LABORATORY PRACTICAL MODULE

Course : MICROBIAL PHYSIOLOGY
Course Code: MAB 1301

Written by:
Microbial Physiology Team

Bachelor Biology Program Study
Faculty of Mathematics and Natural Sciences
University of Jember
2023

TABLE OF CONTENTS

Cover		i
Table of contents		ii
Chapter 1	Bacterial Gram Staining	1
Chapter 2	Bacterial Acid Resistant Coloring	10
Chapter 3	Coloring of Bacterial Capsules and Endospores	15
Chapter 4	Carbohydrate Fermentation	20
Chapter 5	Reduction of Nitrate and Hydrogen Peroxide	22
Chapter 6	Formation of H_2S by Bacteria	26
Chapter 7	Formation Ammonia by Bacteria	28
Chapter 8	Microbial Growth Patterns	30
Chapter 9	Abiotic Factors Affecting Microbial Growth	35
Chapter 10	Biotic Factors Affecting Microbial Growth	45
Chapter 11	References	50

1. BACTERIAL GRAM STAINING

Theory

Differential staining requires the use of at least four chemical reagents applied sequentially to a heat-fixed smear. The first reagent is called the primary dye with the function to color all cells. The second dye is a mordant used to intensify the color of the primary dye. To form color contrast, the third reagent used is a color removal agent. Based on the chemical composition of cellular components, decolorizing agents can remove primary dyes from entire cells or only from specific cell structures. The final reagent, the counterstain, has a color that contrasts with the color of the primary dye. After color removal, if the primary dye is not removed, the counter dye cannot be absorbed, and the cells or their components will be absorbed, maintaining the color of the primary dye. If the primary dye is removed, the decolorized cellular components will accept and take on the contrasting color of the counter dye. In this way, cell types or cell structures can be distinguished from each other based on the dye left behind.

The most important differential stain used in bacteriology is the Gram stain, named after Dr. Hans Christian Gram. Gram divides bacterial cells into two major groups, gram-positive and gram-negative, which makes it an important tool for the classification and differentiation of microorganisms. Figure 1 shows Gram-positive and Gram-negative cells. The Gram stain reaction is based on differences in the chemical composition of bacterial cell walls. Gram-positive cells have a thick peptidoglycan layer, while the peptidoglycan layer in gram-negative cells is much thinner and is surrounded by an outer layer containing lipids. Peptidoglycan is a polysaccharide consisting of two chemical subunits that is only found in bacterial cell walls. These subunits are N-acetylglucosamine and N-acetylmuramic acid. In some organisms, peptidoglycan layers form close together, they are cross-linked by short chains of peptides via the enzyme transpeptidase, resulting in the shape

and rigidity of the cell wall. In gram-negative bacteria and some other gram-positive bacteria, such as *Bacillus*, cross-linking of the peptidoglycan layer is direct because the bacteria do not have short peptide tails. Early experiments showed that gram positive cells lose their cell walls due to the activity of lysozyme or penicillin and will stain gram negative. Gram staining uses four different reagents.

The following is a description of the reagent and its mechanism of action. Figure 2 shows the microscopic appearance of cells at each step of the Gram staining procedure.

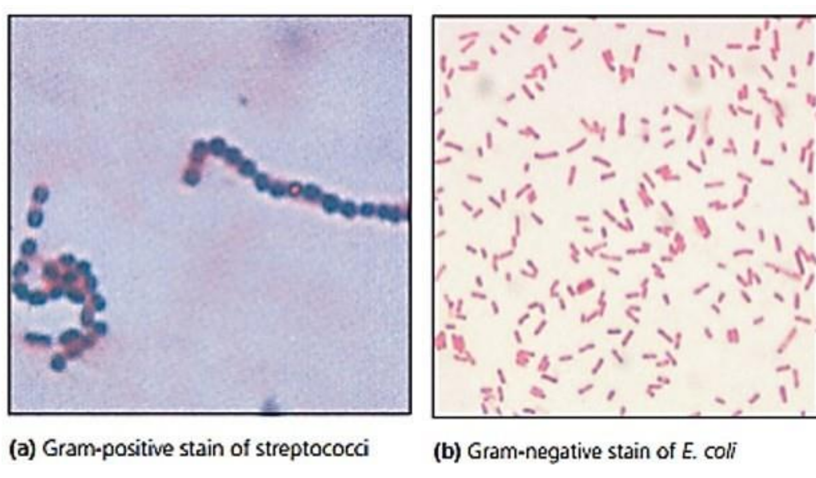


Figure 1.1 Cells from Gram staining

Primary Dye

Crystal Violet (Hucker's): This violet dye is used first and colors all cells purple.

Mordant

Gram's Iodine: This reagent not only functions as a lethal agent but also as a mordant, a substance that increases the affinity of cells to the dye. The reagent does this by binding to the primary dye, thereby forming an insoluble complex. The resulting crystal – purple – iodine (CV-I) complex functions to intensify the color of the stain. At this point, all cells will appear purple-black.

Color Removal Agent

Ethyl Alcohol, 95%: This reagent has double function, as a protein dehydration agent and as a lipid solvent. Its action is determined by two factors, lipid concentration and the thickness of the peptidoglycan layer in the bacterial cell wall. In gram-negative cells, alcohol increases cell wall porosity by dissolving lipids in the outer layer. Thus, the CV-I complex can be more easily removed from thinner peptidoglycan layers and smaller peptidoglycan layers that have high cross-links. Therefore, the washing effect of alcohol facilitates the release of unbound CV-I complexes, leaving the cells colorless or unstained. The thicker peptidoglycan layer on gram-positive cells is responsible for tighter retention of the CV-I complex, as its pores shrink against the dehydrating effect of alcohol. Thus, the tightly bound primary staining complex is difficult to remove, and the cells remain purple. Note: Be careful not to over-remove the dye color with alcohol.

Counter coloring

Safranin: This is the final reagent used for pink staining of previously decolorized cells. Since only gram-negative cells are decolorized, they can now absorb the reverse dye. Gram-positive cells retain the purple color of the primary dye.

The preparation of smears required for staining is as follows:

1. The most critical phase of the procedure is the decolorization step, which is based on the easiness of the CV-I complex released from cells. Remember that excessive decolorization will result in loss of the primary dye, causing gram-positive organisms to appear gram-negative. However, insufficient decolorization will not completely remove the CV-I complex, causing gram-negative organisms to appear gram-positive. Strict adherence to all instructions will help overcome some of the difficulties, but individual experience and practice are the keys for correct decolorization.

2. Very important, between applications of the reagent, the slides are washed thoroughly under running water or water applied with an eye dropper. This removes excess reagent and prepares the slide for subsequent reagent application.
3. The best Gram stain preparations are made from fresh cultures (i.e., no older than 24 hours). As the culture ages, particularly in the case of gram-positive cells, the organism tends to lose its ability to retain the primary dye and may appear as gram-variable; i.e. some cells will appear purple, while others will appear pink.

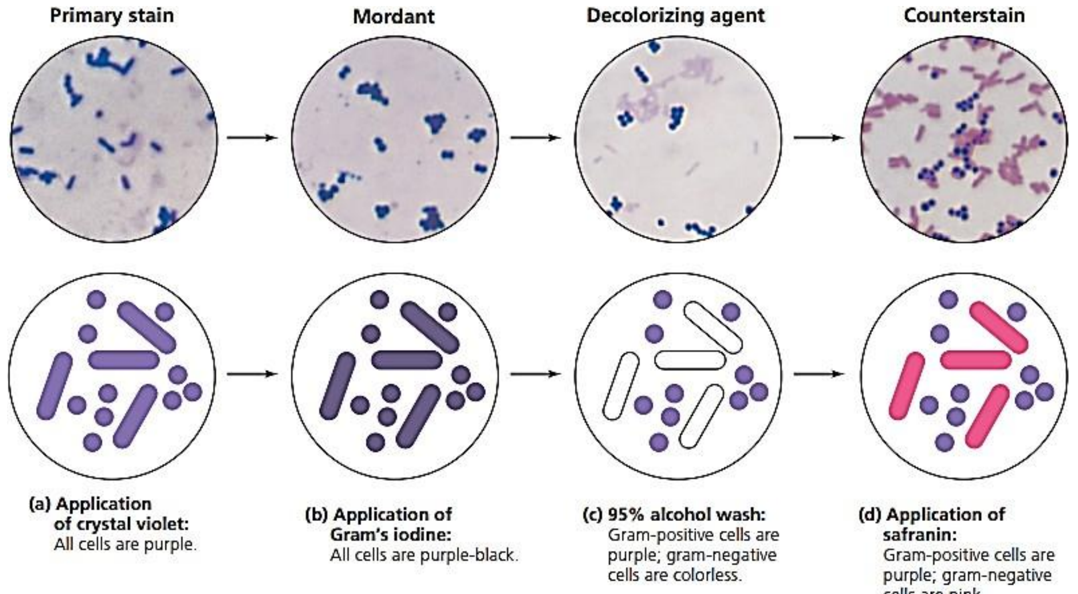


Figure 1.2 Microscopic observation of cells following the steps of the Gram staining procedure

Practical Objectives

1. Students are able to explain the chemical and theoretical basis for differential staining procedures
2. Students are able to explain the chemical basis for Gram staining

3. Students are able to carry out procedures to differentiate between the two main groups of Gram-positive and Gram-negative bacteria.

