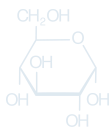


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First edition: 2023

Perpustakaan Negara Malaysia Cataloguing-in-Publication Data
Workbook: Basic Food Microbiology Analysis / Norshazila Senawi/ Ikhmal Hisham Abdul Halim / Norshafiqah Salim
e ISBN **978-967-2742-27-2**



Editor:

Mohamad Azlan Yusuff Abdul Rahim & Liyana binti Ibrahim

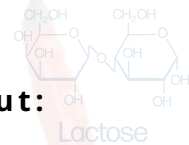
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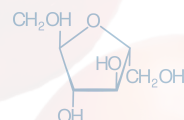
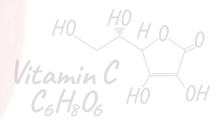
Cover Design and Interior Layout:

Norshazila Senawi
Ikhmal Hisham Abdul Halim



Published by:

Politeknik Nilai
Kementerian Pendidikan Tinggi Malaysia
Kompleks Pendidikan Bandar Enstek
71760 Bandar Enstek
Negeri Sembilan



Preface

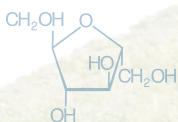
Welcome to "Basic Food Microbiology Analysis", a comprehensive guide to fundamental of laboratory techniques.

In the realm of food analysis, understanding the fundamentals is the cornerstone of ensuring the safety and quality of the food we consume. "Basic Food Analysis" is designed to be your companion on this journey, providing a comprehensive exploration of key laboratory techniques essential for anyone venturing into the field of food science.

This book covers from the initial stages of sample collection and preparation to the intricate techniques of standard plate count, determination of coliforms (*Escherichia coli*) using the Most Probable Number (MPN) method, biochemical analysis of bacteria, and the artistry of the Gram staining technique, each chapter unfolds a vital aspect of the laboratory analysis process.

The chapters are structured to provide a step-by-step guide through each crucial aspect of basic food analysis.

We want to express our utmost gratitude and appreciation to everyone who has assisted in completing this e-book. We sincerely hope that this book will be helpful for its audience.



Fructose

Glucose

Vitamin K

Vitamin C

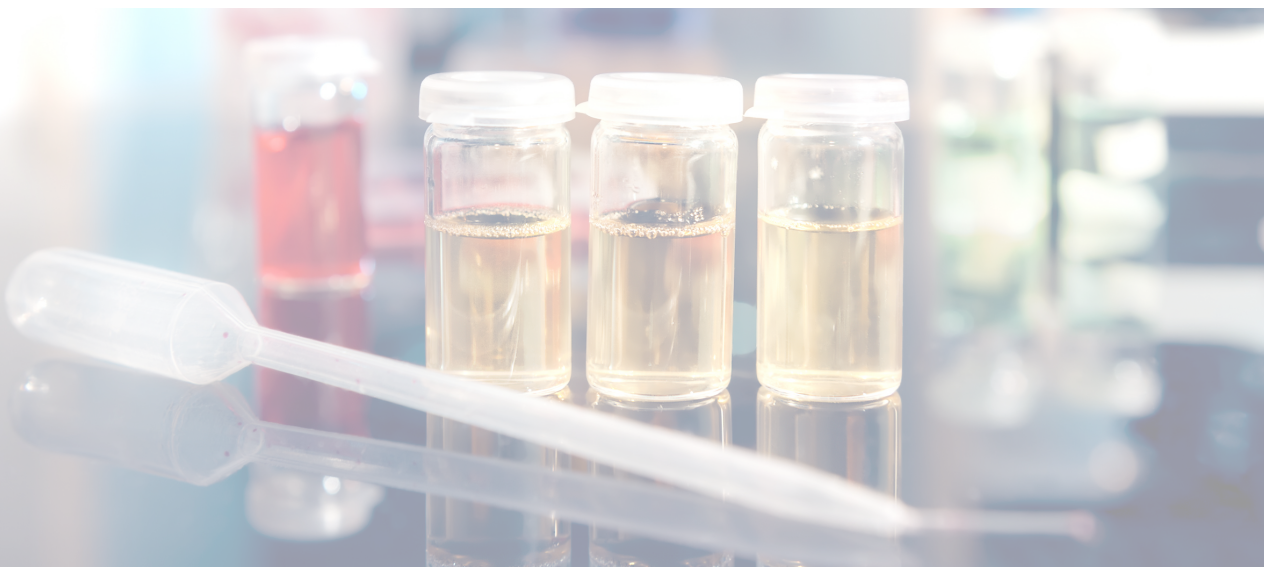
C₆H₈O₆

alpha-tocopherol

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Sample Collection and Preparation



1.1 Sampling Techniques

In food microbiology analysis, sampling is a crucial step since it directly affects the precision and dependability of the results. When samples are taken properly, they are typical of the entire batch or lot of food, enabling accurate analysis and the identification of potentially dangerous microbes. In order to preserve the integrity of the sample and avoid contamination, it is essential to set up the equipment and containers for the sampling procedure in food microbiology.

Following are general instructions on how to prepare the containers and apparatus:

a. Choose the right containers:

Containers used to place samples should be:

- Leak-Proof
- Wided Mouthed (must be suitable to protect against microbial contamination)
- Clean
- Dry

b. Gather the necessary materials:

Collect all the containers and apparatus needed for the sampling task. These may include sterile sample containers, swabs, pipettes, sampling scoops, and any other equipment required for sampling method.

c. Check for sterility:

Make sure that all containers and apparatus are sterile. This is important to avoid sample contamination, which could affect the test results. Sterility can be achieved through autoclaving, chemical sterilization, or using pre-sterilized, disposable containers and tools.

d. Properly label containers:

Clearly label each container with essential information, including sample identification, date, time, and any other relevant details. Proper labelling helps prevent mix-ups and ensures traceability.



Figure 1.1: Example of label for container.

1.2 Sample Preparation

1.2.1 Solid sample

Materials and equipments

- Sample
- Peptone water
- Stomacher bags
- Sterile pipettes

Procedures:

1. Prepare a sterile stomacher bag and weigh the sample.
2. Add a suitable volume of sterile diluent to the stomacher bag.
(e.g.: 9 ml of diluent for 1 g of sample)
3. Seal the stomacher bag securely.
4. Run the stomacher machine for about 1-3 minutes.
5. Dilute the sample in a series of dilutions with sterile peptone water.

1.2.2 Liquid sample

Procedures:

1. Measure the volume of the liquid sample to determine the appropriate dilution factor.
2. Add an appropriate volume of sterile diluent to the liquid sample (e.g., 1 ml of liquid sample to 9 ml of diluent for a 1:10 dilution).
3. Gently mix the liquid sample and diluent by inversion or gentle shaking to ensure uniform distribution.
4. Transfer a known volume of the liquid sample to sterile containers for dilutions.
5. Dilute the liquid sample in a series of dilutions with sterile diluent.

