Bent glass
c. Bunsen burner
d. Inoculation loop
e. Rolled tissue
f. Marker pen

g. Vortex
h. Micropipette
i. Yellow/blue tip
j. Mixed culture of isolated microbes
k. 10 mL solid NA media in a test tube
l. 10 mL solid NA media in a Petri dish
m. 70% alcohol solution
n. 5 mL sterile distilled water in a test tube

**Procedures**

1. Pure culture isolation technique using the four quadrant scratch method.
a. Select a single colony in the mixed culture to isolate.
b. Aseptically, take the colony using an inoculation loop and transfer it to NA media in a Petri dish with the four-quadrant technique (Figure 1).
c. The first step was carried out by dividing four parts of NA media in a Petri dish as shown using markers (1, 2, 3, and 4).
d. One colony is taken using an inoculation loop and scratched into area one.
e. Next, the inoculation loop is burned using Bunsen and scratched from area one to two and further scratches are made on area two without touching area one.
f. This process is done successively until the scratches reach area four.
g. Next, incubated in an incubator at 30°C for two days, then colony growth is observed.
h. The indicator of the success of this process is that a single colony is obtained that is separate from the others.
2. Pure culture isolation technique using the spread plate method.
a. One colony is taken using an inoculation loop and placed in 5 mL of sterile distilled water and homogenized using a vortex to obtain a culture suspension.
b. Next, 100 µL of the culture suspension is taken using a micropipette, poured into NA medium in a Petri dish and spread evenly using an L-shaped bent glass.
c. Next, incubated in an incubator at 30°C for two days, then colony growth is observed.
d. The indicator of the success of this process is that a single colony is obtained that is separate from the others.
3. Pure culture isolation technique using the pour plate method.
a. A total of 100 µL of culture suspension (from Step 2) was taken using a micropipette and poured into liquid NA media in a test tube (temperature 40°C-50°C)
b. Then homogenize by rotating the Petri dish to resemble the number eight.
c. Incubate in an incubator at 30°C for two days, then observe the growth of the colony. The indicator of the success of this process is that a single colony is obtained that is separate from the others.

## V. MORPHOLOGICAL CHARACTERIZATION OF BACTERIA

**Macroscopic Morphology of Bacteria**

Bacterial cultures grown in a medium will show different characteristics. The culture characteristics are used to differentiate bacteria. Bergey's Manual of Systematic Bacteriology describes the cultural characteristics of all known bacteria. These characteristics are based on the character of the bacteria on solid nutrient agar media in Petri dishes and tilted media in tubes, on nutrient broth (NB) and nutrient gelatin (NG) media.

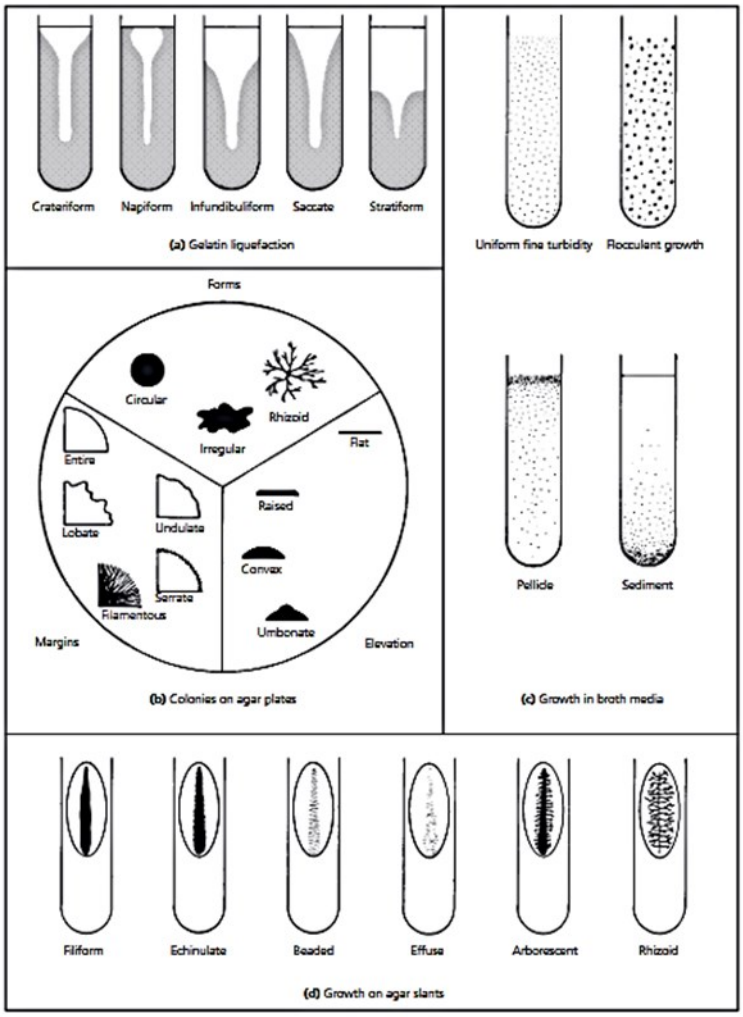



Figure 5. Characteristics of bacterial culture on media (Cappuccino & Welsh, 2019)

The characteristics of the bacterial cultures on various media are presented in Figure 5.

A. Character of bacteria on slant Nutrient Agar media in a test tube

Bacteria are grown on this media by inoculating a loop of bacteria straight on the agar surface. Bacterial characteristics can be observed in this media.

1. Abundance of growth

Growth can be differentiated between none, minor, moderate, and a lot.

2. Pigmentation
Bacteria can produce pigment that can be seen on the bottom surface of the colony. Some bacteria produce extracellular pigments that produce color in the media and some bacteria do not produce pigments.
3. Optical Characteristics
This character is marked by the ability of bacteria to partially transmit. The characteristics are opaque (no partial transmission), translucent, and transparent (full transmission).
4. Shape
Display of colony growth on the inoculated bacterial culture line.
There are filiform (thread-like growth with smooth edges), echinulate (thread-like growth but irregular), beaded (nonconfluent to semi-confluent), effuse (spreading growth), arborescent (tree-like growth), rhizoid (root-like growth).
5. Consistency
The consistency is dry (not moist), buttery (moist and shiny) and mucoid (slimy and shiny).

B. Bacterial characteristics on Nutrient Agar media in Petri dishes.
The character of a colony grown on NA media in a Petri dish can be observed based on the following characteristics:
1. Size: pinpoint, small, moderate, or large.
2. Colony pigmentation: colony color.
3. Form of colonies:
a. Circular: unbroken, peripheral edge.
b. Irregular: notched, peripheral edge.
c. Rhizoid: like roots, spreading growth.
4. Margin: the appearance of the outer edge of the colony
a. Entire: sharply defined, even.
b. Lobate: marked indentations.
c. Undulate: wavy curve.
d. Serrate: thread-like, the ends spread out.
e. Filamentous: threadlike, spreading edge.
5. Elevation: the degree of colony growth above the agar surface.
a. Flat: elevation not discernible.
b. Raised: slightly elevated.
c. Convex: shaped like a dome (dome-shaped elevation).
d. Umbonate: raised, with elevated convex central region.