Materials and tools

1. *Escherichia coli* culture (24 hours) on slanted NA media
2. *Staphylococcus aureus* culture (24 hours) on slanted NA media
3. *Bacillus subtilis* culture (24 hours) on slanted NA media
4. Crystal purple dye
5. Gram iodine (Mordant)
6. 95% ethyl alcohol solution
7. Safranin dye
8. Oil immersion
9. Microincinerator or Bunsen burner
10. Inoculation loop or inoculation needle
11. Coloring tray
12. Objects
13. Blotting paper
14. Lens paper
15. Microscope

Procedure

Smear Preparation

1. Take four clean objects.
2. Using aseptic technique, prepare smears of each of the three organisms, and on the remaining slides, prepare smears containing a mixture of *S. aureus* BSL-2, and *E. coli*. Do this by placing a drop of water on it, and then transferring each organism separately to the drop of water with a cooled, sterile loop needle. Mix and distribute both organisms through circular movements of the inoculation loop.

Note: If bacteria are taken from liquid culture, a drop of water is not necessary. Place one bacterial suspension loop needle directly on the object.

3. Let the smear dry and then fix it with heat in the usual way.

Gram stain

1. Gently pour the smear with crystal violet and let it for 1 minute.
2. Wash gently with tap water.
3. Gently moisten the smear with iodine Gram mordant and let it for 1 minute.
4. Wash gently with tap water.
5. Decolorize with 95% ethyl alcohol. Note: Do not over-remove color. Add drops of reagent until the alcohol is almost clear, showing only a blue tinge.
6. Wash gently with tap water.
7. Counterstain with safranin for 45 seconds.
8. Wash gently with tap water.
9. Dry with blotting paper and check under immersion oil.
10. As you observe each slide under oil immersion, complete the table provided in the Lab Report.
    A. Draw a representative microscopic plane.
    B. Describe cells according to their morphology and arrangement.
    C. Describe the color of the stained cells.
    D. Classify organisms based on Gram reactions: gram positive or gram negative

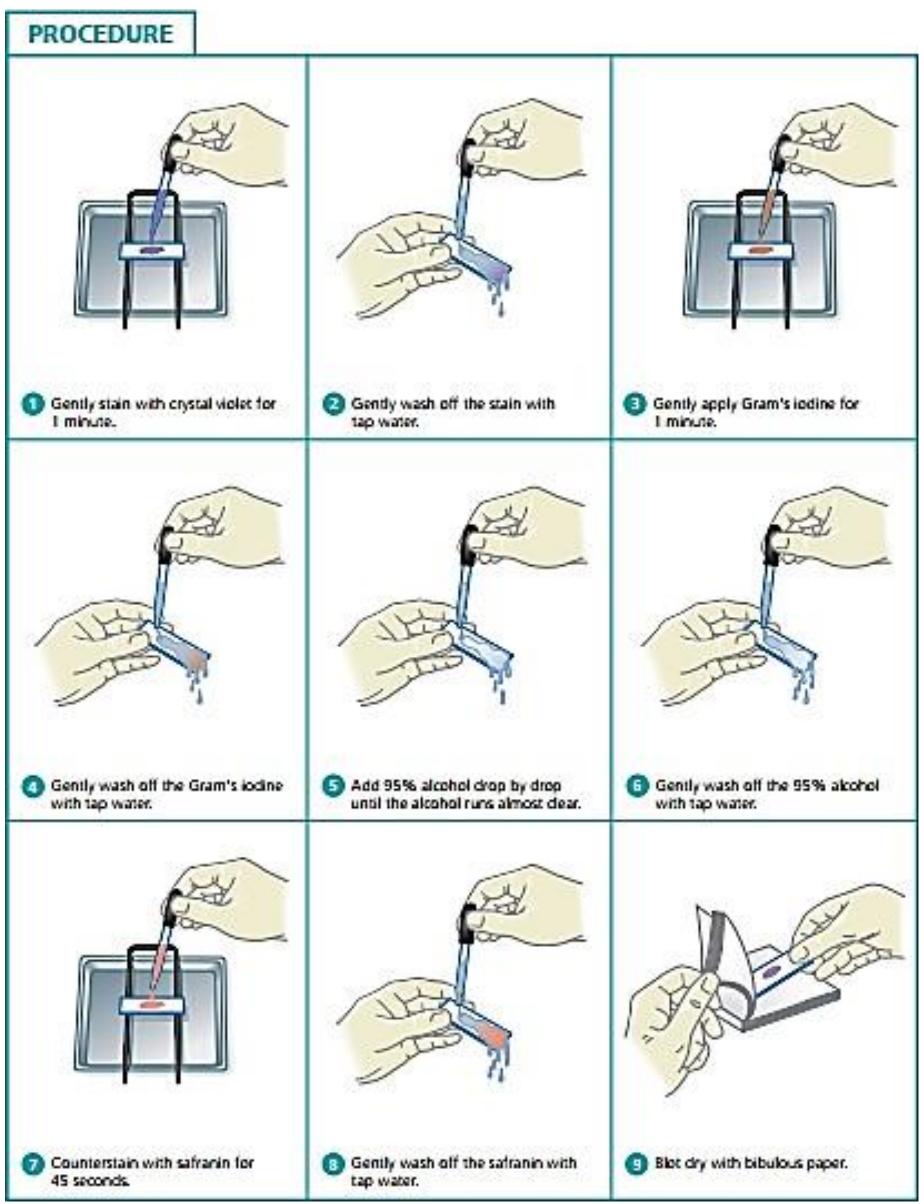


Figure 1.3 Gram staining procedure

Tips for success

1. Proper slide preparation is the key to successful staining. Incorrect heat fixation will affect the number of bacteria that will be present during staining. Fixation that is not hot enough or too short will not allow the cells to attach to the object properly, and the cells will be washed out during the staining

step. On the other hand, overheating will result in the destruction of cells and attached cell debris. If any, only a few cells remain intact for the staining process.

2. The timing of the color removal step is perhaps the most important aspect of the procedure. Excessive decolorization with the wrong alcohol solution, or allowing slides to decolorize for too long, will remove the CV-I complex by causing extensive damage to the cell membrane and cell wall, even in gram-positive cells. Otherwise, removing the color in too short a period of time will not remove enough of the CV-I complex. Cells exposed to safranin will appear darker in color, and can be confused with light purple and gram-positive cells.
3. The age of the culture or colony applied can affect the Gram staining results. Best Gram-stained preparations are made with fresh cultures that are no more than 24 hours old. As the culture ages, particularly in the case of gram-positive cells, the organism tends to lose its ability to retain the primary dye and may appear gram variable; that is, some cells will appear purple, while other cells will appear purple and appear pink.

Observation and Results

	E. coli	***B. cereus***	***S. aureus***	***Mixture***
Draw a representative field.				
Cell morphology: Shape Arrangement Cell color Gram reaction	 __________ __________ __________ __________	 __________ __________ __________ __________	 __________ __________ __________ __________	 __________ __________ __________ __________

Discussion

1. What are the advantages of the differential staining procedure over simple staining techniques?
2. State the purpose of each of the following reagents in the differential staining procedure.
   A. Primary dye
   B. Mordant
   C. Decoloring agent
   D. Counterstain
3. Why must the primary color and counterstain color contrast?
4. Which is the most important step in performing the Gram staining procedure? Explain.