Standard Plate Count Techniques



Introduction

The **standard plate count** is a foundational element in microbiological analysis, employs two widely embraced techniques for isolating and quantifying microbial colonies: the pour-plate and spread plate methods. In the pour-plate technique, the original microbial sample undergoes meticulous dilution to achieve a suitable population reduction. Subsequently, small volumes of the various diluted samples are introduced to sterile Petri plates, where they harmoniously unite with a liquid medium maintained at a temperature between 45° to 50°C. Once the agar solidifies, each microbial cell becomes immovably anchored, forming distinct colonies if the sample dilution is optimal. In the absence of chaining or cell clusters, the total count of colonies mirrors the number of viable microorganisms in the diluted sample. To cultivate pure cultures, colonies flourishing on the surface or subsurface can be transferred to fresh medium. Conversely, the spread plate technique, another essential element in microbiological isolation, involves applying a diluted microbial sample onto an agar plate and meticulously spreading it across the surface using a glass rod. With the right sample dilution, individual cells (CFUs) are strategically placed at sufficient intervals on the agar, enabling their evolution into distinctive colonies. These techniques, both foundational and sophisticated, serve as indispensable tools in the arsenal of microbiologists, enabling the precise enumeration and isolation of microbial populations for diverse applications in research, quality control, and beyond.

2.3 Pour Plate Method

2.3.1 Materials and equipments

- Nutrient agar or another suitable agar medium
- Peptone water
- Stomacher
- Stomacher bag
- Sterile Petri dishes
- Dilution tubes
- Sterile pipettes
- Bunsen burner
- Sterile spreader or glass rod
- Sample

2.3.2 Procedures

a. Pre-lab preparation

1. Prepare a sterile pipette and petri dish.
 - Preheat the oven to the desired sterilization temperature (e.g., 160°C to 180°C).
 - Allow the oven to reach and stabilize at the set temperature.
 - Place the glass pipettes and Petri dishes in canister.
 - (Ensure that the glassware is arranged in a way that allows hot air to circulate around each piece).
 - Carefully place the arranged glassware into the preheated oven.
 - (Ensure that the glassware is evenly spaced to allow for uniform heat distribution).
 - Close the oven door and set the timer for the recommended sterilization duration (2 hours).



2. Label the bottom of each sterile Petri dish with the necessary information including sample, dilution, and date.



Make sure that the glassware is arranged in a way that allows hot air to circulate around each piece

c. Preparation of the sample & first dilution

1. Weigh into a tared stomacher bag at least 25g of sample material (or pipette 25 ml of liquid sample) representative of the food specimen.
2. Add 9 times as much dilution fluid as sample (225ml) to give a 1 in 10 dilution (10^{-1})
3. Place the bag into the stomacher and operate the machine for 1-3 minutes.

b. Preparation of stock culture for the sample

1. Prepare samples inoculated into peptone water (10 ml).
2. Incubate at 35°C for 24 hours for samples inoculated into peptone water.

c. Preparation of serial dilution

The degree of dilution should be carefully tracked to compute the concentration of bacteria in the undiluted samples. The following equation can be used to illustrate the dilution factors in general

$$\text{Dilution factor} = \frac{\text{Weight/ Volume to be diluted}}{\text{Final Weight/ Volume of diluted product}}$$

$$\begin{aligned}\text{Dilution factor} &= \frac{25 \text{ mL of sample}}{250 \text{ (Final Volume)}} \\ &= \frac{1}{10} \\ &= 10^{-1}\end{aligned}$$



When performing serial dilutions, work in a sterile area and use proper aseptic technique to minimize the risk of introducing contaminants.

Procedures:

1. Prepare dilution bottles based on the factor of dilution intended and label each bottle. (For this procedure, prepare 6 dilution bottles containing 9 ml of peptone water to obtain 1×10^{-6} dilution factor).
2. Perform the dilution process by first pipetting 1 ml of sample from the stock culture into the first bottle labelled 1×10^{-1} . Shake the dilution bottle to mix the sample.
3. Using a new pipette, transfer 1 ml of sample from the dilution bottle labelled 1×10^{-1} into the dilution bottle labelled 1×10^{-2} . Shake the bottle to mix the sample.
4. Continue the dilution process to get 10^{-6} (Refer Figure 2.1).

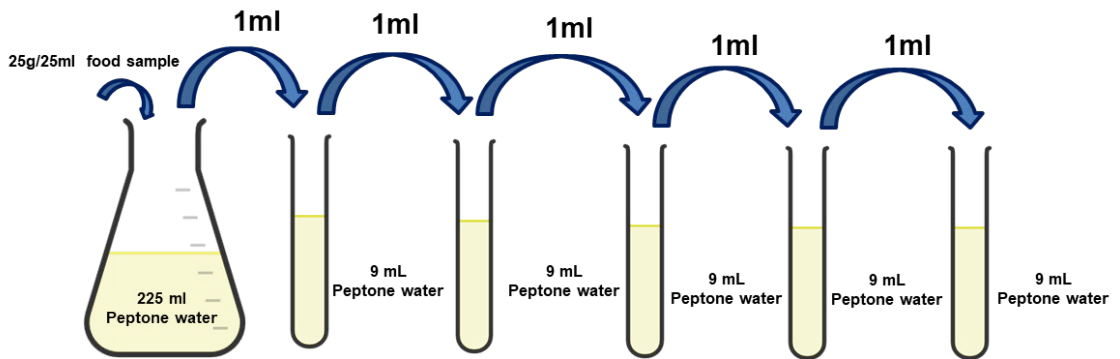


Figure 2.1: Serial dilution process

d. Transfer of inoculum

1. Pipette 1ml of sample from each dilution into petri dishes. (The use of pipette for samples must follow the same procedure as serial dilution).
2. Pour molten medium (45°C) to each plate. Duplicate for each dilution. Perform "8" movement.
3. Leave the plates until the agar medium has set.
4. Incubate all the plates at 37°C for 24-48 hours (or at the optimum temperature and for the period specified for the organism to be estimated).

e. Plate Election and Colony Counting

1. Choose the unspread plate. In standard plate count, consider a colony spread out if it covers more than half of the plate area.
2. Spread colony will be counted as one colony.
3. Choose the plate with the colony that spread uniformly.
4. Count the plate that contains 25-250 colonies.
5. Count all the colonies on the surface plate including pinpoint colony.

