Introductory Bacteriology Lab Manual

INTRODUCTORY BACTERIOLOGY LAB MANUAL

For Students in BIOL-3001

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CONTENTS

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ABOUT THIS MANUAL

This is an introductory bacteriology lab manual used in the Chemical Laboratory Technology and Environmental Technology advanced diploma programs at Fanshawe College. These labs provide students with an opportunity to demonstrate lab skills. Students will isolate an organism from the environment, then, using controlled experiments, determine the identity of the organism using morphology, physiology and molecular methods.

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LABS

This manual contains the following labs:

- LAB 1 Microbiology Lab Safety and General Procedures
- LAB 2: Basic Techniques
- LAB 3: Bacterial Growth
- LAB 4: Isolation Exercises
- LAB 5: Gram Stain and Potassium Hydroxide String Test
- LAB 6: Bacterial Media
- LAB 7: Unknown Molecular Lab Part I
- LAB 8: Unknown Molecular Part II: Agarose Gel and PCR purification
- LAB 9: Bacteriological Analysis of Water
- LAB 10: FOOD LAB
- LAB 11: UNKNOWN LAB

LAB 1 MICROBIOLOGY LAB SAFETY AND GENERAL PROCEDURES

This lab has important safety information that will impact you throughout the course. Please read thoroughly, then complete the quiz on FOL to test your understanding.

Learning Objectives

- Review the safety precautions taken in a level 2 microbiology lab.
- Distinguish biohazard waste disposal practices of different materials.

Lab D3000-1 and -3 are biosafety level 2 labs. Safety procedures for microorganisms associated with human diseases or regarded as opportunistic pathogens are mandatory when working in this laboratory. Bacterial cultures should be handled with caution, and proper aseptic techniques should be followed to prevent contamination. For your laboratory procedures, you are required to come to the laboratory on time with the following:

- Your laboratory goggles
- Blue laboratory coat. This will be stored in a zip-top plastic bag (handed out during the first lab session) while you are not in lab.
- Hardcover lab notebook.

Laboratory Safety and Rules for D3000

- Your personal items, along with your partner's items, will be stored in a locker. Reserve a locker through the Security Office in D1018 . The cost is $12 per term. **Please bring a lock** and inform your lab partner of the combination.
- To protect you and other people working in the laboratory from contamination by microorganisms, stains or chemical reagents, you must wear a buttoned-up laboratory coat, gloves and goggles in the laboratory at all times unless otherwise indicated by the instructor.
- Eating, drinking and chewing gum are not allowed in the laboratory. Avoid handling contact lenses, applying make-up in the laboratory, or touching your face. No articles should be placed in your mouth, including pens and pencils.
- Telephone calls and texting are not permitted in the lab during laboratory sessions.

- You can have your phone on you as we will learn how to take pictures of your results safely.
- Be aware of the location of eye wash, shower stations, First Aid kit and Fire extinguisher in the event of an accident that requires the use of any of this equipment.
- Shorts, short pants, sandals or open-toe shoes should not be worn in the laboratory. This is to protect you from any accidental dropping of objects that might results in serious injury.
- Your work area should be disinfected before and after the experiment with the disinfectant provided for that purpose (70% reagent alcohol or isopropyl alcohol). Ensure your work area is cleaned, tidied-up and all equipment is returned to its appropriate storage cabinet at the end of the experiment. Laboratory coats, gloves and goggles should be worn when cleaning and disinfecting the work surface.
- During most of the laboratory experiments, you will be working with open flames/Bunsen burners, it is important to use extreme caution when working with open flames and with alcohol.
- Read and familiarize yourself with the weekly protocol before coming to the laboratory session.
- Laboratory exercises will be due for submission to the FOL submission folder one week after lab session. Hand it in at the beginning of the next lab. Submissions up to 24 hours late will receive a 25% penalty. Labs handed in after 24 hours late will receive a mark of zero.

Violation of any of these rules at any time during the laboratory session will cause a loss of performance marks and you may be asked to leave the lab.