2. BACTERIAL ACID RESISTANT COLORING

Theory

The characteristic difference between mycobacteria and other microorganisms is the presence of thick, waxy (lipoidal) walls which make penetration by dyes very difficult. Mycobacteria tend to clump, and it is difficult to identify individual cells in smear preparations if this clumping effect occurs. Avoiding or minimizing this phenomenon requires careful smear preparation. Place a small drop of water on an object, suspend the culture in the water, and mix the suspension thoroughly to remove and disperse some of the cells. Once the dye penetrates, however, it cannot be easily removed even with the use of a strong acid-alcohol as a decolorizing agent (unlike the 95% ethyl alcohol used in Gram staining). Due to this property, these organisms are called acid-fast, while all other microorganisms, which are easily decolorized by acid-alcohol, are not acid-fast. Acid fast staining uses three different reagents which are listed below along with a description of their purpose.

Carbol Fuchsin Primary Staining

Unlike cells that are easily stained by common aqueous dyes, most mycobacterial species are not resistant to common dyes such as methylene blue and crystal violet. Carbol fuchsin, a dark red dye in 5% phenol dissolves in the lipoidal material formed. Most of the mycobacterial cell walls, able to penetrate these bacteria, and are retained. Application of heat further enhances penetration, which drives carbol fuchsin through the lipoidal wall and into the cytoplasm. This heat application is used in the Ziehl-Neelsen method. The Kinyoun method, a modification of the Ziehl-Neelsen method, avoids the use of heat by adding a wetting agent (Tergitol®), which reduces the surface tension between the

mycobacterial cell wall and the stain. After application of the primary stain, all cells appear red.

Decolorizing Agent (color remover) Acid-alcohol (3% HCl 95% ethanol):

Before decolorization, the smear is cooled, which allows the waxy cell substance to harden. In acid-alcohol applications, acid-fast cells are resistant to decolorization, because the primary stain is more soluble in the cellular wax than in the decolorizing agent. In this case, the primary dye is retained and the mycobacteria will remain red. This does not occur in non-acid-fast organisms, which do not have cellular wax. Primary stains are more easily removed during decolorization, leaving these cells colorless or unstained.

Counterstain Methylene blue (counterstain):

This dye is used as a final reagent to stain previously decolored cells. Since only non-acid-fast cells decolorize, they can now absorb the counterstain and take on the blue color, while the acid-fast cells retain the red color as the primary dye.

Practical Objectives

1. Students are able to explain the chemical basis of acid-resistant staining.
2. Students are able to carry out procedures to differentiate bacteria into acid-resistant and non-acid-resistant groups.

Materials and tools

1. *Mycobacterium smegmatis* culture (72-96 hours)
2. *Staphylococcus aureus* culture (18-24 hours)
3. Carbol fuchsin
4. Acid-alcohol
5. Methylene
6. Bunsen burner

7. Hot plate
8. 250 ml beaker
9. Inoculation loop
10. Object glass
11. Absorbent paper
12. Lens paper
13. Coloring tub
14. Microscope

Procedure

Smear preparation

1. Provide three clean glass slides.
2. Using aseptic technique, prepare a bacterial smear of each organism plus a smear of a third mixture of *M. smegmatis* and *S. aureus*.
3. Let the smear dry and then heat it in the usual way.

The coloring steps are as shown in Figure 2.1

1. Initial coloring
    a. Drizzle the smear with carbol fuchsin and place it in a glass of water located on a warm hot plate, let the preparation steam for 5 minutes. Note: Do not allow the smear to evaporate; Refill the smear as needed. Additionally, prevent the smear from boiling by adjusting the hot plate temperature.
    b. For the non-heated method, drip the smear with carbol fuchsin containing Tergitol for 5 to 10 minutes.
2. Wash with tap water. Heated objects must be cooled before washing.
3. Decolorize with acid-alcohol, adding the reagent drop by drop until the alcohol flows almost clear with a slight red tinge.
4. Wash with tap water

5. Drip with counterstain with methylene blue for 2 minutes.
6. Wash with tap water
7. Dry with absorbent paper and examine under immersion oil.

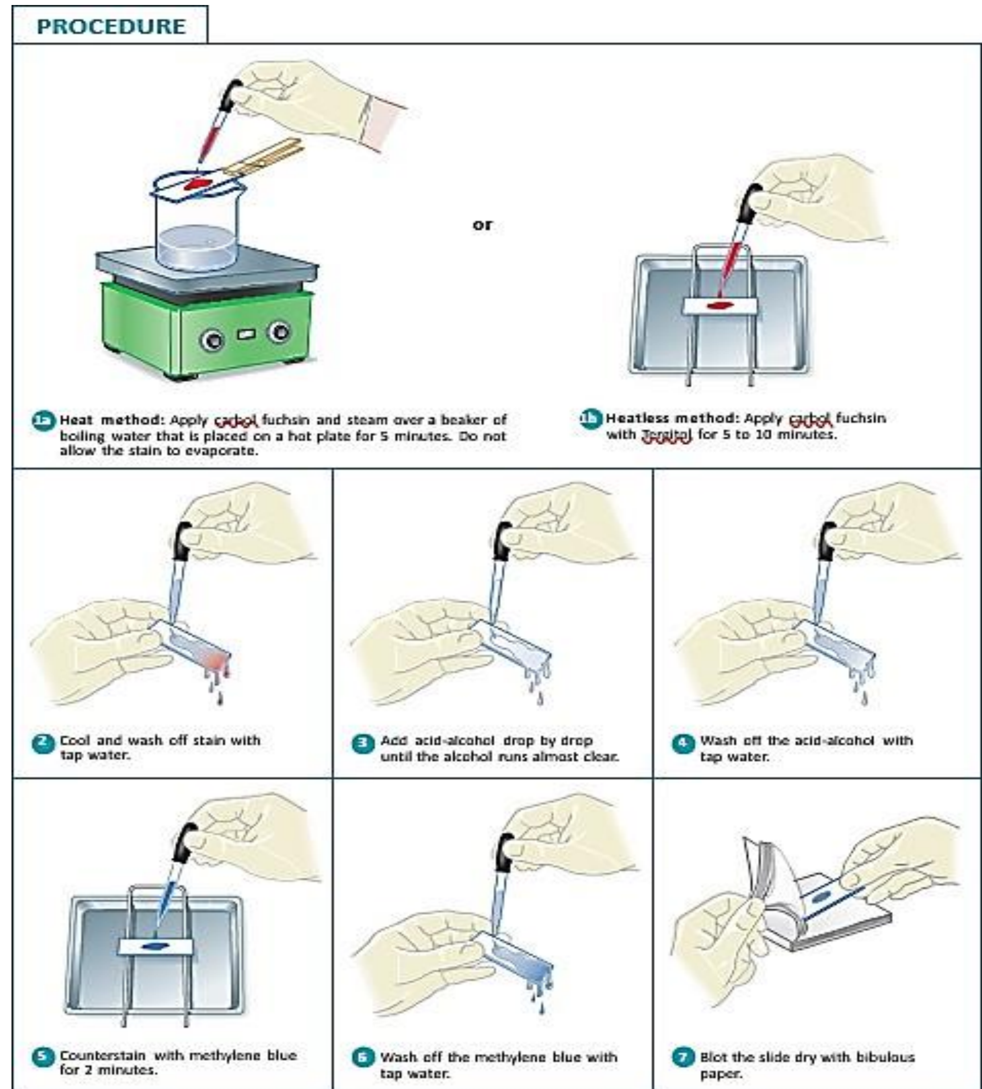


Figure 2.1. Acid fast staining procedure

Discussion

a. Draw a representative microscopic plane for each preparation.
b. Describe the cells according to their shape and sequence.
c. Describe the color of the stained cells.

d. Classify organisms based on the nature of their reactions: acid-fast or non–acid-fast.

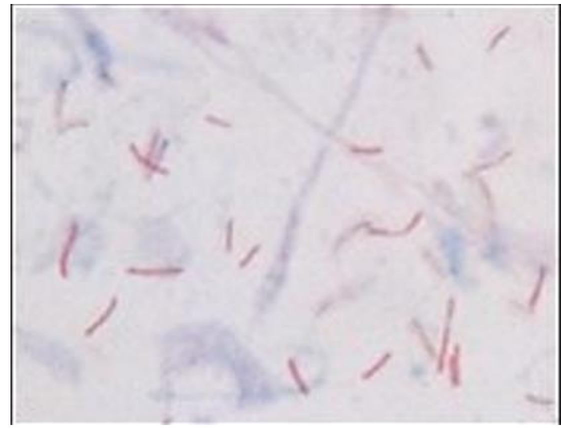


Figure 2.2. Acid-fast staining of mycobacteria

3. COLORING OF BACTERIAL CAPSULES AND ENDOSPORES

Theory

Bacteria are single-celled organisms with a complex structure. In general, it can be divided into three regions, namely the appendix (flagella and pili), the envelope (capsule, cell wall and plasma membrane) and the cytoplasm (DNA, RNA and ribosomes). Certain bacteria, especially those belonging to the genus *Bacillus* and *Clostridium*, form a structure in the cell in specific places, called endospores.