f. Calculation and Report

1. Record the results and refer to guidelines for calculation to compute and record colony counts.

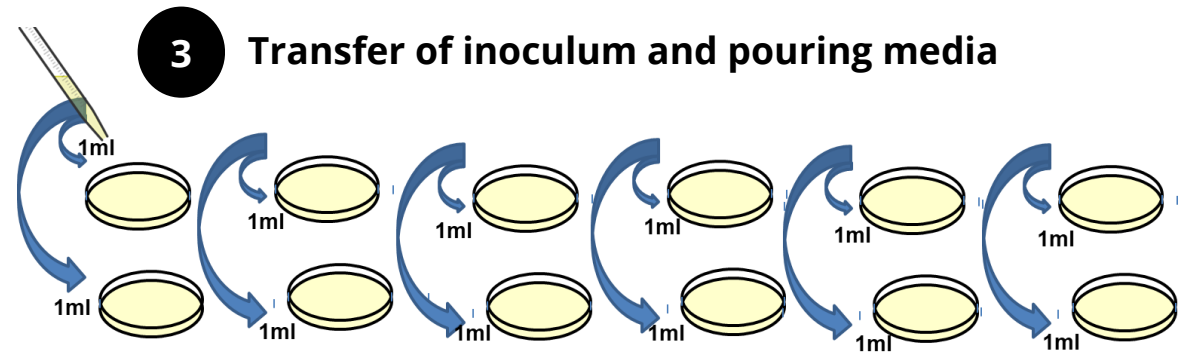
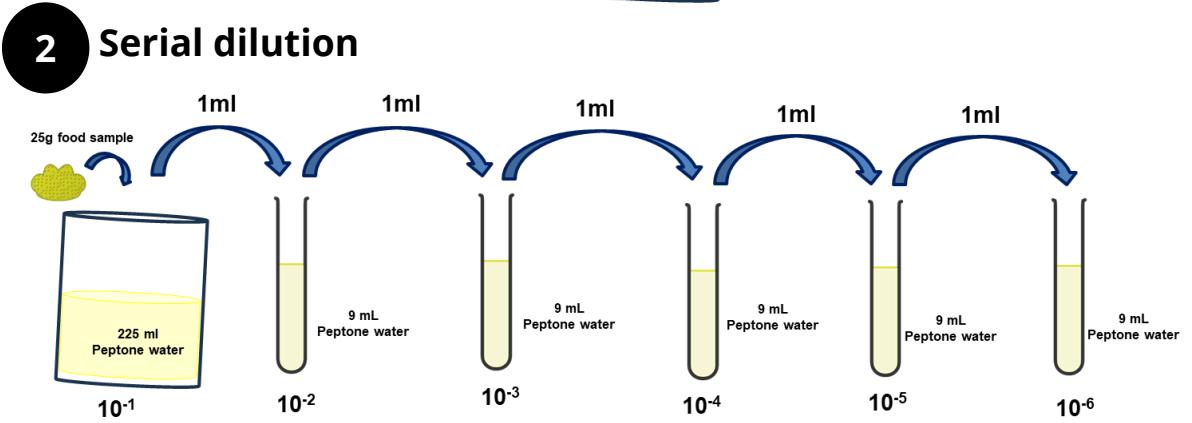
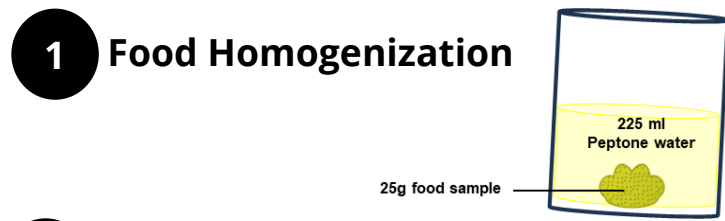


Figure 2.2: Procedure for pour plate method.

2.4 Spread Plate Method

2.4.1 Materials and Equipments:

- Nutrient agar or another suitable agar medium
- Peptone water
- Stomacher
- Stomacher bag
- Sterile Petri dishes
- Dilution tubes
- Sterile pipettes
- Bunsen burner
- Sterile spreader or glass rod
- Sample

2.4.2 Procedures

a. Pre-lab preparation

1. Prepare a sterile pipette and petri dish.
 - Preheat the oven to the desired sterilization temperature (e.g., 160°C to 180°C).
 - Allow the oven to reach and stabilize at the set temperature.
 - Place the glass pipettes and Petri dishes in canister.
 - (Ensure that the glassware is arranged in a way that allows hot air to circulate around each piece).
 - Carefully place the arranged glassware into the preheated oven.
 - (Ensure that the glassware is evenly spaced to allow for uniform heat distribution).
 - Close the oven door and set the timer for the recommended sterilization duration (2 hours).



2. Label the bottom of each sterile Petri dish with the necessary information including sample, dilution, and date.



Make sure that the glassware is arranged in a way that allows hot air to circulate around each piece

c. Preparation of the sample & first dilution

1. Weigh into a tared stomacher bag at least 25g of sample material (or pipette 25 ml of liquid sample) representative of the food specimen.
2. Add 9 times as much dilution fluid as sample (225ml) to give a 1 in 10 dilution (10^{-1})
3. Place the bag into the stomacher and operate the machine for 1-3 minutes.

d. Preparation of the sample & first dilution

1. Prepare the dilution bottles from 10^{-1} to 10^{-6} .
2. Perform the dilution process of the food sample (Figure 2.1).
3. Shake the bag vigorously by hand and pipette 1 ml into a 9 ml dilution blanks to gives a 1 in 100 dilution (10^{-2}). Make sure the pipette does not touch the diluent. Discard the pipette.
4. Shake the 10 ml suspension vigorously. Pipette 1 ml into 9 ml blank to give a 10^{-3} .
5. Continue the dilution process to get 10^{-6} .

e. Inoculation and pouring of plates

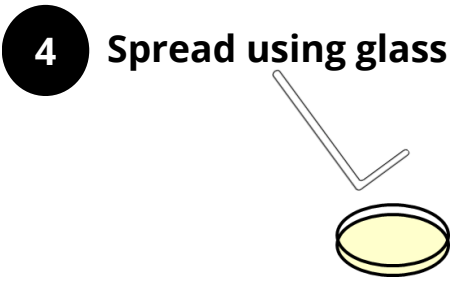
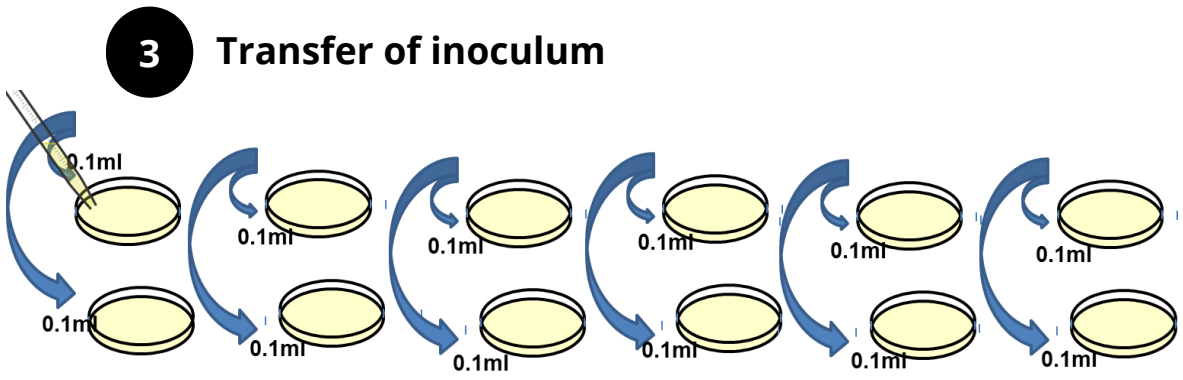
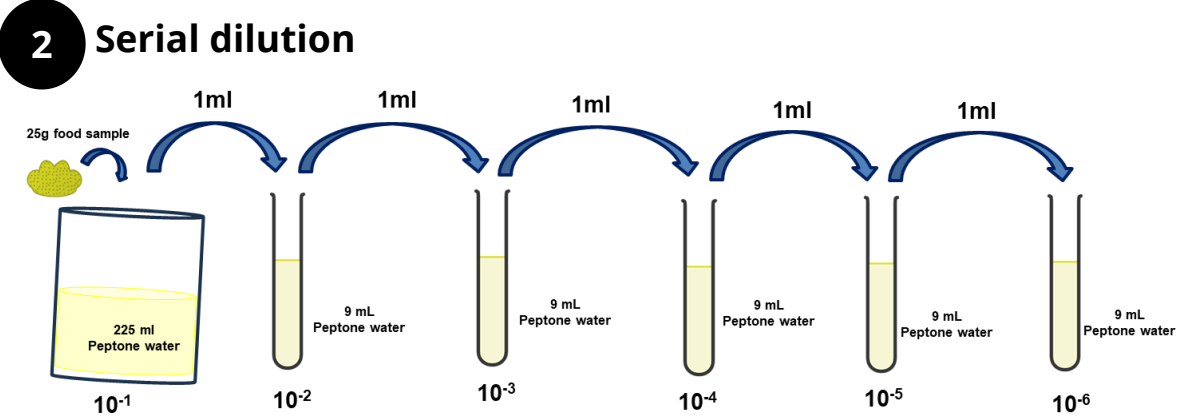
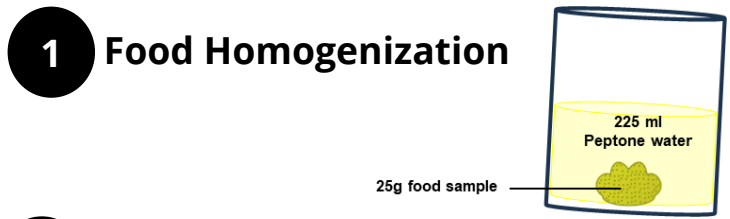
1. Pour 10-15ml of molten medium (45°C) to each plate. Duplicate for each dilution. Allow the plates until the medium to set.
2. Pipette 0.1ml of food sample from each dilution into petri dishes. Discard the pipette.
3. Spread the inoculums gently and evenly over the surfaces of the plates with a hockey stick. Discard the hockey stick. (Note: Care should be taken not to break the surface of the medium as this could result in irregular counts).
4. Continue until the necessary number of dilutions has been plated and spread.
5. Allow the plates to stand until the inoculums on each has been completely absorbed, which should be between 15minutes of spreading.
6. Incubate all the plates at 37°C for 24-48 hours.
7. Count all the colonies.