Good Microbiological Practices and Procedures

Best Practices in the Microbiology Lab

1. To ensure safe work and control of biological risk.
2. Never store food or drink, or personal items such as coats and bags in the laboratory.
3. Activities such as eating, drinking, smoking, and applying cosmetics are only to be performed outside the laboratory.
4. Never put materials, such as pens, pencils or gum, in the mouth while inside the laboratory, regardless of whether gloves are worn or not.
5. Wash hands thoroughly, preferably with warm running water and soap, after handling biological material and/or animals, before leaving the laboratory or when hands are known or believed to be contaminated.
6. Ensure open flames or heat sources are never placed near flammable supplies and are never left unattended.
7. Ensure that cuts or broken skin are covered before entering the laboratory.
8. Ensure that supplies are stored safely and according to storage instructions to reduce accidents and incidents such as spills, trips and falls.
9. Ensure proper labelling of all biological agents.
10. Protect written documents from contamination using barriers (such as plastic coverings or a physical space on the lab bench that doesn't have biologicals), particularly those that may need to be removed from the laboratory.

11. Ensure that the work is performed with care and without hurrying. Avoid working when fatigued.
12. Keep the work area tidy, clean and free of non-essential objects and materials.
13. Prohibit the use of earphones, which can distract personnel and prevent equipment or facility alarms from being heard.
14. Cover or remove any jewelry that could tear gloves, easily become contaminated or become fomites. Cleaning and decontamination of jewelry or spectacles should be considered, if such items are worn regularly.
15. Refrain from using portable electronic devices (for example, mobile telephones, tablets, laptops, flash drives, memory sticks, cameras when not specifically required for the laboratory procedures being performed.
16. Keep portable electronic devices in areas where they cannot easily become contaminated or act as fomites that transmit infection. Where close proximity of such devices to biological agents is unavoidable, ensure the devices are either protected by a physical barrier or decontaminated before leaving the laboratory

Technical Procedures

Controlling risk through safe conduct of laboratory techniques.

Avoiding inhalation of biological agents

1. Use good techniques to minimize the formation of aerosols and droplets when manipulating specimens. This includes refraining from forcibly expelling substances from pipette tips into liquids, over-vigorous mixing, and carelessly flipping open tubes. Where pipette tips are used for mixing, this must be done slowly and with care. Brief centrifuging of mixed tubes before opening can help move any liquid away from the cap.
2. Avoid introducing loops or similar instruments directly into an open heat source (flame) as this can cause spatter of infectious material. Where possible, use disposable transfer loops, which do not need to be resterilized. Alternatively, an enclosed electric microincinerator to sterilize metal transfer loops can also be effective.

Avoiding ingestion of biological agents and contact with skin and eyes

1. Wear disposable gloves at all times when handling specimens known or reasonably expected to contain biological agents. Disposable gloves must not be reused.
2. Avoid contact of gloved hands with the face.
3. Remove gloves aseptically after use and wash hands as outlined below.
4. Shield or otherwise protect the mouth, eyes and face during any operation where splashes may occur, such as during the mixing of disinfectant solutions.
5. Secure hair to prevent contamination.
6. Cover any broken skin with a suitable dressing.
7. Prohibit pipetting by mouth.

Avoiding injection of biological agents

1. Wherever possible, replace any glassware with plastic-ware.
2. If required, use scissors with blunt or rounded ends rather than pointed ends.
3. If glassware must be used, check it on a regular basis for integrity and discard it if anything is broken, cracked or chipped.
4. Never use syringes with needles as an alternative to pipetting devices.
5. Never re-cap, clip or remove needles from disposable syringes.
6. Dispose of any sharps materials (for example, needles, needles combined with syringes, blades, broken glass) in puncture-proof or puncture-resistant containers fitted with sealed covers. Disposal containers must be puncture-proof/-resistant, must not be filled to capacity (three-quarters full at most), must be never reused and must not be discarded in landfills.