Capsule

Capsules may serve as reserve food, protection against phagocytosis (both within the host and in nature), or protection against dehydration. The ability to produce capsules is a genetic trait, but its production is greatly influenced by the composition of the medium in which the cells are grown. Medium composition can also influence capsule size. Capsule size varies according to the type of bacteria and can also differ between different strains within a species. In some types of bacteria, the presence of a capsule is an indication that the organism is virulent (for example, *Streptococcus pneumoniae* or pathogenic pneumococcus), so that the diagnosis of pneumonia and some other diseases is made easier by staining the capsule to determine whether it is present or not. All bacterial capsules appear to be soluble in water.

Capsule composition varies according to the organism. Some are glucose polymers (e.g., dextran in *Leuconostoc mesenteroides*), amino sugar polymers (e.g., hyaluronic acid in *Staphylococcus piogenik*), polypeptides (e.g D-glutamic acid polymer in *Bacillus anthracis*) or polysaccharide-protein complexes (e.g., *Bacillus dysentery*).

Without staining, bacterial capsules are very difficult to observe with an ordinary light microscope because they are colorless and have a low refractive index.

Because the bacterial capsule is non-ionic, it cannot be stained using the usual simple staining procedures. The main problem in staining capsules is that if the prepared bacterial smear is fixed with heat according to the usual method, the capsule will be damaged; however, if it is not fixed with heat, the organism will slide off during washing. In most bacteriological work, all we need is to demonstrate the presence or absence of capsules. This goal can be easily achieved by combining negative staining and simple staining procedures (see procedures).

Endospores

Endospores can survive in conditions of nutrient deficiency, are resistant to heat and other physical elements such as freezing, dryness, ultraviolet radiation and chemicals that can destroy bacteria that cannot form spores. This resistance is caused by the presence of a thick and hard spore coat. Endospores are the most resistant life form known so far; the organisms can survive in dust and soil for years. For example, the presence of endospores in dust explains why *Bacillus* is a common contaminant in laboratories.

The nature of endospores requires harsh treatment to color them. The Gram stain procedure, for example, cannot stain it. Only when subjected to sufficient heat treatment, the suitable dyes are able to penetrate the endospores. However, once the dye enters the endospores, it is difficult to remove. There are two methods commonly used, namely the Schaeffer-Fulton method and the Dorner method. The method used here is the first method (see procedure). The Dorner method uses nigrosin and produces red colored spores and colorless sporangium. The size and location of endospores in the cell are characteristics used to differentiate the bacterial species that form them.

Practical Objectives

1. Students are able to explain the basic techniques for staining capsules and endospores

2. Students are able to carry out procedures to differentiate the morphological characters of microbes based on their ability to form capsules and endospores

A. Capsule Staining

Materials and tools

1. *Azotobacter* sp. culture (36 hours) in slanted PDA medium
2. Dye solution: 0.5% crystal violet in water
3. $CuSO_4.5H_2O$ 20% solution
4. Inoculation loop
5. Clean slide
6. Dye tub
7. Absorbent paper or tissue
8. Microscope
9. Bunsen burner

Procedure

1. Aseptically, transfer the bacterial culture in a slant of PDA media to the left end of a clean glass slide.
2. With a second slide that does not have sharp edges, spread the culture along the surface of the first slide by dragging it to the right. By doing so, the smear will be thick on the left end and thinner on the right end
3. Carefully fix your preparation over the fire so that the bacteria stick to the glass slide
4. Place the slide on a wire rack in the dye bath and allow it to cool
5. Flood the bacterial smear with 0.5% crystal violet solution for 1 minute
6. Tilt the slide with tongs and carefully wash the smear with 20% copper sulfate ($CuSO_4.5H_2O$) solution (do not use distilled water) and absorb the excess copper sulfate solution using a tissue.

7. Observe your preparation under a microscope with an objective without a cover glass (start with the lowest power objective and gradually replace it with a higher power objective). If your staining technique is good, the capsule will appear as a pale blue circle surrounding dark blue to purple cells
8. Draw a sketch of the cell with its capsule with appropriate scale and color

B. Endospore Staining

Materials and tools

1. *Bacillus subtilis* culture (72 hours) in NA slant medium
2. *Bacillus thuringiensis* culture (72 hours) in NA slant medium
3. Dye solution: malachite green in water and safranin in water
4. Aquades
5. Clean slide
6. Inoculation loop
7. Bunsen burner
8. Dye tub
9. Hot plates
10. Absorbent paper or tissue
11. Microscope

Procedure (Schaeffer-Fulton method)

1. Make a smear of *B. subtilis* and *B. thuringiensis* bacteria from slanted PDA medium followed by heat fixation
2. Do the spore staining as follows:
    a. Cover the bacterial smear with malachite green while heating for 10 minutes. Adjust the heating, so that it does not boil or dry out, you can add a few drops of coloring to prevent drying.
    b. Wait until the temperature drops then wash with running water.
    c. Add safranin dye and wait for 1 minute.

d. Rinse with running water

4. Observe the preparation under a microscope without a cover glass (start with the lowest power objective and gradually replace it with a higher power objective). Observe the endospores that are still in the cell or those that are free from the vegetative cells. If your staining technique is good, the endospores will appear green, while the vegetative cells are pink.
5. Draw sketches of several cells containing endospores with the appropriate scale and color.

   Note: the position of the endospore in the vegetative cell and the size of the endospore relative to the width of the cell.

4. CARBOHYDRATE FERMENTATION

Theory

Biochemical characters are one of the microbial identification processes besides morphological characters. Carbohydrate fermentation is one of the biochemical character tests.

Carbohydrates can be enzymatically hydrolyzed into simpler sugars by certain bacteria which can occur in aerobic and anaerobic conditions. Furthermore, sugar can be fermented into various substances such as alcohol, acid and gas depending on the type of sugar and bacterial species. Fermentation is a catabolism process that does not require O_2 (anaerobic) and organic materials are not completely oxidized to CO_2. The final metabolic product in carbohydrate fermentation will produce organic acids (lactic acid, formic acid, acetic acid) and gas (hydrogen and carbon dioxide). The formation of acid can be identified by changing the color of the indicator in the medium to yellow with a decrease in pH to acid, while the formation of gas is marked with the presence of bubbles in the Durham fermentation tube.

Practical objectives

Students are able to determine the diversity of microbial metabolism based on their ability to ferment carbohydrates

Materials and tools

1. *Bacillus substilis* culture (24 hours) in liquid nutrient medium
2. *Escherichia coli* culture (24 hours) in liquid nutrient medium
3. *Staphylococcus aureus* culture (24 hours) in liquid nutrient medium
4. Liquid glucose medium with phenol-red (PR) indicator
5. Glucose agar medium with bromothymol blue (BTB) indicator
6. Liquid lactose medium with PR indicator

7. Lactose agar medium with BTB indicator
8. Liquid sucrose medium with PR indicator
9. Sucrose agar medium with BTB indicator
10. Liquid mannose medium with PR indicator
11. Mannose agar medium with BTB indicator
12. Liquid dextrin medium with PR indicator
13. Dextrin agar medium with BTB indicator

Procedure

1. Inoculate the pure culture on each of the 2 tubes of slant nutrient medium. As a control, use medium without inoculum
2. Incubate at 37^0C for 2-3 days
3. Observe and record the results with the signs A = acid, G = gas, AG = acid and gas formed, O = no change

5. REDUCTION OF NITRATE AND HYDROGEN PEROXIDE

Theory

Nitrate is a chemical compound that is often found in waters and plays an important role in plant growth. Nitrate-reducing bacteria utilize nitrate as the final electron acceptor in respiration (anaerobic respiration). Nitrate reduction is carried out by several aerobic and facultative anaerobic bacteria which occurs in the absence of oxygen (Bhurgate and Ingole, 2014). In these microbes, anaerobic respiration is a process where the cells use inorganic compounds, namely nitrate (NO_3^-) or sulfate (SO_4^{2-}) as the final electron acceptor. The nitrate reduction process (denitrification) is the process of reducing nitrate with two electrons to nitrite (NO_2^-), or further reduction to nitric oxide gas (NO), nitrous oxide (N_2O), or dinitrogen (N_2) (Madigan *et al.,* 2018). The nitrate reduction test determines nitrite production in the nitrate reduction process by nitrate reductase (Figure 5.1). This test is carried out by adding sulfanilic acid reagent. Nitrite as a result of nitrate reduction will form a complex with sulfanilic acid to form colorless diazotized sulfanilic acid, and this complex with the addition of α-naphthylamine will form ρ-sulfobenzen-azo-α- naphthylamine which becomes red (Figure 5.2) (Cappucino and Sherman, 2014).