e. Plate Election and Colony Counting

1. Choose the unspread plate. In standard plate count, consider a colony spread out if it covers more than half of the plate area.
2. Spread colony will be counted as one colony.
3. Choose the plate with the colony that spread uniformly.
4. Count the plate that contains 25-250 colonies.
5. Count all the colonies on the surface plate including pinpoint colony.

g. Calculation and Report

1. Refer to guidelines for calculation to compute and record colony counts.



5 Incubate all the plates at 37°C for 24-48 hours

6 Count the colonies

Figure 2.3: Procedure for spread plate method.

Most Probable Number Method (MPN)



Introduction

The Most Probable Number (MPN) test is a statistical method used to estimate the number of coliform bacteria in a water sample. This estimation involves a three-part test: the presumptive, confirmed, and completed tests. In the presumptive test, different dilutions of the water sample are added to fermentation tubes, and after incubation, the presence of gas production indicates the likely presence of coliforms. The confirmed test involves transferring material from positive tubes to selective broth, and gas formation confirms the presence of total coliforms. The completed test further identifies coliforms by streaking samples on specific agar and conducting additional tests. The MPN index, indicating the probable number of coliforms per 100 ml, is determined based on the presumptive test results. An alternative to the classical MPN method is the Presence-Absence (P-A) test, which uses a larger water sample and is based on colour changes indicating coliform presence or absence.

3.1 Materials and Equipments

- Peptone Water
- Mac Conkey broth
- Eosin Methylene Blue agar
- Food sample
- Dilution bottle
- Petri dish
- Durham tube
- Stomacher bag
- Glass pipette

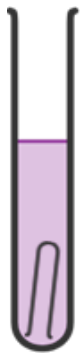
3.2 Procedures

a. Sample Dilution:

1. Weigh into a tared stomacher bag at least 25g of sample material (or pipette 25ml of liquid sample) representative of the food sample.
2. Add 9 times as much dilution fluid as sample (225ml) to give a 1 in 10 dilution (10^{-3}).
3. Place the bag in the stomacher and operate the machine for 1-3 minutes.
4. Prepare serial decimal dilutions (10^{-1} , 10^{-2} , 10^{-3}).

b. Presumptive Stage: A series of MacConkey broth primary fermentation tubes are inoculated with graduated quantities of the sample to be tested.

1. Set up three sets of three tubes containing 9ml of MacConkey. Put in Durham tube inside the bottles. Autoclave at 121°C for 15 minutes.
2. Pipette the sample as much as 1.0 ml from each of three dilutions (10^{-1} , 10^{-2} and 10^{-3}) into the 3 tubes series (make sure no air bubble trapped inside the tube). Mix the contents of the by gently rotation.
3. The inoculated tubes are incubated at 35°C for 24 to 48 hours, at which time the tubes are examined for gas formation.
4. Formation of gas in any amount within 48 hours in the tube indicates a positive presumptive test. The number of positive tubes in each set is used to cross refer to the MPN table. (Confirmation tests may be needed at this stage).



Negative result.

Negative MPN result with no changes in McConkey broth and absence of gas in the Durham tube, suggesting the absence of coliforms in the sample.



Positive result.

Positive MPN result with changes in McConkey broth and gas in the Durham tube, indicating the presence of coliforms in the sample.

Figure 3.1: Interpretation of results for presumptive stage.

c. Confirmation Stage: The confirmation test is performed on all samples showing the positive result in the presumptive stage.

1. Fermentation tubes that show positive result of MacConkey broth are streaked on Eosin Methylene Blue Agar (EMB).
2. Inoculation should be performed as soon as possible after gas formation occurs.
3. The streaked plates are incubated for 24 to 48 hours at 35°C. The coliform colonies developing on EMB agar are dark colonies with green metallic sheen and /or dark purple centers after 24 to 48hr incubation.

CALCULATION

Record the numbers of tubes in each dilution that were confirmed as positive for coliforms and calculate from MPN tables.

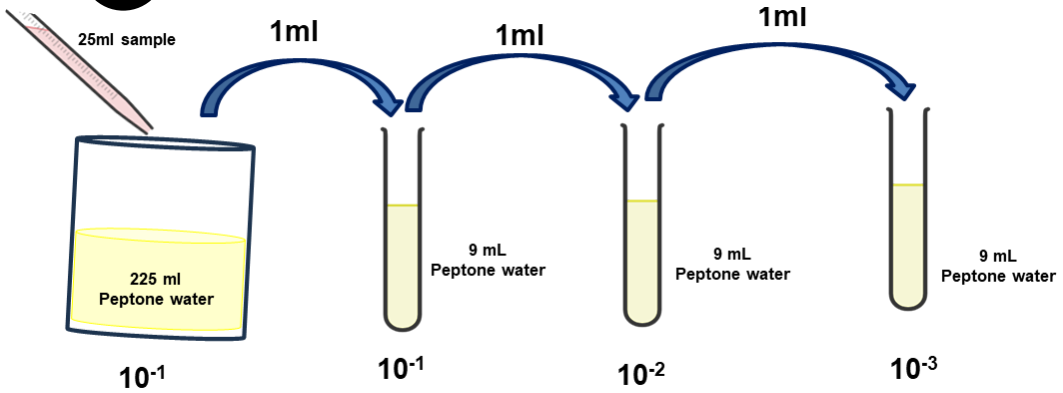
$$\text{MPN / g@ml} = \frac{\text{Result from MPN table} \times \text{Dilution factor of middle set of tubes chosen}}{\text{Dilution factor of middle set of tubes of MPN table}}$$

Table 1: Most Probable Numbers (MPN) per 1g of sample using 3 tubes with each of 0.1, 0.01 and 0.001 g Portions.