Preventing dispersal of biological agents

1. Discard specimens and cultures for disposal in leak-proof containers with tops appropriately secured before disposal in dedicated waste containers.
2. Waste containers are present at every workstation.
3. Regularly empty waste containers and securely dispose of waste. Ensure all waste is properly labelled.
4. Consider opening tubes with disinfectant-soaked pad/gauze.
5. Decontaminate work surfaces with a suitable disinfectant at the end of the work procedures and if any material is spilled.
6. When disinfectants are used, ensure the disinfectant is active against the agents being handled and is left in contact with waste materials for the appropriate time, according to the disinfectant being used.

Personal Protection

1. *Laboratory coats must be worn at all times for work in the laboratory.*

- When putting on lab coats, care should be taken to minimize contact with the outer/exposed side of the material in case the material is contaminated from the previous use.
- When removing lab coats, gloves should be removed first followed by the lab coat. Place the lab coat in the plastic bag then wash your hands.

2. *Appropriate gloves must be worn for all procedures.*

- After use, gloves should be removed aseptically and hands must then be washed.
- Disposable gloves should not be disinfected, for example, with ethanol, before starting work and they should not be reused as exposure to disinfectants and prolonged wear will reduce the integrity of the glove material. If disposable gloves become noticeably contaminated, they should be removed immediately and disposed in the red bin to prevent further contamination of other PPE, equipment and specimens.
- To remove disposable gloves correctly, pinch the thumb and forefinger together on one hand. With the other hand, pinch the material just below the top of the cuff of the glove, and pull the glove down towards the closed thumb and forefinger, turning the glove inside out in the process. Stop once you have reached the thumb and forefinger so that the glove is only partially removed. Repeat on the other hand. At this point the

gloves are both partially removed with the clean undersides of the gloves now forming the outer surface. The gloves can now be removed by touching only the clean underside of the material.
- Used disposable gloves should be discarded in the red bin. Once gloves are removed hands must be washed. See Figure 8.1 on page 35 of the Personal Protective Equipment pdf posted on FOL for a visual of this process.

3. *Eye protection*

- Lab glasses should be put on with clean hands (for example, not after handling microorganisms) to avoid contamination of the face and the eye protection itself.
- Eye protection should be removed after taking off your lab coat with clean hands to avoid contamination of the head.

4. *Hand washing*

- Personnel must wash their hands after handling infectious materials and animals, and before they leave the laboratory working areas.

5. *It is prohibited to wear protective laboratory clothing outside the laboratory, e.g. in the hallway or washrooms.*

- You could contaminate non-lab environments with microorganisms.
- Even if your coat is sterile, your appearance will scare others in the college!

6. *Protective laboratory clothing that has been used in the laboratory must be stored in the plastic bag when not in use.*

Disposal procedures in the lab

We make lots of waste in the lab and it is treated based on if the item is going to be reused and if it is contaminated.

Table 1.1. Waste disposal in the microbiology lab

Waste	Contaminated Y / N	Disposal location	Notes
Test tubes, flasks with culture	Y	In tube rack on side bench	Will be autoclaved by the lab tech. Do not rinse in sink.
Agar plates with culture, other plastics	Y	Orange biohazard bags on benchtop	Will be autoclaved by the lab tech. Pipette tips can go into small waste container first.
Paper towels from drying hands	N	Green bin	
Liquid waste from Gram staining	N	Mixed inorganic waste in fumehood	If staining procedure kills microorganisms, waste will not be contaminated.
Liquid culture waste	Y	Biohazard waste in fumehood	
Slides	Y	Glass tray by carboy	If live-stained or wet mount, it is contaminated
Gloves	Y	Red bin	

LAB 2: BASIC TECHNIQUES

Learning Objectives

The purpose of this lab is to introduce you to skills that you will use throughout the course. You will have a chance to practice these skills today before being evaluated on them later.

- Demonstrate the process of making media and pouring agar plates
- Use a micropipette
- Perform serial dilutions

Introduction

There are many ways to categorize media. One way is to divide media based on whether it is a solid or liquid at room temperature.