Partial Reduction

$$NO_3^- + 2H^+ + 2e^- \xrightarrow[\text{reductase}]{\text{Nitrate}} NO_2^- + H_2O$$

Nitrate | **Hydrogen electrons** | **Nitrite** | **Water**

Figure 5.1 Nitrate reduction process

Figure 5.2. Reaction complex between nitrite and Griess-Ilosvay (Nguyen *et al.,* 2018)

Hydrogen peroxide (H_2O_2) is an intermediate product of the aerobic respiration process which uses oxygen as the final electron acceptor. These compounds are formed when aerobic, facultative anaerobic, and microaerophilic microbes use aerobic respiration during carbohydrate degradation for energy formation. H_2O_2 reducing bacteria produce the enzyme catalase which is able to catalyze the reduction of H_2O_2 into H_2O and O_2 (Figure 5.3). The catalase test is carried out to determine the ability of several microorganisms to decompose hydrogen peroxide by producing catalase. The presence of catalase is characterized by the formation of free oxygen gas bubbles after adding H_2O_2 (Cappucino and Sherman, 2014).

$$\underset{\text{Hydrogen peroxide}}{2H_2O_2} \xrightarrow{\text{Catalase}} \underset{\text{Water}}{2H_2O} + \underset{\text{Free oxygen}}{O_2\uparrow}$$

Figure 5.3 Mechanism of hydrogen peroxide reduction reaction

Practical objectives

Students are able to determine the diversity of microbial metabolism based on their ability to reduce nitrate and hydrogen peroxide.

A. Nitrate Reduction

Materials and tools

1. *Escherichia coli* culture (24 hours) in Nutrient Broth medium
2. *Bacillus subtilis* culture (24 hours) in Nutrient Broth medium
3. *Streptococcus thermophilus* culture (24 hours) in Nutrient Broth medium
4. Nitrate broth media
5. Sulfanilic acid solution
6. α-naphthylamine solution
7. Inoculation loop
8. Bunsen burner
9. Micropipette and micro tip (size 1 mL)

Procedure

1. Inoculate each culture in Nitrate broth media using inoculation loop. As a control, use media without inoculum
2. Incubate at 37°C for 2-3 days
3. After incubation, pour 1 ml of sulfanilic acid solution and 1 ml of α-naphthylamine solution into each tube. If after shaking it forms a red color, it indicates the formation of nitrite
4. Compare the observation results with controls and record the results in tabular form

B. Reduction of Hydrogen Peroxide

Materials and tools

1. *Escherichia coli* culture (24 hours) in Nutrient Broth medium

2. *Bacillus subtilis* culture (24 hours) in Nutrient Broth medium
3. *Staphylococcus aureus* culture (24 hours) in Nutrient Broth medium
4. Slant Nutrient Agar Media
5. 3% H_2O_2 Solution
6. Inoculation loop
10. Bunsen burner
7. Micropipette and microtip (size 1 mL)

Procedure

1. Inoculate each culture on a slanted Nutrient Agar medium using inoculation loop (streak or scratch). As a control, use media without inoculum
2. Incubate at 37°C for 2-3 days
3. Tube method: after incubation, pour 3 ml of 3% H2O2 into each tube on the surface of the media. If H2O2 reduction occurs, O2 bubbles will be visible around the bacterial growth
4. Slide method: after incubation, pour 1 drop of 3% H2O2 on the surface of the glass object. Take 1 tube of bacterial culture and place it in 3% H2O2 solution. If H2O2 is reduced, O2 bubbles will be visible.
5. Compare the observation results with controls and record the results in tabular form.

6. FORMATION OF H_2S BY BACTERIA

Theory

Sulfur (S) is an element that is very necessary for living organisms, including microorganisms. This element is important in the synthesis of amino acids that make up protein. Most of the sulfur is taken up by microorganisms in the form of sulfate (SO_4). Sulfate utilization, depending on the microorganism, can occur in two ways: assimilation and dissimilation. Sulfate reduction in the dissimilation process, sulfate is used as the final electron acceptor in anaerobic respiration and hydrogen sulfide is produced, this occurs in anaerobic bacteria. Sulfate reduction in the assimilation process, sulfate is metabolized into hydrogen sulfide and then into sulfur-containing amino acids, this occurs in facultative aerobic bacteria (Figure 6.1). Amino acids that contain sulfur are cysteine and methionine.

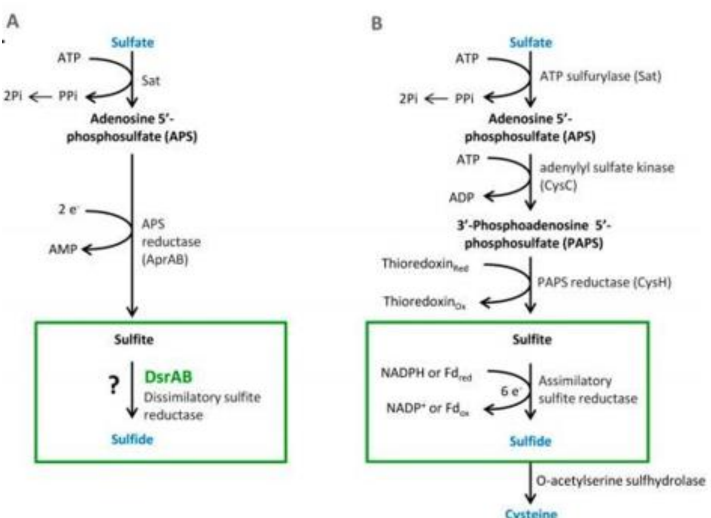


Figure 6.1 The process of dissimilation and assimilation sulfate reduction

Medium Triple Sugar Iron (TSIA) is a differential medium containing sulfate in the form of sodium thiosulfate and three types of sugar, namely glucose, lactose and sucrose. This medium is used to analyze the ability of bacteria to form hydrogen sulfide (H_2S) and ferment carbohydrates. The formation of H_2S by bacterial

metabolic activities is known through the formation of FeS. Hydrogen sulfide will react with Fe^{2+} metal contained in the medium to become FeS (Ferro Sulfide) which is black.

Practical Objectives

Students are able to determine the diversity of microbial metabolism based on their ability to reduce sulfate.

Materials and tools

1. *Escherichia coli* culture (24 hours) Nutrient Broth medium
2. *Bacillus subtilis* culture (24 hours) Nutrient Broth medium
3. *Staphylococcus aureus* culture (24 hours) Nutrient Broth medium
4. *Salmonella typhi* culture (24 hours) Nutrient Broth medium
5. TSIA (Triple Sugar Iron Agar) media (upright)
6. Inoculation loop
7. Bunsen burner

Procedure

1. Inoculate each pure culture by puncture into 1 tube of TSIA medium. As a control, use medium without inoculum
2. Incubate at 37^0C for 2 days
3. After incubation, observe the formation of H_2S which is indicated by the presence of black areas of bacterial growth.
4. Record the observation results in table form

7. FORMATION OF AMMONIA BY BACTERIA

Theory

Protein is a substrate in the medium needed for bacterial growth. In nutrient broth medium, the protein sources used are yeast extract and peptone. Protein utilization by bacteria varies depending on their metabolism. Some bacteria utilize proteins by carrying out ammonification. The ammonification process is the hydrolysis of proteins by releasing amine groups from amino acid compound molecules. The released amine group (NH2) is converted to ammonia (NH3) (Figure 7.1).

$$R\text{-}NH_2 + HOH \xrightarrow[\text{enzimatik}]{\text{Hidrolisis}} R\text{-}OH + NH_3 + \text{Energi}$$

Figure 7.1 Ammonification reaction in bacteria

Potsma test is applied for detection of the formation of NH3 by bacteria. The free NH3 produced by bacteria will bind the MgO reagent and produce the weak base NH3OH which will change the color of litmus paper from red to blue.