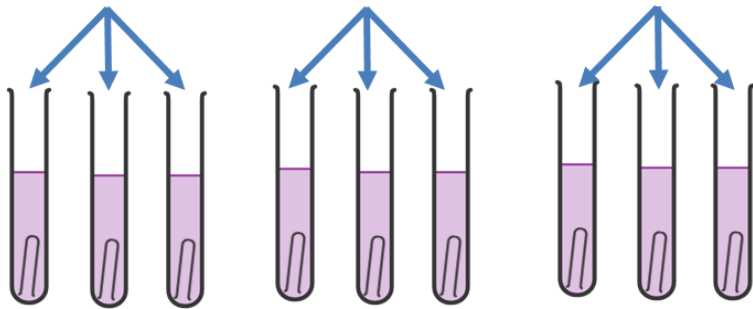
Positive Tube				Positive Tube				Positive Tube				Positive Tube			
0.1	0.01	0.001	MPN	0.1	0.01	0.001	MPN	0.1	0.01	0.001	MPN	0.1	0.01	0.001	MPN
0	0	0	<3	1	0	0	3.6	2	0	0	9.1	3	0	0	23
0	0	1	3	1	0	1	7.2	2	0	1	14	3	0	1	39
0	0	2	6	1	0	2	11	2	0	2	20	3	0	2	64
0	0	3	9	1	0	3	15	2	0	3	26	3	0	3	95
0	1	0	3	1	1	0	7.3	2	1	0	15	3	1	0	43
0	1	1	6.1	1	1	1	11	2	1	1	20	3	1	1	75
0	1	2	9.2	1	1	2	15	2	1	2	27	3	1	2	120
0	1	3	12	1	1	3	19	2	1	3	34	3	1	3	160
0	2	0	6.2	1	2	0	11	2	2	0	21	3	2	0	93
0	2	1	9.3	1	2	1	15	2	2	1	28	3	2	1	150
0	2	2	12	1	2	2	20	2	2	2	35	3	2	2	210
0	2	3	16	1	2	3	24	2	2	3	42	3	2	3	290
0	3	0	9.4	1	3	0	16	2	3	0	29	3	3	0	240
0	3	1	13	1	3	1	20	2	3	1	36	3	3	1	460
0	3	2	16	1	3	2	24	2	3	2	44	3	3	2	1100
0	3	3	19	1	3	3	29	2	3	3	53	3	3	3	>1100

Source: Peeler and McClure, 1992.
 Food Quality Control, Ministry of Health Malaysia

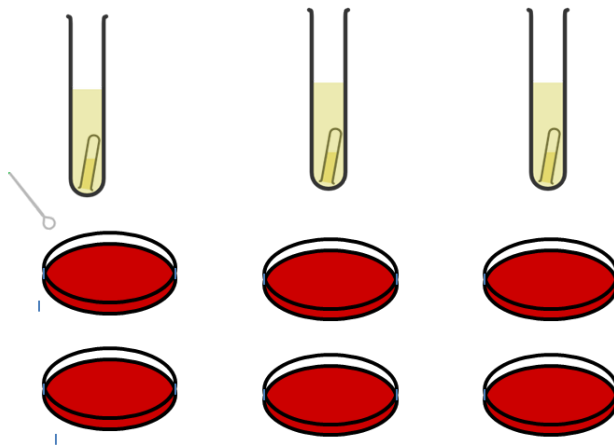
1 Serial dilution



2 Presumptive stage



3 Confirmation stage



4 Incubate all the plates at 35°C for 24-48 hours

5 Record the result

Figure 2.3: Procedure for spread plate method.

Membrane Filter Method



Introduction

This technique is specifically applied to samples containing antimicrobial or heat-sensitive substances. The process entails filtering a liquid sample through a membrane with pores too small for microorganisms to pass through. The microorganisms in the sample are retained on the membrane, which is subsequently placed on a filter pad saturated with liquid medium or on solid medium. Each colony that develops signifies an individual organism originally captured by the filter and incubated. The counted colonies, corresponding to the organisms collected on the filter, are then tallied, enabling the calculation of the number of organisms per liter of water.

4.1 Materials and Equipment

- Membrane Filter (0.45 μm or less)
- Filter flask
- Vacuum pump
- Forceps
- Petri dishes
- Cylinder
- Nutrient agar

4.2 Procedures

1. Homogenate the food sample and make any necessary dilutions if the bacteria concentration is high.
2. Flame the forceps and remove the membrane from the sterile package.
3. Place the membrane filter into the funnel assembly.
4. Flame the pouring lip of the sample container and pour 100ml sample into the funnel.
5. Turn on the vacuum and allow the sample to draw completely through the filter.
6. Rinse the funnel with sterile buffered water. Turn on the vacuum and allow the liquid to draw completely through the filter.
7. Flame the forceps and remove the membrane filter from the funnel.
8. Place the membrane filter onto the nutrient agar.
9. Incubate at 30°C for 24-48 hours.
10. Count the colonies and report the results by using the following equation:

$$\frac{\text{Total colonies}}{100\text{ml}} = \frac{\text{Colonies counted}}{\text{ml sample filtered}} \times 100$$

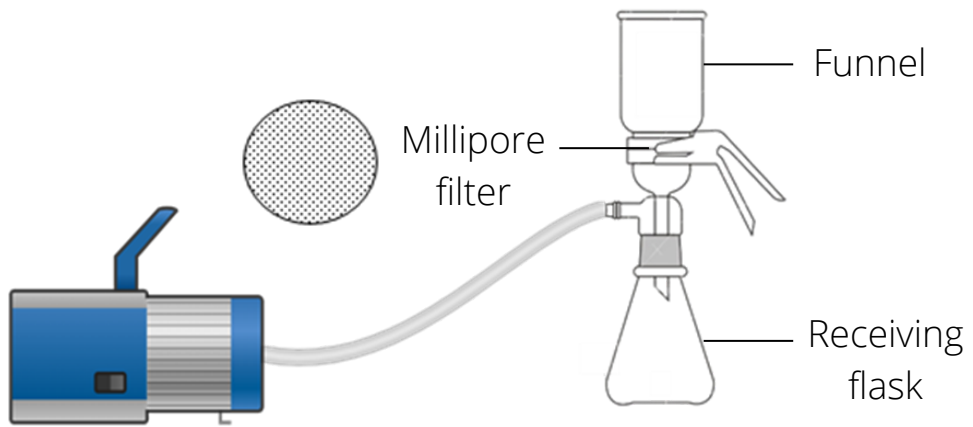


Figure 4.1: Membrane filtration devices.