C. Character of bacteria grown in Nutrient Broth media
1. Uniform fine turbidity: finely dispersed growth throughout.
2. Flocculent: flaky aggregates dispersed throughout.
3. Pellicle: thick, padlike growth on the surface.
4. Sediment: The concentration of growth at the bottom of the broth culture may be granular, scaly, or flocculent.
D. Character of bacteria on Nutrient Gelatin media

The gelatin in the media will be degraded by the gelatinase enzyme to form liquid (liquefaction). The liquefaction pattern of gelatin degradation by bacteria can be distinguished:
1. Crateriform: liquefaction on a surface with a plate shape.
2. Napiform: liquefaction on the surface with round-shaped
3. Infundibuliform: funnel-shaped liquefaction
4. Saccate: elongated, tubular liquefaction
5. Stratiform: the top half of the media undergoes liquefaction

**Practical Objectives**
Students can describe the characteristics of bacterial cultures in the media.

**Method**
**Tools and materials**
a. Stereo microscope
b. Magnifying glass
c. Pure 24-hour cultures of Bacillus cereus, Escherichia coli, Pseudomonas fluorescent, Staphylococcus aureus and Salmonella sp. in slanted NA medium, NA in Petri dishes and NB and NG medium.

**Procedures**
1. Observe the characteristics of the bacterial culture on slanted NA medium, slanted nutrient agar in a Petri dish (can be aided by magnification using a stereo microscope or magnifying glass) and NB medium.
2. Take a photo and write down the characteristics of the bacterial culture (see Figure 1) compared to the characteristics of the bacterial culture in Bergey's Manual of Systematic Bacteriology.

**Microscopic Morphological Characteristics: Simple Staining**
The principle in simple staining is that we stain a bacterial smear with a single reagent, which contrasts the organism and the background differently. Basic staining with positively charged chromogens is preferred because bacterial nucleic acids and specific cell wall components carry negative charges that are highly attractive and bind to cationic chromogens. The purpose of simple staining is to explain the morphology and arrangement of bacterial cells (Figure 6). Methylene blue, crystal violet and carbol fuchsin are the most commonly used basic dyes.

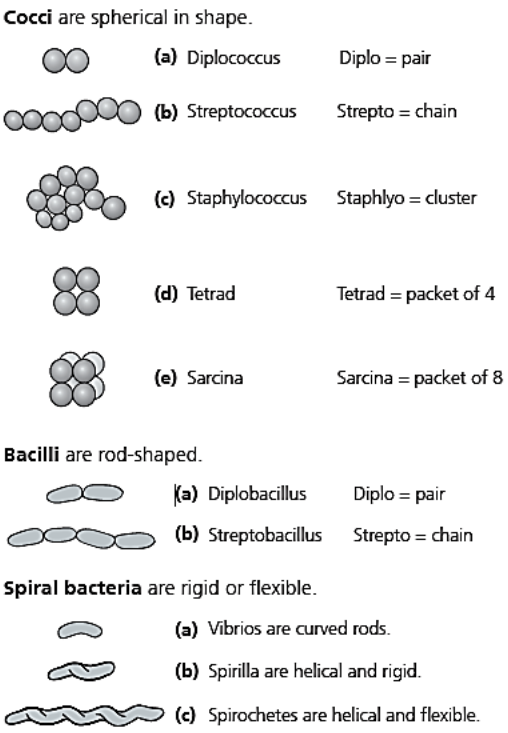



Figure 6. Shape and arrangement of bacteria (Cappuccino & Welsh, 2019)

Simple staining is a relatively quick and practical testing method for the presence, shape determination, or determination of the number of bacteria present in a sample. Generally involving only a single staining step, simple methods are not considered differential or diagnostic and will have limited utility. However, it is a quick procedure to determine whether a clinical sample has the presence of foreign bacterial pathogens.

**Practical Objectives**

Students can carry out simple staining procedures and compare the morphology and arrangement of bacterial cells.

**Method**

**Tools and materials**

a. Bunsen burner
b. Inoculation loop
c. Coloring tray
d. Microscope
e. Lens paper
f. Bibulous paper (highly absorbent)
g. Glass slides
h. 24 hours old of Escherichia coli and Bacillus sp. in slant media
i. 24 hour old Staphylococcus aureus in NB media

j. Alternatively, use a smear prepared in Methylene blue, Crystal violet, and Carbol fuchsin Reagents

**Procedures**

1. Prepare a bacterial smear. Note: All smears must be heated before staining. The Simple Staining in Figure 7 illustrates the following steps: place the slide in a staining tray and drop one of the dyes using the appropriate exposure time: 1) carbol fuchsin, 15 to 30 seconds; 2) crystal violet, 20 to 60 seconds; 3) methylene blue for 1 to 2 minutes.

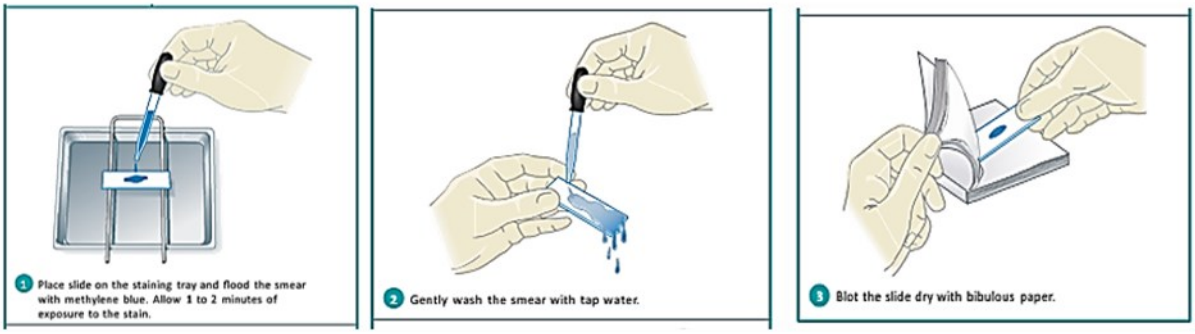



Figure 7. Simple staining procedure (Cappuccino & Welsh, 2019)

2. Wash the dye gently with tap water to remove excess dye. During this step, hold the slide parallel to the water flow; this can reduce the loss of organisms from the preparation.
3. Dry using bibulous paper, but do not clean the slides.
4. Repeat this procedure with the remaining two organisms, using a different dye for each.
5. Examine all colored slides under immersion oil.
6. Draw and explain the morphology of organisms concerning their shape (e.g., bacilli, cocci, or spirilla) and sequences (e.g., chains, groups, or pairs). See the photos in Figure 8.