- **Liquid media**, called broth, is usually the type found in test tubes. Bacteria grow throughout this medium.
- **Solid media** can be solidified with many gelling agents; in this lab, we use agar to solidify media. Solid media can be found in petri plates and in test tubes as deeps or slants.

There are two main ways to make media:

1. The cookbook method involves adding each ingredient separately. This is done when the organism is difficult to grow and needs special nutrients or if the medium is uncommon and not commercially available. This is typically done for **chemically defined media** where the exact chemical composition of each ingredient is known.

2. The other method involves rehydrating a powder of pre-mixed medium in water (like making a cake from a mix!). This is what is done for most commonly used media and **complex** media. The chemical composition of complex media is not precisely defined.

Depending on the medium your group makes, you could be making agar or broth, complex or defined, and cookbook or “cake mix” media. Regardless of the medium you are making, you will follow the same steps.

After the media has been mixed, it must be sterilized in an autoclave. An autoclave is essentially a steam cooker under pressure. It is able to achieve 121 °C for 15 minutes using increased pressure. This kills all life, including spores, in the media. If you mixed media and did not autoclave it immediately, it would quickly start to grow bacteria since the media itself, the glassware and water were not sterile.

If you are making solid media and are dispensing it into tubes before autoclaving, the media must be heated. This

is to melt the agar, otherwise the agar settles to the bottom of the flask and you would have an unequal distribution of agar in your tubes after dispensing the media (some tubes would have little agar and be liquid and some, with too much agar, would be solid).

You will pour some agar plates in the lab this week:

Media Exercise

Materials

- Medium bottle
- Medium recipe (if required)
- Flask
- Weigh boat
- Metal scoopula
- Hot plate
- Magnetic stirrer
- Graduated cylinder
- Hot hands

Method

1. Carefully read your recipe found in documents in the FOL content folder for this lab. The mass of reagents required is usually given per litre. You will likely be making less than a litre. Calculate below the amount of reagents required:

Table 2.1. Reagent Calculation Worksheet [download worksheet]

Volume of medium being made:	
Reagent	**Mass**

2. Using the **top-loading balance**, weigh out each reagent.

- If you are weighing out multiple reagents, empty the weigh boat into the flask and clean the spatula between reagents.
- For this application, you do not need the precision of an analytical balance.
- Clean the balance with a damp paper towel after use as media powders are hygroscopic and become sticky.

3. Using the **graduated cylinder**, measure out the volume of distilled water required. Add gradually to the flask, stopping to mix periodically.

- A graduated cylinder offers a more precise volume measure than a flask or beaker.
- Since the volumes are large (>100 ml), a graduated cylinder is more accurate than using a 25 ml pipette multiple times.

4. Once all the water is added, drop magnetic stir bar into your flask and put it on a hot plate.

a. If you are making broth, stir until the medium is completely mixed.
b. If you are making agar, heat and stir. Turn the hot plate heat to maximum. Watch the flask closely because once it starts to boil, it will quickly overflow the flask, start to burn and make smoke.

> Smoke will set off the sprinkler system. Please don't make smoke.

c. Once the medium becomes transparent and you see small bubbles rising up to the top, remove the flask from the hot plate.

5. If your medium is going into test tubes, use the peristaltic pump to dispense medium into test tubes. If your medium is going into petri plates, place the flask on the cart to be autoclaved.

Note: All media will be autoclaved. Petri plates will be poured after autoclaving.

6. Once agar is autoclaved, it is cooled to 60 °C then poured into petri plates in the BSC.

Pouring Agar Plates

a. Open the lid of the plate like a clamshell (as if it was hinged on the far edge)
b. Pour until the agar has just covered the bottom of the plate.
c. Cover the plate and allow to solidify in a single layer
d. Once solid, the plates can be inverted and allowed to dry before packaging for longer-term storage.