Biochemical Analysis



Introduction

Biochemical tests are a set of laboratory tests used to identify and characterize bacteria based on their metabolic activities. These tests rely on the ability of bacteria to produce or utilize specific enzymes or other biochemical compounds and are commonly used in microbiology to differentiate between different bacterial species.

There are several different types of biochemical tests, including carbohydrate fermentation tests, oxidase tests, catalase tests, and indole tests, among others. Each of these tests provides different information about the metabolic activities of bacteria and can be used to identify and differentiate between different bacterial species.

Carbohydrate Fermentation Tests

This test is used to determine if a bacteria can ferment specific sugars, such as glucose, lactose, and sucrose. The presence of acid or gas production in the media indicates a positive result. This test can help identify the metabolic characteristics of microorganisms and is often used to differentiate bacterial species.

a) Materials and reagents

- Carbohydrate fermentation tubes (with inverted Durham tubes)
- Bacterial culture to be tested
- Sterile loop or inoculating needle
- Incubator
- pH indicator (e.g., phenol red or bromothymol blue)
- Paraffin or mineral oil (for sealing the tubes)

b) Procedures

1. Inoculation

- Sterilize the inoculating loop or needle by heating it in a Bunsen burner flame until red hot. Allow it to cool for a few seconds.
- Aseptically transfer a small amount of the bacterial culture to be tested by touching the loop or needle to the culture.

2. Inoculate the tubes

- Inoculate the carbohydrate fermentation tubes. Each tube should contain a specific carbohydrate as the sole carbon source such as glucose, lactose, or sucrose along with a pH indicator. The indicator may be phenol red, bromothymol blue, or another suitable pH indicator.
- Ensure that you have a control tube containing the same medium without any carbohydrate as negative control and a control tube with a known carbohydrate fermenter as the positive control for comparison.

3. Incubation

- Incubate the inoculated tubes at 35-37°C.

4. Observation

- The tubes were checked for signs of carbohydrate fermentation after 24-48 hours of incubation period.

c) Result interpretation

- A positive result in the carbohydrate fermentation test is indicated by a color change to yellow (acidic) and the presence of gas (indicated by bubbles in the Durham tube).
- A negative result is indicated by no color change (remains red or blue-green) and no gas production.

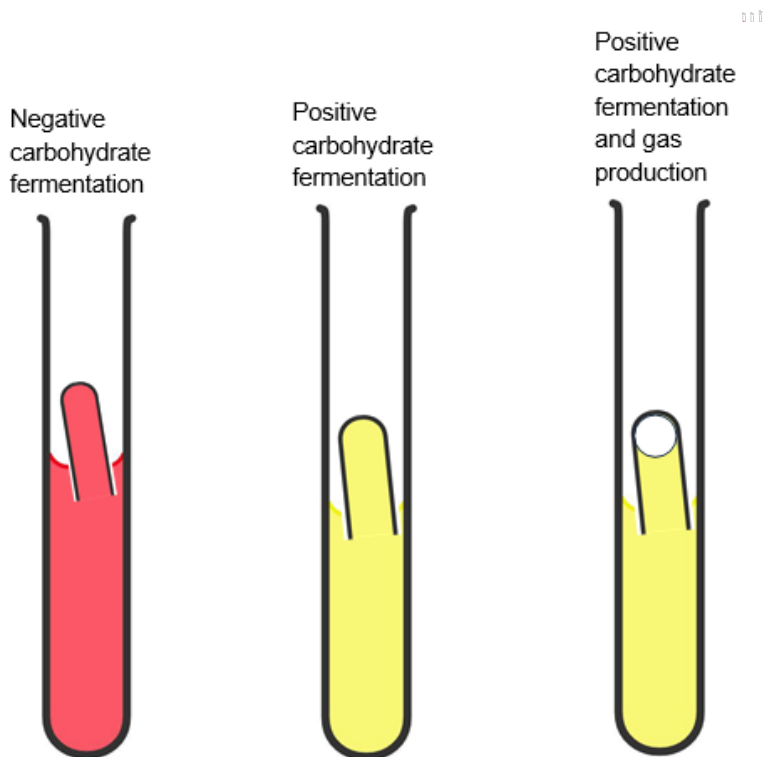


Figure 5.1 No colour changes (left), colour changes (centre) and colour changes with gas production (right)

Oxidase Test

This test is used to determine if a bacteria produces the enzyme cytochrome oxidase. It helps to distinguish between Gram-negative bacteria that are oxidase- positive and those that are oxidase-negative

a)Materials and reagents

- Bacterial culture to be tested
- Sterile wooden applicator sticks or a sterile loop
- Oxidase reagent (e.g., filter paper impregnated with oxidase reagent)

b) Procedures

1. Inoculation

- Aseptically transfer a small amount of the bacterial culture to be tested using sterile loops.

2. Application of oxidase reagent

- Gently press the inoculated loop onto the oxidase reagent(filter paper) to impregnate it with the bacterial sample.
- Ensure good contact between the bacterial culture and the reagent.

3. Color change observation

- Observe the oxidase reagent for a color change. A positive result will display a color change within 10-30 seconds

c) Result interpretation

- Positive Result: A positive oxidase test result is indicated by the appearance of a dark purple or blue color change on the reagent strip.
- Negative Result: A negative oxidase test result is indicated by no color change on the reagent strip, which means the bacterium is oxidase-negative.



Figure 5.2 Negative result (left) and positive result (right)

Catalase Test

This test is used to determine if a bacteria produces the enzyme catalase. Catalase is an enzyme that catalyzes the decomposition of hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). This test distinguishes between bacteria that produce catalase (catalase-positive) and those that do not (catalase-negative).

a) Materials and reagents

- Bacterial culture to be tested
- Sterile wooden applicator sticks or a sterile loop
- Hydrogen peroxide solution (3% or 15%)

b) Procedures

1. Inoculation

- Aseptically transfer a small amount of the bacterial culture to be tested using sterile loops.

2. Application of hydrogen peroxide

- Place the loop with the bacterial culture onto a clean and dry microscope slide.
- Add a drop of hydrogen peroxide(3% or 30%) directly to the bacterial colony or culture

3. Observation

- Look for the appearance of bubbles or effervescence.

c) Result interpretation

- Positive Result: If bubbles or effervescence is observed within a few seconds, it indicates that the bacterium possesses catalase and is catalase- positive. The presence of catalase prevents the accumulation of toxic hydrogen peroxide within the bacterial cell.
- Negative Result: No bubbles are observed. The bacteria is catalase negative.



Figure 5.3 Negative result (left) and positive result (right)

Indole Test

This test is used to determine if a bacteria can produce the enzyme tryptophanase, which can break down the amino acid tryptophan. The indole test is commonly used to distinguish between members of the Enterobacteriaceae family that are indole- positive and those that are indole-negative.

a) Materials and reagents

- Bacterial culture to be tested
- Sterile wooden applicator sticks or a sterile loop
- Kovács' reagent (p-dimethylaminobenzaldehyde)

b) Procedures

1. Inoculation

- Aseptically transfer a small amount of the bacterial culture to be tested using sterile loops.

2. Inoculation to Tryptophan medium

- Inoculate a tube of tryptophan broth or medium with the bacterial culture.
- Incubate the tube at 35-37°C.