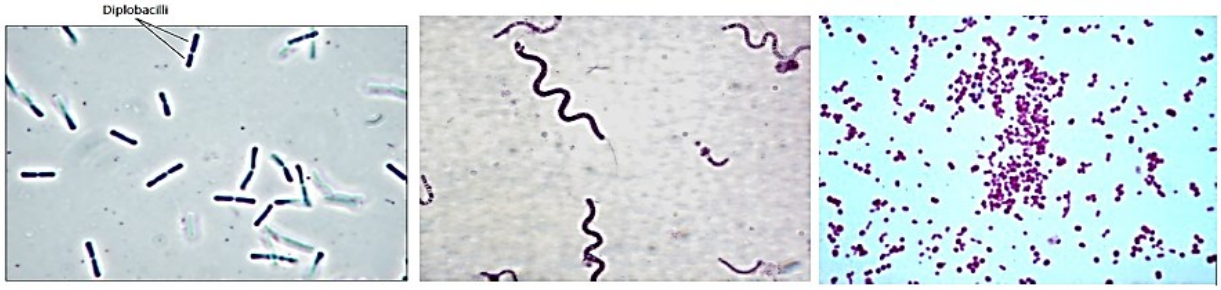



Figure 8. Microscopic image showing bacterial morphology (Cappuccino and Welsh, 2019)
a) Bacillus and dicloxacillin bacteria (rod-shaped) b) Spirilla bacteria (spiral-shaped) c) Coccus bacteria (round-shaped): Staphylococcus

**Characterization of Microscopic Morphology of Bacteria: Negative Staining**

The principle of negative staining is using acid dyes such as India ink or nigrosin. Acid dyes with negatively charged chromogens will not penetrate cells due to the negative charge on the surface of the bacteria. Therefore, colourless cells are easily seen against a coloured background.
There are two practical applications of negative staining. First, we can see their natural size and shape because heat fixation is not required and the cells do not experience the distorting effects of chemicals and heat. Second, we can observe bacteria that are difficult to stain, such as some spirilla. Since heat fixation is not performed during the staining process, it is essential to remember that the organisms are not killed, and the slides should be handled carefully. Figure 9 shows negative staining of bacilli.

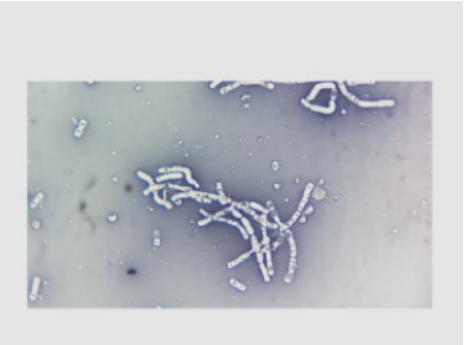


Figure 9. Negative staining with nigrosin: basil 1000x (Cappuccino & Welsh, 2019)

The principle of applying negative staining determines whether an organism has a capsule (the outer layer that makes microorganisms more virulent). However, it can also be used to demonstrate the formation of spores. This technique is often used to identify fungi such as Cryptococcus neoformans, a crucial infectious agent found in bird droppings associated with meningeal and lung infections in humans.

**Practical Objectives**
Students can perform negative staining procedures and explain the benefits of visualization of unstained microorganisms.

**Method**
**Tools and materials**
a. Bunsen burner
b. Inoculation loop
c. Coloring tub
d. Glass slides
e. Lens paper
f. Microscope
g. Slant agar culture of 1) *Bacillus subtilis*, 2) *Pseudomonas fluorescens*, 3) *Staphylococcus aureus,* 4) Twenty-four hour old *Salmonella typhimurium*.
h. Nigrosin Reagent

**Procedures**

Figure 10 illustrates the steps 1 to 4.

1. Place a small drop of nigrosin close to one end of a clean slide.
2. Using an aseptic technique, place the inoculum of bacterial culture in a drop of nigrosin and mix.
3. Place the slide against the droplet of the suspended organism at a 45° angle and allow the droplet to spread along the edge of the slide.
4. Push the slide away from the droplet of suspended organisms to form a thin smear. Air dry. Note: Do not heat the slide.
5. Repeat steps 1 to 4 to prepare the other culture slides.
6. Examine the slide under oil immersion, and record the observation in a laboratory report.

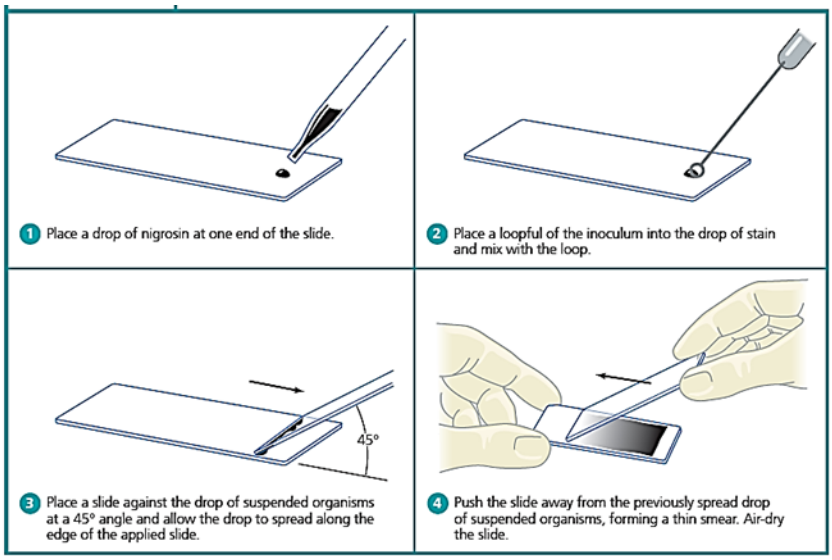



Figure 10. Negative staining procedure (Cappuccino & Welsh, 2019)

## VI. MORPHOLOGICAL CHARACTERIZATION OF ACTINOMYCETES

Actinomycetes are a group of unicellular organisms with mycelia, which reproduce through division or by particular spores or conidia. These microorganisms are closely related to actual bacteria, often considered at a higher taxonomic level as filamentous bacteria. During their growth, these microorganisms form only substrate (vegetative) mycelium or two types, namely substrate (vegetative) and aerial mycelium (as the part that forms spores/sporogenous).

At the beginning of the description, actinomycetes were often defined as unicellular microorganisms, 1 μm in diameter, forming filaments, monopodial branching, rarely dichotomous, producing colonies with a finger structure. The two generally recognized forms of reproduction are (a) fragmentation, or formation of conidia, and (b) segmentation. Both types of spores grow well in the medium and form branched mycelia.

Actinomycetes are generally recognized as a large, heterogeneous group of microorganisms consisting of several genera and many species. Actinomycetes vary in morphology, physiology, biochemical activity, and role in natural processes.

The morphology of actinomycetes can be easily observed using slide cultures incubated in a humid chamber. The mycelium attached to the cover of the object glass, which is placed at an angle to the growing culture, can be transferred to the object glass and observed at high magnification.