Practical Objectives

Students are able to determine the diversity of microbial metabolism based on their ability to produce ammonia.

Materials and tools

1. *Escherichia coli* culture (24 hours) in nutrient broth medium
2. *Bacillus subtilis* culture (24 hours) in nutrient broth medium
3. *Staphylococcus aureus* culture (24 hours) in nutrient broth medium
4. *Salmonella typhi* culture (24 hours) in nutrient broth medium
5. Nutrient broth medium, pH 7.2
6. MgO

7. Litmus Paper
8. Inoculation loop
9. Bunsen burner

Procedure

1. Inoculate the pure culture in liquid nutrient medium, 1 tube each. As a control, use medium without inoculum
2. Incubate at 37^0C for 2 days
3. After incubation, add 0.1 gram of MgO, place red litmus paper at the mouth of each tube so that the litmus paper is sandwiched by the cotton cap.
4. Place the tubes in boiling water for 5 minutes
5. Observe the color change of the litmus paper. If the litmus paper turns blue, it indicates the presence of ammonia.

8. MICROBIAL GROWTH PATTERNS

Theory

Growth is the result of cell division and is the final stage in microbial life. In the field of microbiology, growth is defined as an increase in the number of cells. Microbial growth can be determined by calculating the microbial population using either direct or indirect methods. Measurement of microbial growth can be used to determine microbial growth patterns.

Microbial growth patterns in closed culture (Batch Culture) provide an overview of the growth phases during a certain incubation period. In bacteria, the growth phases include the lag phase (adaptation phase), exponential (logarithmic) phase, stationary phase and death phase. The length of time for each phase of each bacteria varies depending on the type of bacteria and the culture conditions used. In fungi, the growth phases include the lag phase, exponential phase, acceleration phase, exponential phase, deceleration phase, stationary phase and death phase.

Practical Objectives

1. Students are able to construct microbial growth patterns by calculating microbial populations based on cell number (cells/mL) using the direct method
2. Students are able to construct microbial growth patterns by calculating microbial populations based on the number of colonies (CFU/mL) using the indirect method.

A. Counting Microbial Populations Using Indirect Methods

Materials and tools

1. *Escherichia coli* culture (24 hours) on nutrient agar media
2. *Bacillus subtilis* culture (24 hours) on nutrient agar media

3. *Staphylococcus aureus* culture (24 hours) on nutrient agar media
4. *Streptococcus thermophilus* culture (24 hours) on nutrient agar media
5. *Pseudomonas fluorescens* culture (24 hours) on nutrient agar media
6. Nutrient Broth media
7. Physiological salt (NaCl 0.85%)
8. Nutrient agar medium
9. Alcohol 70%
10. Micropipette
11. Microtip
12. Test tube
13. Incubator
14. Erlenmeyer flask
15. Micro centrifuge tube
16. Shaker incubator
17. Vortex
18. Bunsen burner

Procedure

1. Inoculate 1 loop of bacterial culture in 25 mL of NB media and incubate at 30°C in an incubator shaker at a speed of 150 rpm for 10 hours.
2. At 0, 4 and 8 hours, calculate the cell density, starting with making a culture dilution series.
3. At hour 0, carry out a 10-1 dilution by taking 1 ml of culture and placing it in 9 ml of sterile physiological saline.
4. Carry out the next dilution by taking 100 µl of the 10-1 dilution and placing it in a sterile microcentrifuge tube containing 900 µl of sterile physiological salt to obtain a 10-2 dilution. Next, carry out graded dilutions until you get a dilution of 10-6.

5. Inoculate 2 µl of each dilution 10-1 to 10-6 via drop plate in NA medium and incubate in an incubator at 30°C for 24 hours.
6. At the 4th hour of growth observation, inoculation is carried out in a culture dilution series of 10-1 to 10-8 using a drop plate in NA medium and incubated in an incubator at 30°C for 24 hours.
7. At the 8th hour of growth observation, inoculation is carried out in a culture dilution series of 10-1 to 10-12 using a drop plate in NA medium and incubated in an incubator at 30°C for 24 hours.
8. At the 10th hour of growth observation, inoculation is carried out in a culture dilution series of 10-4 to 10-12 using a drop plate in NA medium and incubated in an incubator at 30°C for 24 hours.
9. Microbial population growth is determined by counting the number of colonies that grow using a colony counter. The number of cells/ml is determined based on the number of colonies per mL (CFU/mL) using the following formula:

   Number of cells/ml (CFU/ml) =

   Number of colonies per plate x $\frac{1000\ \mu l}{2\ \mu l}$ x $\frac{1}{f.dilution}$
10. Data is displayed in graphical form with the number of cells per mL (CFU/mL) as the y-axis and incubation time (days) as the x-axis
11. Using data on the number of cells at the 4th and 8th hours, calculate the number of generations and generation time of the bacteria.

Number of Generations (n) = 3.3 (log Nt-log No)

n = Number of generations

Nt = Final number of cells

N0 = Initial number of cells

Generation Time (g) = t/n

g = generation time (time unit)

t = time interval

n = number of generations

B. Counting Microbial Populations Using the Direct Method

Materials and tools

1. *Trichoderma* sp. culture (5 days) on potato dextrose agar media
2. *Aspergillus niger* culture (5 days old) on potato dextrose agar media
3. 0.05% Tween-80 solution
4. 70% Alcohol
5. Hemocytometer
6. Micropipette
7. Microtip
8. Inoculation loop
9. Test tube
10. Erlenmeyer flask
11. Incubator
12. Microscope
13. Vortex
14. Hand counters
15. Bunsen burner

Procedure

1. Observation of the hemocytometer on a microscope with 400x magnification
    a. Place the hemocytometer on the microscope
    b. Adjustable magnification up to 400x
    c. Observe the box on the hemocytometer

d. Calculate the volume of large, medium and small boxes on the hemocytometer

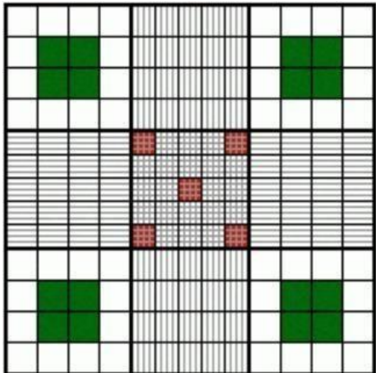
2. Counting mold spores using a hemocytometer

   a. Add 5 ml of Tween-80 0.05% to each culture little by little

   b. Scrape with a inoculation loop until all the fungus is released from the PDA media (make sure there is no media carried away), pour into a sterile 50 mL Erlenmeyer flask and homogenize using a vortex.

   c. Pour 20 µL of each culture into the hemocytometer using a micropipette and cover using a glass cover.

   d. Count the average number of spores from 5 medium boxes under a microscope (spores/ml). If the spores are too dense to count then dilute them.



   e. Count the number of spores using the following formula:

$$\textbf{Jumlah Spora (sel/mL)} = \frac{n}{La \times d} \times \frac{1}{fp}$$

Information:

n = average number of spores from 5 boxes

d = depth (0.1 mm)

La = Base area (1/25 $mm2$)

Fp = dilution factor (at the measurement stage above retail factor = 5).

9. ABIOTIC FACTORS AFFECTING MICROBIAL GROWTH

Microbial growth generally depends and is influenced by environmental factors. Changes in environmental factors can result in changes in morphological and physiological characteristics. This is because, apart from requiring suitable nutrients for their growth, microbes also need environmental factors that allow their optimum growth.

Abiotic factors that influence growth include temperature, pH, osmotic pressure, and ultra violet.

A. Effect of temperature

Theory

Temperature is an environmental factor that is important for the survival of bacteria. Low temperatures can slow down cellular metabolism, while temperatures that are too high can denature enzyme proteins. The best temperature for the life of living things is called the optimum temperature.

Based on the optimum temperature for growth, bacteria can be grouped into 4, namely psychrophiles (low optimal temperature), mesophiles (medium optimal temperature), thermophiles (high optimal temperature), and hyperthermophiles (very high optimal temperature). Psychrophilic bacteria can grow at an optimal temperature of 15° C or lower, a maximum growth temperature below 20° C, and a minimum growth temperature of 0° C or lower. Mesophyll bacteria can grow in the temperature range of 20-45°C. Thermophilic bacteria grow at temperatures of 45-80°C. Hyperthermophilic bacteria live at an optimum temperature of 80° C or greater (Madigan, 2019).

Practical Objectives

Students are able to determine the effect of temperature on microbial growth.