3. Observation

- Check the tube for the presence of indole production after 24-48 hours.
- No visible change in the medium may indicate a negative result, while a color change may indicate a positive result.

4. Addition of Kovac's reagent

- To confirm the result, add a few drops of Kovács' reagent to the surface of the medium.

5. Observation of Kovac's reagent

- The presence of a red and pink color ring indicates a positive indole test result.

c) Result interpretation

- Positive Result: The appearance of a red or pink color ring in the medium after the addition of Kovács' reagent indicates that the bacterium is indole-positive, meaning it can produce indole from tryptophan.
- Negative Result: The absence of a red or pink color ring suggests that the bacterium is indole-negative, meaning it does not produce indole

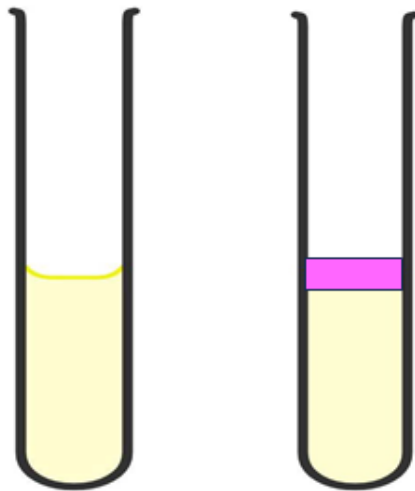
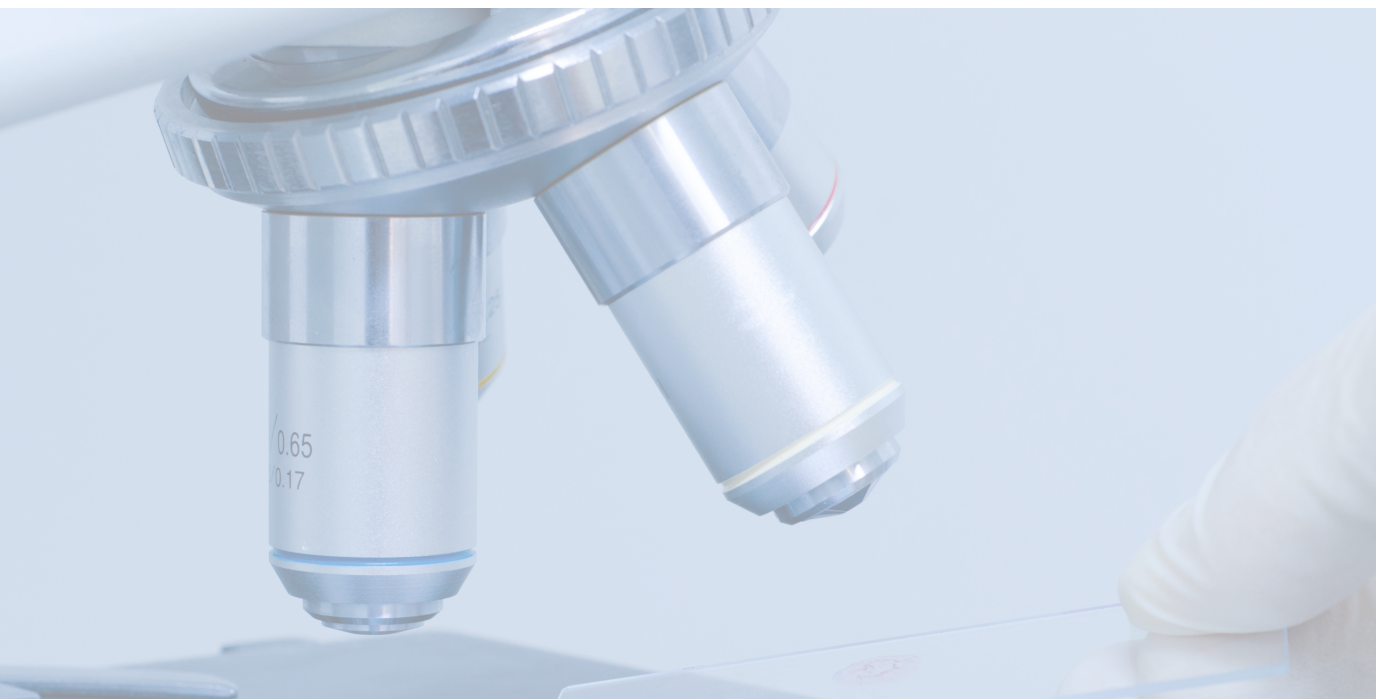


Figure 5.4 Negative result (left) and positive result (right)

Gram Staining



Introduction

Gram staining is a basic technique used in microbiology to categorize and differentiate bacteria into two primary groups: Gram-positive and Gram-negative. It was developed by Danish bacteriologist Hans Christian Gram in the 1880s. Gram staining serves several important purposes which are:

(a) cell wall differentiation: The primary objective is to distinguish between different types of bacterial cell walls. Bacterial cell walls play a vital role in the structural integrity and defense mechanisms of bacteria.

(b) classification: It helps classify bacteria into two major groups based on the characteristics of their cell walls. This classification has profound implications in microbiology, clinical diagnosis, and the selection of appropriate antibiotics.

Procedures

a) Preparation of Bacterial Smear:

- Place a drop of water on a clean microscope slide.
- Using a sterile loop or inoculating needle, aseptically transfer a small amount of the bacterial culture to the water on the slide.
- Spread the bacterial culture evenly over the slide, creating a thin, and uniform bacterial smear.
- Allow the smear to air-dry completely.
- Heat-fix the smear by gently passing the slide through a Bunsen burner flame several times, which helps to adhere the bacteria to the slide.

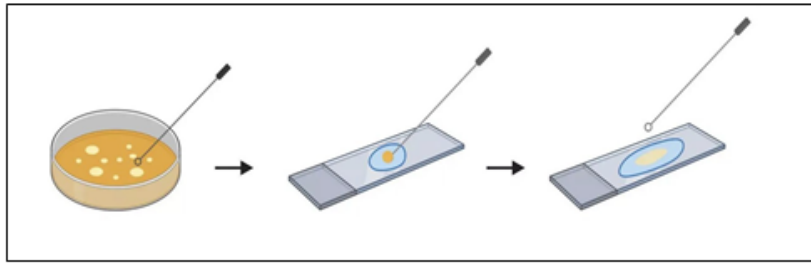


Figure 6.1 Steps in bacterial smearing

b) Gram staining procedure

1. Crystal Violet Staining (Primary Stain):

- Cover the dried, heat-fixed bacterial smear with crystal violet stain. Ensure that the entire smear is covered.
- Allow the stain to sit on the smear for about 1 minute.

2. Gram's Iodine Treatment (Mordant):

- Gently rinse the slide with Gram's iodine solution, ensuring the entire smear is covered.
- Let the iodine solution sit for about 1 minute. The iodine acts as a mordant and forms a complex with crystal violet within the bacterial cells.

3. Alcohol Decolorization:

- Carefully rinse the slide with alcohol or acetone by gently pouring the decolorizing agent over the smear. Tilt the slide slightly to ensure complete coverage.
- Watch the slide carefully. The decolorization time can vary depending on the bacterial species and the specific Gram stain kit or method being used.
- For Gram-negative bacteria, the crystal violet-iodine complex is removed from the cell wall, causing the cells to become colorless.
- For Gram-positive bacteria, the thick peptidoglycan layer retains the crystal violet-iodine complex, and the cells remain purple.