Morphological characters are still widely used to characterize genera, for example, the presence or absence of spores in the substrate mycelium or the formation of special spore vesicle zoospores or spore boxes. The morphological features of actinomycetes include the presence of mycelium, conidia, sporangia, and other structures.

1. Mycelium

Mycelium can be stable or temporary; if it breaks, the parts and their motility must be observed (Oerskovia sp. releases flagellate elements). The mycelium formed is either substrate mycelium and air is formed, or only substrate mycelium (very common), or only air hyphae (very rare-Sporichthya). The mycelium may form inserted vesicles that do not contain spores (Intrasporangium) or many spores (Frankia).

2. Conidia

This term is used for asexual spores that are neither intercalary chlamydospores nor sporangiospores. Actinomycetes form conidia in various ways:

a. Single conidia are found in several genera

The genus Thermoactinomycetes (Group 28), is known for its heat-resistant endospores. Single non-thermostable conidia were found in the genera Saccharomonospora and Promicrospora (Group 22), Micromonospora (Group 24.) and Thermomonospora (Group 27). Meanwhile, genera Frankia, Dactylosporangium, and Intrasporangium members sometimes form terminal vesicles confused with spores. Also, other organisms, such as actinomadurae, will form single vesicles when grown under unsuitable conditions.

b. Conidia pair

Longitudinal pairs of conidia are characteristic of the genus Microbispora (Group 26), which form only in aerial mycelium.

c. Short chains of conidia

Although it is difficult to determine how long a short chain of conidia is, conidia chains of up to 20 spores are usually considered short. Representatives of the following genera form chains of conidia: Nocardia, Pseudonocardia, and Saccaromonospora (Group 22); Streptoverticillium and Spirochtya (Group 25), Actinomadura and Microtetraspora (Group 26); Streptoallotechus (Group 27); and Glycomyces (Group 29). These morphological types may also be grouped into the genera Amycolata and Amycolatopsis (Group 22) and Catellatospora (Group 24). Some Streptomycetes of the Microellobosporia type form short chains, which are surrounded by a sheath and can be observed using a light microscope.

d. Long chain of conidia

Several genera of Actinomycetes have long chains of conidia, namely Nocardia, Nocardiales, Pseudonocardia, Saccharopolyspora, Actimopolyspora, and Amycolatopsis (Group 22); Streptomyces and Streptoverticillium (Group 25); Actinosynnema et al. (Group 27); and Kibdelsporangium, Kitasatospora, Glycomyces, Saccharothrix (Group 29).

e. Conidia on hyphae fuse into cinemata, releasing motile spores (Actinosynnema, Group 27).

3. Sporangia

Sporangia are spore sacs. The sac-shaped structure of the sporangia is a place where the spores develop and bind together until they are released so that the sporangia are empty and become a membrane. The membrane is not integrated into the spore. Sporangia originate from (a) well-developed aerial hyphae or the surface of colonies with few or no aerial hyphae (Actinoplanes et al. (Group 24); Planobispora et al. (Group 26); or (b) especially in agar media (Kineospora, Group 25).

4. Other structures

Some actinomycetes form unusual structures. What has been mentioned are the spores in synnemata found in the genus Actinosynnema. These organisms in Group 23 form masses of spores resulting from multiple plane divisions, not from perpendicular divisions in the hyphal axis. The structures on which the spores lie are called multilocular sporangia.

Some actinomycetes will form spherical structures in aerial hyphae. It is not a condensed water droplet like a curved chain of spores, or the structure may contain hyphae embedded in an amorphous matrix (Kibdelosporangium, Group 29).

Sclerotia are globose formed by some Streptomycetes. These skelotia do not contain spores but rather cells containing lipids. These germinate completely, as pseudosporangia from kibdelosporangia.

The aerial morphology and surface growth of several genera in groups 22-29 are shown schematically in the figure below. Morphology is useful for identification of some genera but not all.

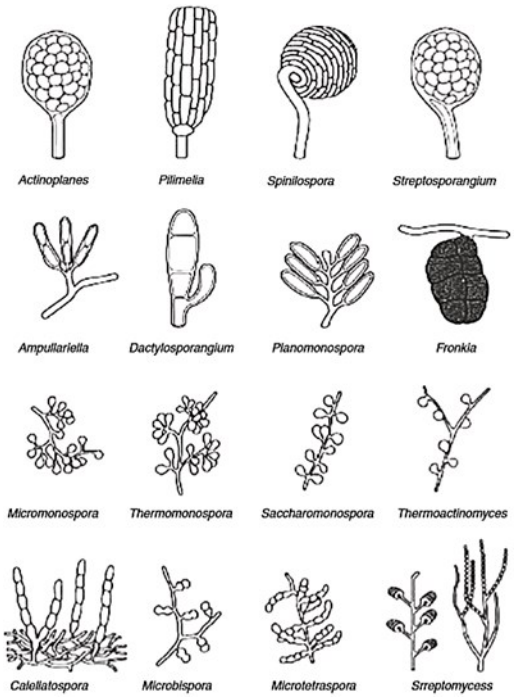



Figure 11. Structure of actinomycete spores (Li et al., 2016).

**Macroscopic Morphological Characterization of Actinomycetes**

**Practical Objectives**

Students can recognize the colony morphology of various actinomycetes genera.

**Method**

**Tools and materials**

a. Actinomycete culture
b. Yeast Malt Extract Agar (YMA/ISP-2) media
c. The YMA media is tilted in the test tube
d. Sterile Petri dishes
e. Magnifying glass

**Procedure**

1. Melt the upright medium in a water bath and aseptically pour it into a sterile Petri dish; wait until it cools and solidifies.
2. Inoculate a small amount of actinomycetes culture on two plates and one YMA slant agar tube.
3. Incubate both plates upside down at room temperature for 2 to 7 days or more.
4. Observe the changes in the colony, which shows the nature of the colony colour and pigmentation as well as the shape of the colony, which includes compact, leathery, conical appearance, dry surface, often covered by air mycelium and observe the presence of spores.
5. Actinomycetes, which produce two types of mycelium, namely substrate or vegetative and air mycelium, usually form special reproductive cells known as spores or conidia.

## Microscopic Morphological Characterization of Actinomycetes

### Practical Objectives

Students can describe the microscopic morphology of actinomycetes cells.

### Method

### Tools and materials

a. Actinomycetes culture
b. YMA cup media
c. Sterile distilled water
d. Sterile Petri dishes
e. Sterile object glass and cover glass
f. Sterile toothpick
g. Microscope
h. Inoculation loop
i. Bunsen burner

### Procedure

1. Slide the object glass

a. Cut a thin block of agar from a Petri dish filled with agar, place it in a sterile microscope slide, then inoculate and cover it with a sterile cover glass.

b. After incubation in a humid chamber, observe the culture slide directly with a microscope, observing the mycelium in the air and the substrate in the agar.