Materials and tools

1. *Bacillus subtilis* culture (24 hours) in slant nutrient medium agar
2. *Escherichia coli* culture (24 hours) in slant nutrient medium agar
3. Nutrient agar media (tilt)
4. 70% Alcohol
5. Inoculation loop
6. Incubator
7. Test tube
8. Refrigerator
9. Test tube rack
10. Bunsen burner

Procedure

1. Inoculate 1 dose each of *B. subtilis* and *E. coli* cultures on nutrient agar slants, each culture is inoculated in 4 different tubes
2. Separate each tube containing the culture and incubate each culture at room temperature, refrigerator, 37^0C and 55^0C for 2 days
3. Note the growth in each tube and use the signs for growth as follows:

   +++= grows extraordinary

   ++ = growing well

   + = grows slightly

   - = does not grow

Table 9.1 Effect of temperature on microbial growth

Bacterial spesies	**Cell culture growth (turbidity of cell culture suspension)**				
	Repetition	**Temperature at 4°C**	**Room temperature (± 25°C)**	**Temperature at 37°C**	**Temperature at 55°C**
Escherichia coli	1				
	2				
	3				
	4				
	5				
Bacillus subtilis	1				
	2				
	3				
	4				
	5				

B. Osmotic pressure

Theory

Osmotic pressure is one of the abiotic factors that influences microbial growth because the concentration of the environment outside the microbial cell will affect the osmoregulation of the microbial cell. Osmoregulation is the process of regulating fluid concentration and balancing fluid intake and output by living cells or organisms. If microbes are placed in a hypotonic environment, their cells will experience plasmolysis, and in a hypertonic environment they will experience crenation, whereas in an isotonic environment, the microbial cells will maintain their shape because the concentration between the outside of the cell and inside the cell is the same.

Practical Objectives

Students are able to determine the effect of osmotic pressure on microbial growth.

Materials and tools

1. *Bacillus subtilis* culture (24 hours) in nutrient broth medium
2. *Escherichia coli* culture (24 hours) in nutrient broth medium
3. Medium nutrient, upright
4. Sterile distilled water
5. 30% NaCl solution
6. 40% sucrose solution
7. 70% Alcohol
8. Sterile test tubes
9. Test tube rack
10. 10 ml and 1 ml pipettes
11. Sterile petridishes
12. Ruler
13. Incubator
14. Bunsen burner

Procedure

1. Fill 2 sterile test tubes each with 10 ml of 40% sucrose solution
2. Put 1 ml of 40% sucrose solution into a test tube containing 9 ml of sterile distilled water and mix until homogeneous (a 4% sucrose solution is formed), make 2 tubes
3. Also make 0.4% sucrose solution in 2 tubes each
4. In the same way, make 30%, 3% and 0.3% NaCl solutions, 2 tubes each
5. Melt the nutrient agar medium and cool to a temperature of 50^{0}C
6. Pour the nutrient agar medium into 6 sterile petri dishes respectively
7. After solidified, invert the petri dish and with a pencil the glass is marked into 6 parts

8. Inoculate each *B. subtilis* and *E. coli* into the series of solutions that have been made and homogenize. Immediately after inoculation, scratch each on each sector that has been marked
9. Repeat the work again 30, 60, 90, and 120 minutes, after the bacterial suspension is in solution
10. Incubate at 37^0C for 2 days
11. Observe the growth of bacteria in each sector of each petridish
12. Observation results are entered in the observation table
13. Use the following signs for growth:

    +++ = extraordinary growth

    ++ = growing well

    + = grows slightly

    - = does not grow

Table 9.2 Effect of osmotic pressure (NaCl solution) on microbial growth

Isolate Name	**Concentration**	**Repetition**	**Incubation time**		
			0 minute incubation	**60 minutes after incubation**	**120 minutes after incubation**
	30 %	1			
		2			
		3			
		4			
		5			
	3%	1			
		2			
		3			
		4			
		5			
	0,3%	1			
		2			
		3			
		4			
		5			

Table 9.3 Effect of osmotic pressure (sucrose solution) on microbial growth

Isolate Name	Concentration	Repetition	Incubation time		
			0 minute incubation	60 minute after incubation	120 minute after incubation
	40 %	1			
		2			
		3			
		4			
		5			
	4%	1			
		2			
		3			
		4			
		5			
	0,4%	1			
		2			
		3			
		4			
		5			

C. Oligodynamic Power

Basic theory

Other abiotic factors that influence microbial growth are heavy metals, where some metals have oligodynamic power, namely the ability to have a lethal or death effect on microbes. The most toxic and toxic forms of heavy metals are their ionic groups, for example: Pb^{2+}, Cd^{2+}, As^{3+} and others. Heavy metal ions will denature proteins in bacterial cells by binding to their reactive groups so that the proteins become inactive, for example the oligodynamic force of copper (Cu) where copper ions will induce the formation of Reactive Oxygen Species (ROS) which causes cell damage and copper ions will bind to DNA, cellular proteins and enzymes so that cells are damaged and die.

Practical Objectives

Students are able to understand the effect of oligodynamic forces on microbial growth.

Materials and tools

1. *Bacillus substilis* culture (24 hours) in nutrient broth medium
2. *Escherichia coli* culture (24 hours) in nutrient broth medium
3. Nutrient agar media, upright
4. 10% nitric acid solution
5. 70% Alcohol
6. Sterile Petridishes
7. Copper metal (Cu)
8. Lead metal (Pb)
9. Beaker glass
10. Vernier caliper
11. Incubator
12. Bunsen burner
13. Tweezers
14. Inoculation loop

Procedure

1. Clean the copper metal by soaking it in a 10% nitric acid solution
2. Wash with sterile water until all the acid is washed off
3. Inoculate each culture of *B. subtilis* and *E. coli* using the sprinkling method on nutrient agar medium. This method is carried out by pouring 1 mL of *B. subtilis* and *E. coli* culture each into a sterile petri dish then pouring the thawed nutrient agar (which is still lukewarm/at a temperature of approximately 40°C) into the containing petri dish. Culture aseptically, shake

the petri dish as necessary to form a figure 8 so that it is evenly distributed before the medium solidifies

4. Divide the petridish in half with a marker and ruler
5. Place the metal in the middle of each petridish containing the culture
6. Part 1 for copper metal (Cu) and part 2 for lead metal (Pb)
7. Incubate at 37ºC for 2 days
8. Draw and measure the respective growth inhibition zones by lead metal and copper metal

Table 9.4 Effect of oligodynamic forces on microbial growth

Isolate name	**Repetition**	**Metal**	
		Pb (Lead)	**Cu (Copper)**
	1		
	2		
	3		
	4		
	5		
	1		
	2		
	3		
	4		
	5		

Information:

+ (capable of being inhibited by heavy metals marked with a clear zone)

- (not able to be inhibited by metal, indicated by no clear zone)

D. Effect of UV Rays

Theory

One way to control the growth of pathogenic bacteria is by using ultraviolet (UV) radiation. Ultraviolet radiation is an energy source that has the ability to penetrate the cell walls of microorganisms and change the composition of their nucleic

acids. Ultraviolet radiation absorbed by proteins in cell membranes will cause cell membrane damage and cell death.

UV light is used because it is effective in killing bacteria without causing a bad impact on the environment. The effectiveness of ultraviolet light in killing bacteria is influenced by several factors. These factors include the size of the room, the intensity of the light used, the distance from the light source to the bacteria, the length of exposure time, and the type of bacteria itself. UV rays have very low penetration power, even a thin layer of glass can block most of the UV rays, so UV rays are only effective for controlling microorganisms on surfaces that are directly exposed to UV rays.

Practical Objectives

Students are able to explain the effect of UV light on microbial growth.

Materials and tools

1. *Bacillus substilis* culture (24 hours) in nutrient broth medium
2. *Escherichia coli* culture (24 hours old) in nutrient broth medium
3. Nutrient agar media, upright
4. 70% Alcohol
5. Sterile petridishes
6. Tin paper / aluminum foil
7. Test tube
8. Test tube rack
9. Bunsen burner
10. Laminar Air Flow (LAF)
11. Incubator

Procedure

1. Inoculate each culture of *B. subtilis* and *E. coli* using pour plate method. This method is carried out by pouring 1 mL each of *B. subtilis* and *E. coli* culture into a sterile petridish, then pouring the melted nutrient agar into the petridish containing the culture aseptically, shaking the petridish as necessary so that it is evenly distributed before solidifying.
2. Place a piece of tin foil on top of the culture in the media that has solidified and incubate for 3 hours (in a closed room or enclosure)
3. The petridish is exposed to ultraviolet light in laminar air flow for 3 hours with the petridish open.
4. Take the piece of tin foil aseptically, then cover the petridish again.
5. Incubate at 37^0C for 2-3 days

10. BIOTIC FACTORS AFFECTING MICROBIAL GROWTH

Theory

Environmental factors that influence microbial growth apart from abiotic factors are also biotic factors. Biotic factors are associations or living together between microbes. Associations can be in the form of symbiosis, synergism, antagonism and cytotropism.