4. Counterstaining with Safranin:

- Rinse the slide with safranin stain, ensuring that the entire smear is covered.
- Allow the safranin to sit on the smear for about 1 minute.

5. Wash and Drying:

- Rinse the slide gently with water to remove excess safranin.
- Blot the slide carefully with absorbent paper to remove excess moisture.
- Allow the slide to air-dry completely.

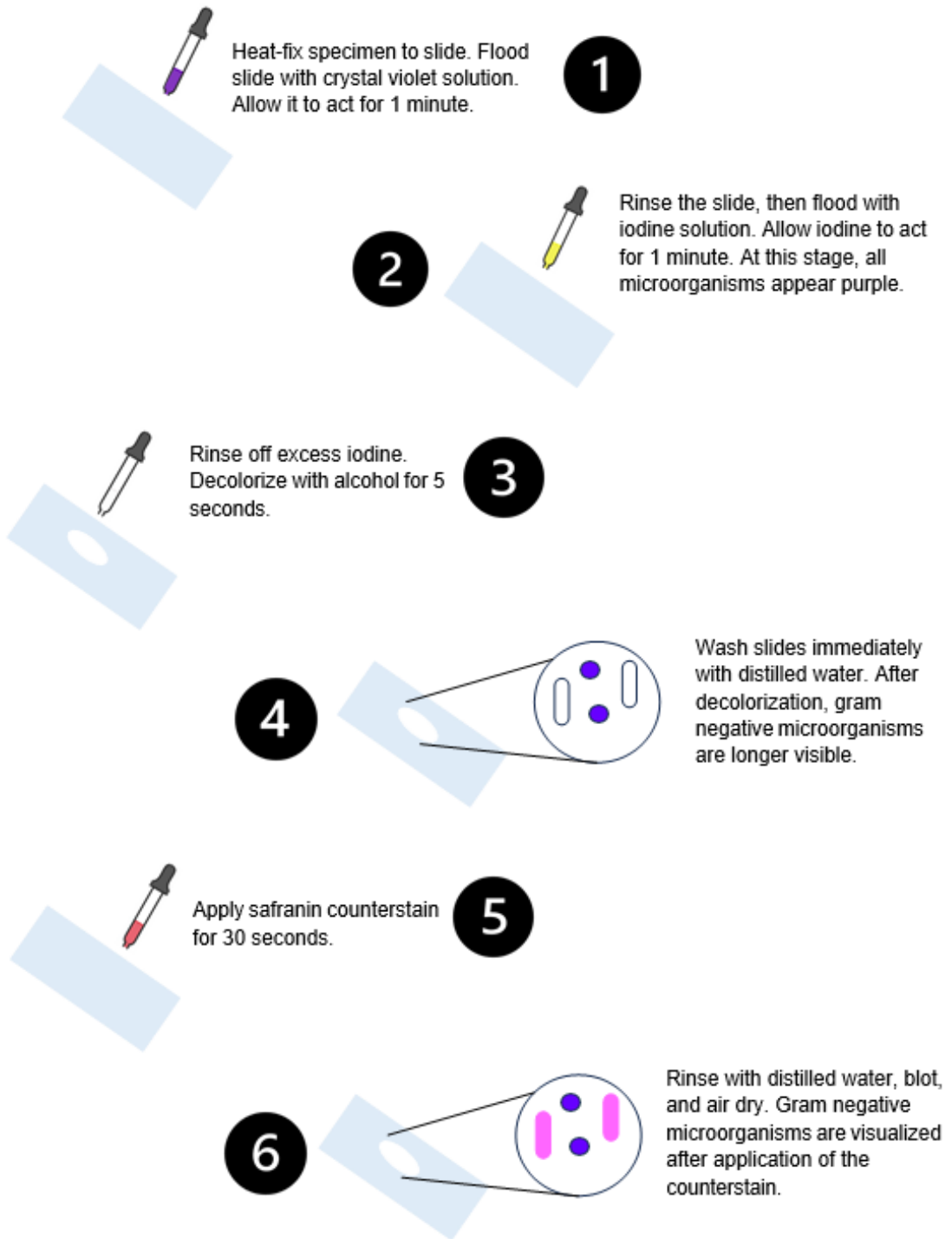


Figure 6.2 Steps in Gram staining

Interpretation of Results

1. Characteristics of Gram-Positive bacteria

- Appearance: Gram-positive bacteria will appear purple or violet under the microscope.
- Retained Stain: The purple color is retained because the thick peptidoglycan layer traps the crystal violet-iodine complex. This complex resists removal during the decolorization step.

2. Characteristics of Gram-Negative bacteria

- Appearance: Gram-negative bacteria will appear pink or red under the microscope.
- Lost Stain: During the decolorization step, the thinner peptidoglycan layer cannot hold onto the crystal violet-iodine complex, leading to the loss of the purple stain. The cells take up the counterstain (safranin), giving them a pink or red appearance.

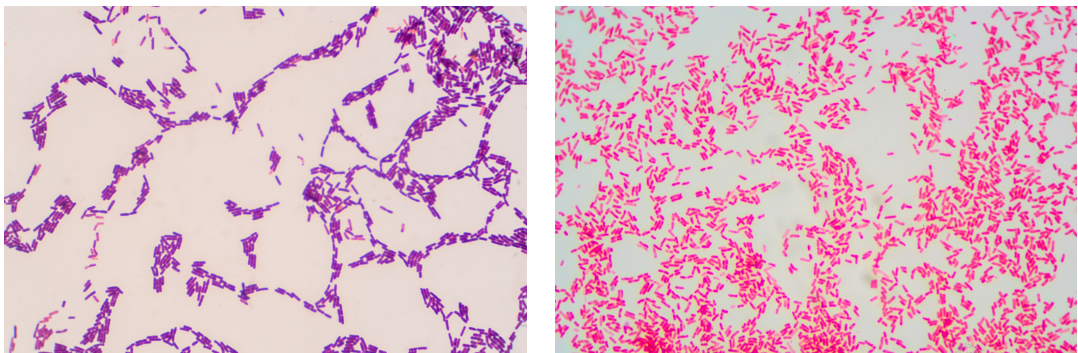


Figure 6.3 Gram-positive bacteria (left) and Gram-negative bacteria (right)

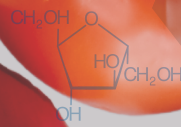
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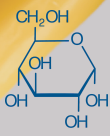
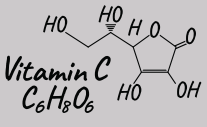
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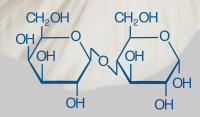
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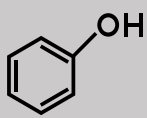
Fructose



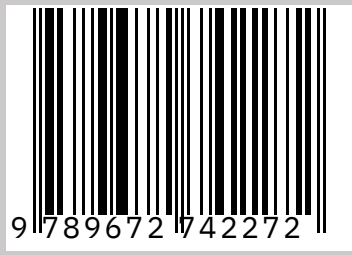
Glucose



Lactose



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