2. Tilt the glass cover to observe the morphology of actinomycetes

a. Inoculate an agar plate with a small amount of actinomycetes culture and mount the slide cover at an angle near the scratch.

b. After incubation, attach an object glass cover to the object glass. The top surface faces the bottom and then drips with water.

## VII. CHARACTERIZATION OF YEAST CELLS

Yeast is a non-filamentous unicellular fungus. The growth characteristics of yeast resemble bacteria when grown on the surface of artificial laboratory media. The size of yeast colonies is 5 to 10 times larger than bacteria. Figure 12 illustrates a yeast colony. In microscopic observation, yeast cells can be ellipsoidal, round, or, in some cases, cylindrical (Figure 13a). Yeasts differ from fungi, i.e. they do not have aerial hyphae or supporting sporangia.

Figure 12. Yeast colonies growing on media in the laboratory (Cappuccino & Welsh, 2019)

Yeasts reproduce asexually by budding or by fission. Buds form from parent cells that pinch off, producing daughter cells (Figure 13b). Division occurs in certain yeast species, such as in the genus *Schizosaccharomyces*. Division occurs when the parent cell elongates, its nucleus divides, and the cell divides evenly into two daughter cells.

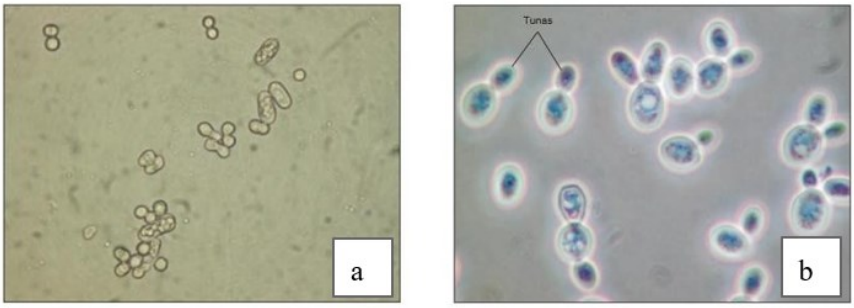



Figure 13. The microscopic structure of (a) S. octosporus yeast cells and (b) S. cerevisiae cells forming shoots resulting from vegetative reproduction (Cappuccino and Welsh, 2019)

Some yeasts can also undergo sexual reproduction when two sexual spores conjugate, producing a zygote, or diploid cell. The nuclei of these cells divide by meiosis, producing four new haploid nuclei (sexual spores), called ascospores contained in a structure called the ascus (Figure 14b). When the ascus ruptures, the ascus spores are released, conjugate, and start the cycle again.


Figure 14. Yeast structure (a) vegetative reproduction by budding and (b) sexual reproductive structure.

Yeast is essential for many reasons. Saccharomyces cerevisiae is known as bread yeast and is used as a leavening agent in dough. Two main yeast strains, *S. carlsbergensis* and S. cerevisiae, are used for brewing beer. The wine industry relies on wild yeasts (present in grapes) for the spontaneous fermentation of grape juice. Meanwhile, *Saccharomyces ellipsoideus* is used as a fermentation inoculum. Yeast's high vitamin content makes it very valuable as a food supplement.

**Practical Objectives**

Students can describe the growth characteristics and types of reproduction used to identify various genera of yeast.

**Method**

**Tools and materials**

a. Bunsen Lamp
b. Inoculation loop
c. Object glass
d. Cover glass
e. Drop pipette
f. Microscope
g. Whiteboard marker
h. Saccharomyces cerevisiae
i. Candida albicans
j. Lactophenol-cotton-blue solution

**Procedure**

1. Apply a tube of yeast culture in a few drops of lactophenol-cotton-blue solution placed on a microscope slide and cover with a cover slip.
2. Examine all yeast wet slide preparations at low and high magnification, noting the shape and presence or absence of buds.
3. Record observations on the observation table.

## VIII. MOLD SLIDE CULTURE TECHNIQUE

Determining the genus and species of a mould culture is not easy; this requires identification. The first stage in identifying mould recognizes the morphological characteristics both macroscopically and microscopically. Macroscopic mould morphology can be seen from the colour of the colony, the condition of the colony surface, the presence or absence of liquid spots on the colony's surface, and the presence or absence of radial lines or concentric circles (zonation). Meanwhile, microscopically, you can see the shape and size of the sporangium, sporangiospores, and the form of mycelial branching.

**Practical Objectives**

Students can recognize the morphology of molds microscopically and identify them.

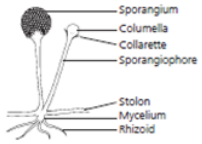
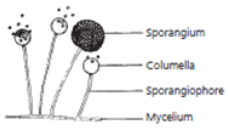
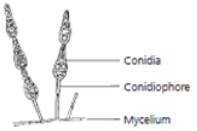
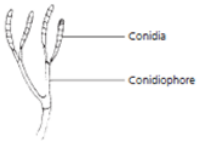
**Method**

**Tools and materials**

a. Object glass
b. Cover glass
c. Petri dish containing sterile tissue
d. Ent needle
e. Inoculation loop
f. Fan
g. Tweezers
h. Dropper bottle
i. Painting tub
j. Bunsen burner
k. Microscope
l. Tissue
m. One culture of mold isolate was prepared by the laboratory on an SA plate
n. SA medium
o. 70% alcohol solution
p. Sterile distilled water.

**Procedure**

1. Aseptically, a small amount of SA agar is placed on both ends of a sterile glass object using an inoculation loop.
2. Each agar is inoculated with the mold provided using an Ent needle.
3. A sterile cover glass is placed on the top of the agar piece, and then the culture is incubated in a sterile Petri dish containing a piece of sterile filter paper moistened with sterile distilled water. Mold will grow on the object glass and cover the glass after incubation for three days.
4. First, dry the bottom surface of the culture slide with tissue. Observe under a microscope, first with a weak objective lens and then with a strong magnification lens.
5. Note and draw everything observed, such as mycelium branched or not, septumized or not, smooth or rough, sterigma, conidia, spores, conidiophores, sporangiophores, columella, vesicles, as in Table 1.

| DIAGRAM | COLONIAL MORPHOLOGY | MICROSCOPIC APPEARANCE |
|---|---|---|
| **Molds**<br>  <br><br>*Rhizopus:*<br>Black bread mold; common laboratory contaminant | Rapidly growing white-colored fungus swarms over entire plate; aerial mycelium cottony and fuzzy | Spores are oval, colorless, or brown; nonseptate mycelium gives rise to straight sporangiophores that terminate with black sporangium containing a columella; rootlike hyphae (rhizoids) penetrate the medium |
|  <br><br>*Mucor:*<br>Food contaminant | Resembles the colonies of *Rhizopus* except that it lacks rhizoids and collarettes. Sporangiophore arises directly from mycelial mat.<br>Note: Branching sporangiophores may occur with *Mucor.* | Spores are oval; nonseptate mycelium gives rise to single sporangiophores with globular sporangium containing a columella; there are no rhizoids |
| **Molds**<br>  <br><br>*Alternaria:*<br>Normally found on plant material; also found in house dust | Grayish-green or black colonies with gray edges rapidly swarming over entire plate; aerial mycelium not very dense, appears grayish to white | Multicelled spores (conidia) are pear-shaped and attached to single conidiophores arising from a septate mycelium |
|  <br><br>*Fusarium:*<br>Found in soil; also likely in eye infections | Woolly, white, fuzzy colonies changing color to pink, purple, or yellow | Spores (conidia) are oval or crescent-shaped and attached to conidiophores arising from a septate mycelium; some spores are single cells, some are multicelled |