Commensalism is an ecological relationship between two microbes in which one species benefits from the association without affecting the growth of the other microbe.

Synergism is an association or living relationship between two microbial species that do not interfere with each other. However, their respective activities actually benefit each other.

Antagonism is when one party harms another party. Such organisms may be very useful, because they often produce antibiotics or other inhibitory substances that can inhibit the growth of other microbes.

Practical Objectives

Students are able to explain the nature of the association between microbes during their growth.

A. Commensalism

Materials and tools

1. *Clostridium sporogenes* culture (24 hours) in nutrient broth medium
2. *Staphylococcus aureus* culture (48 hours) in liquid thioglycollate medium
3. Nutrient agar medium, upright
4. Crystal violet
5. Aquades
6. Ethanol

7. Iodine
8. Safranin
9. Inoculation loop
10. Sterile 1 ml volume pipette
11. Object glasses
12. Coloring tub
13. Spray bottle
14. Bunsen burner
15. Tripod
16. Microscope

Procedure

1. Inoculate 1 loop each of *C. sporogenes* and *S. aureus* cultures in 1 tube of the same nutrient broth medium
2. Inoculate 1 loop culture each of *C. sporogenes* and *S. aureus* in 1 tube of the same nutrient broth medium
3. Incubate each culture at 37ºC for 2 days
4. Observe whether the tubes become cloudy
5. Make a Gram's stain of each turbid tube
6. Write the observation results in the table as follows:

Table 10.1 Commensalism relationship between microbes

Bacteria	Turbidity	Gram staining
C. sporogenesis		
S. aureus		
C. sporogenus dan *S. aureus*		

B. Synergism

Materials and tools

1. *Staphylococcus aureus* culture (24 hours) in nutrient broth medium
2. *Proteus vulgaris* culture (24 hours) in nutrient broth medium
3. *Escherichia coli* culture (24 hours) in nutrient broth medium
4. Three tubes of liquid lactose fermentation medium with bromothymol blue (BTB) indicator in Durham tubes
5. Three tubes of liquid sucrose fermentation medium with bromothymol blue (BTB) indicator in Durham tubes
6. 70% Alcohol
7. Inoculation loop
8. Test tube
9. Test tube rack
10. Bunsen burner
11. Incubator

Procedure

1. Inoculate 1 loop of *S. aureus* in the lactose liquid fermentation medium
2. Inoculate 1 loop of *P. vulgaris* in the lactose liquid fermentation medium
3. Inoculate 1 loop each of *S. aureus* and *P. vulgaris* in liquid lactose fermentation medium
4. Put a mark on each tube
5. Inoculate 1 loop of *S. aureus* in liquid lactose fermentation medium
6. Inoculate 1 loop of *E. coli* in liquid lactose fermentation medium
7. Inoculate 1 loop each of *S. aureus* and *E. coli* in liquid lactose fermentation medium
8. Put a mark on each tube
9. Incubate all tubes at 37^{0}C for 2 days

10. Write the results of the observations in the table as follows:

Table 10.2 Synergistic relationship between microbes

Bacteria	Liquid lactose		Bacteria	Liquid sucrose	
	Acid	Gas		Acid	Gas
S. aureus			*S. aureus*		
P. vulgaris			*E. coli*		
S. aureus dan *P. vulgaris*			*S. aureus* dan *E. coli*		

C. Antagonism

Materials and tools

1. *Bacillus subtilis* culture (24 hours old) in nutrient broth medium
2. *Sarcina lutea* culture (24 hours old) in nutrient broth medium
3. *Escherichia coli* culture (24 hours old) in nutrient broth medium
4. Nutrient agar medium
5. Inoculation loop
6. Sterile petridishes
7. Pipette volume of 1 mL
8. Water bath
9. Incubator
10. Bunsen burner

Procedure

1. Melt 4 tubes of nutrient agar medium in a waterbath
2. Cool it to a temperature of 50^0C
3. Inoculate 0.1 ml of *E. coli* culture each on 2 melted nutrient agar tubes
4. Inoculate 0.1 ml of *S. lutea* culture each on 2 melted nutrient agar tubes

5. Pour the inoculated medium into 4 sterile petri dishes aseptically, shake the petridishes as necessary so that they are evenly distributed before solidifying.
6. Mark and name the bacteria on each petridish
7. Take 1 loop of *B. substilis* culture and streak it on the surface of the nutrient agar medium that has been inoculated with *E. coli* in a petridish
8. Without taking any more inoculant, scrape off the remaining *B. subtilis* culture that is already in the loop on the surface of the nutrient agar medium that has been inoculated with the *E. coli* culture in another petridish.
9. In the same way, inoculate 1 loop of *B. subtilis* culture on the surface of the nutrient agar medium that has been inoculated with *S. lutea* in a petridish
10. Incubate all tubes at 37^0C for 2 days
11. Observe for a zone where there is no growth (inhibition zone) around the *B. substilis* culture scratch
12. Write the observation results in the table as follows:

Table 10.3 Antagonistic relationships between microbes

The 1st bacteria in agar nutrient medium	The 2nd bacteria grown (scratched) on medium	Inhibition zone
E. coli	*B. substilis*	
S. lutea	*B. substilis*	

REFERENCES

Cappuccino, J.G. and Welsh, C. 2020. Microbiology: A Laboratory Manual. Pearson.

Deacon J.W. 2006. *Fungal Biology 4th ed.* Blackwell Publishing Ltd. Victoria. Australia

Deng, Y.J. and Shiao, Y.W. 2016. Synergistic growth in bacteria depend on substrate complexity. *Journal of Microbiology.* 54(1): 23-30.

Figuiredo, A.R.T and Jos K. 2020. Cooperation and Conflict within the Mocrobiota and Their Effects on animal Hosts. *Frontiers in Ecology and Evolution*. 8(132): 1-15.

Hatti-kaul, R., Lu C., Tarek, D., and Hesha, E.E. 2018. Lactic acid bacteria from starter culture to producers of chemicals. *Journals Investing in Science.* 365 (20): 1-21.

Herigstad, B., Hamilton, M. and Heersink, J. 2001. How to optimize the drop plate method for enumerating bacteria. *Journal of Microbiological Methods.* 44(2): 121-129.

Jawetz, m. and Adelberg's. 2018. *Medial microbiology* 28e. New York Publisher Service.

Jutono, J., Soedarsono, S., Hartadi, S., Kabirun, S., and Suhadi, D. 2019. *General Microbiology Practikum Guidelines for University*. UGM Press. Yogyakarta.

Kenny, D.J. and Balskus, E.P. 2018. Engineering chemical interactions in microbial communities. *The Royal Society of Chemistry.* 47: 1705-1729.

Madigan, M.T, Martinko, J.M and Parker, J. 2019. *Biology of Microorganisms*. Prentice-Hall.

Powedchagun, P., Hideyuki, S. and Sirait. R.2011. Characterization of a probiotic *bacillus* S11 bacterium of black tiger shrimp *Penaeus monodon. Songklanakarin J. Sci. Technol.* 33(1): 1-8.

Rifai, M.R., Hening W. and Agus, S. 2020. Synergism and antagonism of several types of bacterial isolates that are consosrsium. *Biolova.* 1(1): 21-26.

Suganthi C., Mageswari, A. Karthikeyan, S., Anbalagin M., Sivakumar, A., and Gothandam, K.M. 2013. Screening and optimizing of protease production from a halotolerant *Bacillus licheniformis* isolated from saltern sediment. *Journal of Genetic Engineering and Biotechnol.* 11(1): 47-52.

Tshikantwa, T.S., Muhammad W.U. Feng, H. and Guang, Y. 2018. Current Trends and Potential Application of Microbial Interactions for Human Welfare. *Frontiers in Microbiology*. 9:1-19.

Yusuf, Y. 2018. *Food and Nutrition Chemistry.* Jakarta: Edu Center Indonesia.