Using a Micropipette

Micropipettes are used to measure volumes less than 2 ml. Each micropipette will accurately pipette a defined volume range; different micropipettes require different tips. Do not try to use a pipette outside of its range. This can damage it. You will be using an air-displacement micropipette frequently in the lab since many experiments require small volumes. See page 6 of the Gilson pdf posted on FOL for terminology.

In our lab, you can find the following micropipettes:

Table 2.2. Available Micropipettes

Pipette	Volume dispensed	Immersion depth
P2	0.1 µL – 2 µL	1 mm
P10	0.5 µL – 10 µL	2-3 mm
P20	2 µL – 20 µL	2-3 mm
P200	20 µL – 200 µL 50 µL – 200 µL (older style)	2-4 mm
P1000	100 µL – 1000 µL 200 µL – 1000 µL (older style)	3-6 mm

You can tell which pipette you have by looking at the top of the push button.

Steps for Using a Micropipette

See page 17 of the Gilson pdf posted on FOL for a pictorial representation of the steps below. Figure 2.1 below shows the volume displays of the pipettes.

1. ***Set the volume*** by turning the volume adjustment knob.
    - Open a tip box and with one gentle twisting motion, put a tip on the top holder. Do not tap.
    - The volume adjustment knob can be the same as the push button, or it might be a black dial (called a thumbwheel) at the top of the pipette body.
    - For best pipetting accuracy: finish adjusting the volume clockwise. This means if you are decreasing the volume, don't go past the target volume. If you are increasing the volume, go past the target volume then dial down to the target without overshooting.
    - NEVER adjust a pipette outside of the volume range indicated. This can damage the pipette.
2. ***Prepare for aspiration****:* Holding the pipette vertically, smoothly press the push button to the first stop.
    - Push button can also be called a plunger.

3. ***Aspirate the sample***: Immerse the tip in the sample at the correct depth. With control, release the push button to aspirate the liquid into the tip. Wait one second to allow all the liquid to enter the tip before moving.
    - Immersion depth of the tip is critical. Too shallow creates vortexes which cause incorrect volumes to be aspirated. Too deeply causes droplets to form on the outside of the tip, which also cause incorrect volumes to be dispensed.
    - ALWAYS hold the pipette vertically once liquid is in the tip. If not, the liquid can enter the pipette body, causing contamination and damage to the components.
4. ***Dispense***: Rest the tip at an acute angle to the receiving vessel so the liquid runs down the walls of the vessel. Smoothly press the push button to the first stop to release the sample. Wait one second then press to the second stop to release any remaining sample. Release the tip into the tip dispense waste. Do not re-use tips. This leads to inaccurate results.

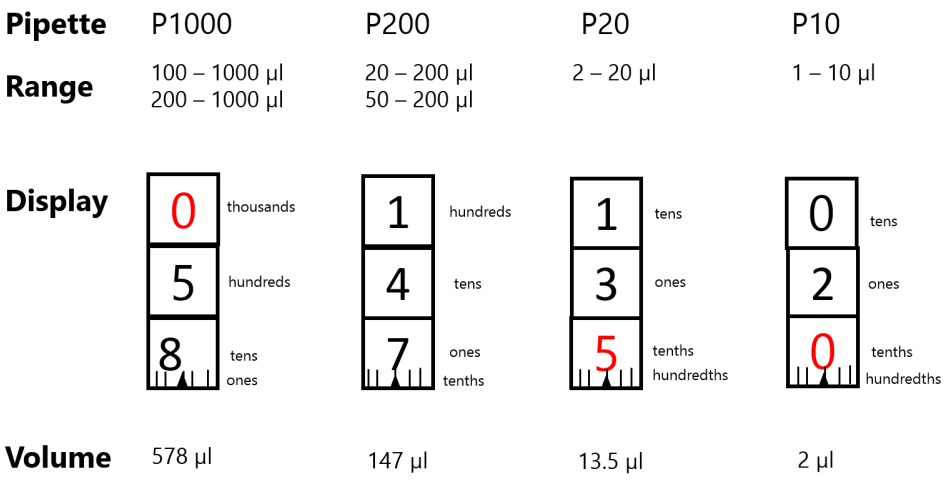

Figure 2.1. The four pipettes in our lab each have a different volume range and display.