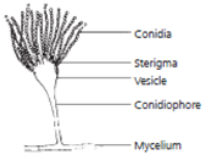
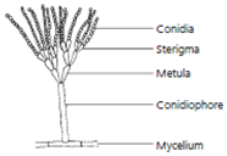
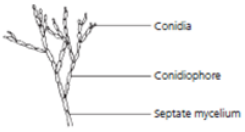
| DIAGRAM | COLONIAL MORPHOLOGY | MICROSCOPIC APPEARANCE |
|---|---|---|
|  <br><br>*Aspergillus:*<br>Plant and animal pathogens; some species used industrially | White colonies become greenish-blue, black, or brown as culture matures | Single-celled spores (conidia) in chains developing at the end of the sterigma arising from the terminal bulb of the conidiophore, the vesicle; long conidiophores arise from a septate mycelium |
|  <br><br>*Penicillium:*<br>Antibiotic-producing citrus fruit contaminant; soil inhabitant | Mature cultures usually greenish or blue-green | Single-celled spores (conidia) in chains develop at the end of the sterigma arising from the metula of the conidiophore; branching conidiophores arise from a septate mycelium |
| **Molds**<br>  <br><br>*Cladosporium:*<br>Dead and decaying plants | Small, heaped colonies are greenish-black and powdery | Spores (conidia) develop at the end of complex conidiophores arising from a septate mycelium that is usually brownish |

Table 1. Colony morphology and microscopic characteristics of mold (Cappuccino & Welsh, 2019)

## IX. MORPHOLOGY OF MOLDS AND YEASTS

Knowing the name of the genus and species of mold culture is not easy, so an identification stage is required. The first stage for identification is the introduction of morphological characteristics, both microscopic and macroscopic morphology. The color of the colony, the surface of the colony, the presence or absence of radial lines or concentric circles (zonation) and the presence or absence of liquid dots (exudate drops) on the surface of the colony are characters that need to be noted and considered in macroscopic colony identification. Furthermore, the shape and size of the sporangium, sporangiospores, mycelial branching form, branching and conidial head shape are often accurate considerations for determining the species. A beginner can certainly have difficulty distinguishing between the Genus *Penicillium* and the Genus *Paecilomyces*. However, by looking at the development of the colony macroscopically and the nature of the branching of the conidial heads microscopically, the two genera can clearly be distinguished.

Yeast is a single-celled fungus and does not form mycelium, however, some species can form pseudo-mycelium. The morphology of yeast is simpler than mold, but its size is larger than bacteria. Important morphological characteristics of yeast include: cell shape (oval, round like a sausage, elliptical), cell size, shape and number of buds, shape and number of spores in the ascus, shape/formation and presence or absence of pseudomycelium are very important for identification.

### Morphology of Thread Molds

### Macroscopic Observation

### Practical Objectives

Students can recognize the colony morphology and growth characteristics of each type of mold on the medium.

### Method

### Tools and materials

a. Sterile Petri dishes
b. Inoculation loop
c. Bunsen burner
d. Pure culture of mold; Aspergillus; Rhizopus; Penicillium; and Mucor
e. PDAs media

### Procedure

1. Heat the prepared upright PDA media in a sterile Petri dish until it melts aseptically, and wait until it cools and solidifies.
2. Transfer a small amount of the culture of each mold species to the surface of the media
3. Incubate for 4-5 days at room temperature
4. Observe every day the color changes that appear on the colony, the condition of the colony's surface (flat, mountainous like flour, grainy or cotton-like), whether there are radial lines, the presence of concentric lines or circles (zonation), the presence or absence of exudate drops as well as the color, whether there is a distinctive odour and the shape and color of the colony.

5. Record the data observed.

**Microscopic Observation**
**Practical Objectives**
Students can recognize morphological characteristics microscopically to differentiate one species from another.
**Method**
**Materials and tools**
a. Pure culture of R. oryzae; A. oryzae; Penicillium sp.
b. Object glass and cover glass
c. Lactophenol solution or lactophenol cotton blue
d. Inoculation loop
e. Preparation needle
f. 70% alcohol solution
g. Bunsen burner

**Procedure**
1. Clean the object glass and cover it with 70% alcohol until they are free from fat, then drop a few drops of lactophenol solution or lactophenol cotton blue on the surface of the object glass.
2. Take a small amount of the colony with an inoculation loop, place it in a drop of lactophenol and spread it carefully with the preparation needle. Make sure all the wet mycelium is exposed to lactophenol.
3. Cover the preparation with a cover glass so there are no air bubbles. Wipe off excess lactophenol with blotting paper.
4. Observe with a microscope using an objective lens with 10x magnification and then with 40x magnification. To see the morphology of conidia or spores, use an objective lens with 100x magnification with immersion oil.
5. Note and draw everything observed, such as whether the mycelium branched or not, septumized or not, smooth or rough, sterigma, conidia, spores, conidiophores, sporangiophores, columella, vesicles, etc.

**Yeast Morphology**
**Yeast Cell Staining**
**Practical Objectives**
Students can recognize the various forms of yeast cells and differentiate dead cells from living cells

**Method**
**Tools and materials**
a. Pure culture (72 hours) of Saccharomyces cerevisiae; Candida sp.; Hansenulla sp. in the medium
b. Methylene blue (MB) solution
c. Object glass and cover glass
d. Inoculation loop

e. 70% alcohol solution (w/v)
f. Bunsen burner

**Procedure**
1. Clean the slide using 70% alcohol until it is grease-free
2. Drop a little MB on the object glass.
3. Take a small amount of the yeast culture with a loop, place it in the MB drop, and cover it with a cover slip, ensuring no air bubbles.
4. Observe under a microscope using 40x and 100x magnification using immersion oil.
5. Draw and note cell shape, the presence or absence of budding, the number of shoots in each cell, and the presence or absence of pseudo mycelium.

**Staining Yeast Spores**
**Practical Objectives**
Students can stain, see the various shapes of yeast spores, and count the number of spores in the ascus.

**Method**
**Tools and materials**
1. Object glass.
2. Pure culture (72 hours) of S. cerevisiae; Candida sp.; Hansenulla sp. on carrot slant agar or carrot slice medium
3. Crystal violet aniline paint solution (paint A)
4. Safranin paint solution (paint B)
5. Acid alcohol solution

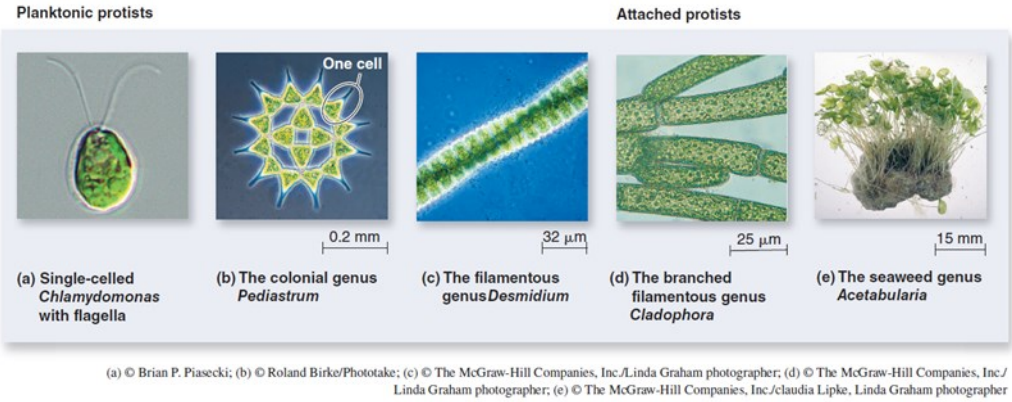
**Procedure**
1. Suspend the pure yeast culture in sterile distilled water, then spread one loop over an object glass covering an area of approximately 1 cm2.
2. Air dry, fix with a Bunsen burner for 5-7 times over a fire.
3. Cover the culture stain with paint A and heat it for 3 minutes, ensuring it doesn't dry out.
4. Wash with running water.
5. Dissolve with acidic alcohol.
6. Wash with running water
7. Drip with paint B for 10-15 seconds (exactly)
8. Wash with running water and air dry
9. Observe with a microscope at strong magnification with immersion oil
10. Draw and give a complete description of the yeast cells and their spores.

## X. MORPHOLOGICAL CHARACTERIZATION OF ALGAE

Protists can be easily grouped according to their ecological role as algae, protozoa, and fungus-like protists. The term algae (Latin for "seaweed") applies to about ten groups of protists, most of which are photosynthetic species. Despite the general features of photosynthesis, algae do not form monophyletic groups descending from a common ancestor. However, it is polyphyletic (that is, it has more than one common ancestor), and the details of its phylogeny are still being researched).

Algae are photosynthetic organisms, eukaryotic and usually do not have multicellular sex organs. The major groups of algae are differentiated in part by their energy storage products, cell walls, and colour resulting from the type and abundance of coloured pigments (substances that absorb light) in their plastids. Biologists often group the algae based on the color of these pigments—for example, green, brown, and red algae.

Algae are also differentiated based on their cellular organization (Figure 15). Unicellular algae species appear as single, unattached cells that may or may not be motile. Filamentous algae species occur as chains of cells attached end to end. These filaments may have few or many cell lengths and may be unbranched or branched in various patterns. Colonial algae occur as groups of cells attached in a nonfilamentous manner. For example, a colony may include several to many cells stuck together as a sphere, flat sheet, or other three-dimensional shape.




(a) © Brian P. Piasecki; (b) © Roland Birke/Phototake; (c) © The McGraw-Hill Companies, Inc./Linda Graham photographer; (d) © The McGraw-Hill Companies, Inc./Linda Graham photographer; (e) © The McGraw-Hill Companies, Inc./claudia Lipke, Linda Graham photographer

Figure 15. The diversity of algal body types reflects their habitat.
(a) The single-celled flagellate genus *Chlamydomonas* resides in lake phytoplankton. (b) The colonial genus *Pediastrum* consists of several cells arranged in a lacy star shape which helps this algae stay buoyant in the water. (c) Genus *Desmidium* is filamentous and occurs as rows of twisted cells. (d) *Cladophora* is a genus of branching filaments that grow attached to surfaces near shore large enough to be seen with the naked eye. (e) The relatively large seaweed genus *Acetabularia* lives on rocks and coral debris in shallow tropical seas.

**Practical Objectives**
Students can describe unicellular algae.

**Method**

**Tools and materials**

a. Microscope
b. Drop pipette
c. Object glass
d. Cover glass
e. Samples of rice field water, soil, rivers, river soil/sand, and ponds
f. Methylcellulose solution 1-10% (w/v)
g. Lugol's solution

**Procedure**

1. A drop of water that is thought to contain live unicellular algae is placed on a glass object and then covered with a glass cover. This procedure is repeated on a new glass object where one drop of methylcellulose has been placed first. After that, a drop of the same sample water was added.
2. The preparation is observed with a microscope.
3. Based on the structure, determine the appropriate type of algae

a) Chlamydomonas

1) Preparation showing a Chlamydomonas specimen, if the movement is too fast, make a new preparation by placing one or two drops of methylcellulose on the slide and adding a drop of water containing Chlamydomonas.
2) Stir gently and add the cover glass.
3) Note the stigma, which appears as a light-absorbing reddish spot at the anterior end of the cell.

b) Volvox

1. The preparation showing the Volvox specimen is observed with a microscope under low magnification, observing large, hollow and round colonies.
2. Another structure observed is the presence of flagella on the surface.

c) Diatoms

1) The preparations showing Diatom specimens were observed for their shell types; some were broken, and some were intact.
2) a clean, insoluble, porous shell mass is another structure observed.

d) Dinoflagellates (supergroup Chromalveolata)

Preparations showing dinoflagellate specimens were observed for flagella and flagellar longitudinal and transverse grooves.

e) Euglenoids (Excavata Supergroup)

1) The preparation showing the Euglena specimen is observed for the presence of colored eye spots near the base of the flagella
2) To slow down Euglena, adding a drop of methylcellulose to the preparation is necessary.
3) Another characteristic observed is the movement and changes in the organism's shape.

## XI. PROTOZOA

Protozoa are a large and diverse group of unicellular eukaryotic organisms. Protozoan taxonomy is continually updated as new technologies allow classification based on molecular characteristics. For our discussion of protozoa, we follow a more traditional taxonomic scheme, dividing them into four groups based on locomotion:

1. Sarcodina:

The motility results from the flow of ectoplasm, producing protoplasmic projections called pseudopods (false legs). Prototypic amoebas include the free-living *Amoeba proteus* and the parasite *Entamoeba histolytica*.

2. Mastigophora:

It has one or more whips, thin structures called flagella. Free-living members include the genera *Cercomonas*, *Heteronema* and *Euglen*a, which are photosynthetic protists that can be classified as flagellated algae. Parasitic forms include *Trichomonas vaginalis*, *Giardia intestinalis* (formerly called *Giardia lamblia*), and *Trypanosoma species*.

3. Ciliophora:

Short, hair-like strands called cilia, whose synchronous beating drives organisms to movement. A characteristic example of a free-living member of this group is *Paramecium caudatum*, and an example of a parasite is *Balantidium coli*.

4. Sporozoa:

Unlike other members of this phylum, sporozoa do not have locomotor organ cells in their adult stages; however, immature forms show some movement. All members of this group are parasites. The most significant members of the genus *Plasmodium* are malaria parasites in animals and humans.

**Practical Objectives**

Students can describe protozoa found in water samples.

**Method**

**Tools and materials**

a. Microscope
b. Glasses and covers
c. Pasteur pipette or dropper pipette
d. Well, water, pond water, rice field water, river water, sewer or sewage water
e. *Amoeba*, *Paramecia*, *Euglena* and *Stentor* preparations (adjusted to availability in the laboratory).
f. Methylcellulose solution 1-10% (v/v)

**Procedure**

1. One drop of a pool water sample is taken from the bottom of the water sample and placed in the middle of a clean glass object.
2. One drop of methyl cellulose was added to the sample to slow down the movement of protozoa.
3. The slide is then covered using a coverslip in the following way to prevent the formation of air bubbles.

a. Place one edge of the coverslip on the outer edge of the culture drop.
b. Once the culture drop has spread along the inside edge of the coverslip, carefully lower the coverslip onto the slide.
c. The slide culture is observed under a microscope at the lowest to highest magnification and observes the presence of different protozoa. Record the results of observations in a report.

## XII. BACTERIOPHAGE OBSERVATION

Bacteriophages are viruses that infect bacteria and can kill the bacterial cells directly or integrate viral DNA into the chromosomes of the host bacteria. Like other viruses, bacteriophages also have similar components in the form of a protein coat and nucleic acid in the form of ssRNA, dsDNA and dsRNA (ss: single strand, ds: double strand); the shape of the nucleic acid strands is generally linear, circular or segmented.

Judging from the behaviour of bacteriophages, some bacteriophages can insert their nucleic acid with the nucleic acid of the host bacteria and also be able to directly cause the lysis of the host bacteria by producing several enzymes that play a role in lysing the bacterial cells. The results of bacterial lysis by viruses can be observed using Plaque Assay.

Bacterial virus isolates (bacteriophages) can be obtained from various natural sources, including soil, intestinal contents, raw unprocessed waste, and some insects, such as cockroaches and flies. Isolation of viruses from this environment is not easy, because phage particles are usually present in low concentrations. Therefore, isolation requires a series of steps:

1. Collection of samples containing phages in the source.
2. Add enriched susceptible host cell culture to the sample to increase the number of phage particles for subsequent isolation.
3. After incubation, centrifugation of enriched samples to obtain concentrated particles.
4. The supernatant fluid is filtered through a bacteria-retaining membrane filter.
5. The bacteria-free filtrate is inoculated into the growth area of susceptible host cells grown on soft agar plate media.
6. Incubation and observation of the culture for the presence of phage particles, characterised by plaque formation in the area of bacterial growth on the agar plate.

**Practical Objectives**

Students can observe the formation of plaque by bacteriophages in bacterial cultures.

**Method**

**Tools and materials**

a. Erlenmeyer flask
b. Filter membrane
c. Beaker Glass
d. Centrifuge
e. Bunsen burner
f. 1 mL micropipette and tip
g. Test tube rack
h. Label
i. Incubator
j. Sterile Pasteur pipette
k. Five Petri dishes containing Nutrient Agar (NA) media (1.5% agar)
l. Five test tubes containing 3 mL of soft NA media (0.5% Agar)
m. *Escherichia coli* (24 hours) culture in Nutrient Broth (NB) media
n. T2 coliphage (water sample from Bedadung River)

**Procedure**

1. Enrichment of Waste Water Samples

a. Add 5 ml of E. coli culture (24 hours) grown in NB media to 45 mL of wastewater sample in a sterile 250 mL Erlenmeyer flask.

b. Incubated the culture at 37℃ for 24 hours.

2. Filtration and Seeding

a. After being infected with phage, put 50-100 mL of the E. coli culture into microtubes of 2 mL each and centrifuged at 2500 rpm for 20 minutes.

b. After the centrifuge, the supernatant is carefully poured into a 125 ml beaker.

c. Filter the supernatant with a filter membrane to obtain a bacteria-free supernatant.

d. Melt the NA soft agar media in five test tubes in boiling water and cooled to a temperature of 45℃ so the media remains melted.

e. Label the five reaction tubes containing NA soft agar media with 1, 2, 3, 4, and 5, respectively.

f. Using a micropipette, aseptically add 0.1 mL of E. coli liquid culture to each of the five soft NA agar tubes.

g. Add 1, 2, 3, 4, and 5 drops of the filtrate to each labelled soft agar tube with a sterile Pasteur pipette. Mix and pour each tube of softened agar into an agar dish corresponding to the label.

h. The agar in the cup is allowed to harden.

i. All culture dishes were incubated in an inverted position for 24 hours at 37℃.

3. Observation method

a. Observe all culture plates for plaque formation, which indicates the presence of phagecoli (coliphages) in the culture.

b. Indication of the presence of plaque is marked plus (+) or no negative (-) in each culture in the practical report table. If there is an indication of plaque, the number is counted.

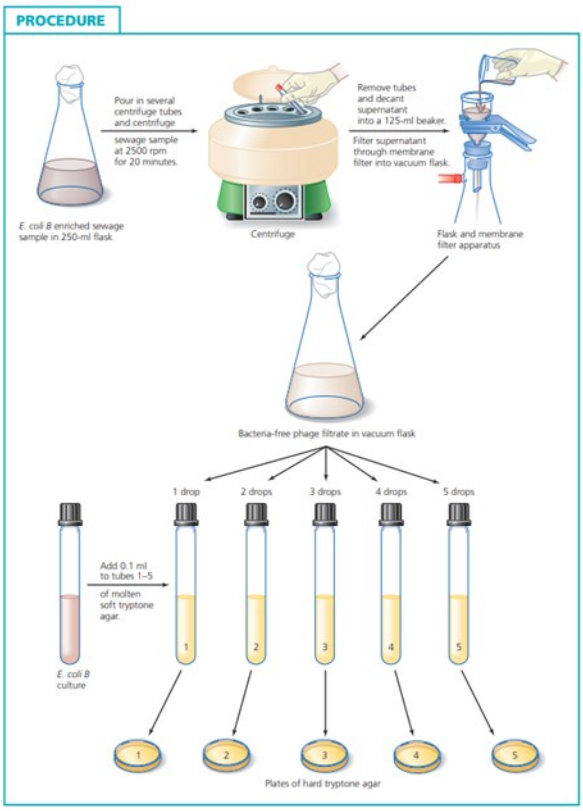



Figure 16. Wastewater sample enrichment, filtration, and seeding (Cappuccino & Welsh, 2019)

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