Making Dilutions

Since bacteria are small, there are often billions of cells per milliliter. In order to count the number of cells in a culture, we need to dilute the culture to a countable number of cells. Then simple math is done to determine how many cells are in the original culture, based on the dilutions that were done. When making dilutions, the **stock solution** refers to the substance being diluted (cells in our labs) and **diluent** refers to the solvent the substance is diluted into (water or broth). Figure 2.2 shows this process.

To dilute a culture, you usually make 10-fold dilutions as follows:

- 100 µl of culture into 900 µl sterile water = 1 in 10 or 10^{-1}
- 100 µl of 10^{-1} dilution into 900 µl sterile water = 1 in 100 or 10^{-2}
- 100 µl of 10^{-2} dilution into 900 µl sterile water = 1 in 1000 or 10^{-3}
- This is repeated until the 1 in 100 million 10^{-8} dilution is achieved.

- This is typically diluted enough that all cultures will be in a countable range by this point.

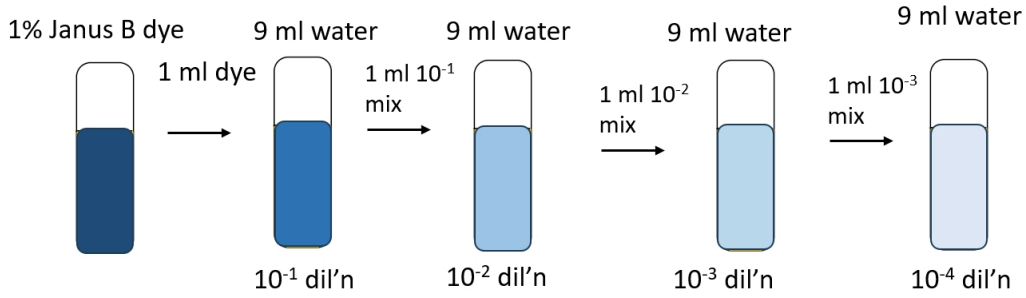

Figure 2.2 Making Dilutions with a stock solution (dye) and diluent (water).

1 ml dye into 9 ml broth = 10^{-1} dilution factor

Formula:
a) Total volume x dilution factor = **volume of stock solution**
b) Total volume – stock solution = **diluent**

e.g. 10^{-1} dilution of a stock solution in 5 ml total volume
a) 5 ml x 0.1 = 0.5 ml = 500 µl stock solution
b) 5 ml – 0.5 ml = 4.5 ml diluent

As you can see, you are making one dilution on top of another dilution. These are called serial dilutions because they are in series.

You will practice making dilutions using a micropipette with a dye solution in this lab before using bacteria in the following labs.

Dilution Exercise

Objective

Pipetting accuracy will be measured by determining the mass and absorbance of the solutions pipetted.

Materials

- Deionized water
- 3 per group member Glass test tubes and rack
- 3 x 2 ml microfuge tubes and rack per group member
- 10 ml pipette and pump
- P1000 and P200 Micropipettes and tips
- 1% aqueous solution of Janus B dye

Method

Each group member will perform these activities to get practice.

Dilute a dye solution

1. Label one test tube 10^{-1}, a second tube 10^{-2}, and a third tube 10^{-3}

Serial Dilutions

2. Tubes 10^{-1}, 10^{-2} and 10^{-3} will be part of one dilution series.

    a. Determine how much water will go into each of the three tubes. The final volume is 10 ml and the dilutions are 1 in 10.
    b. Add the water to each tube.

How much water and dye is added to the 10^{-1} tube?

Table 2.3. Volume Worksheet [Download Worksheet]

Volume of dye	µl	P1000
Volume of water	ml	10 ml pipette
Final volume	10 ml	

3. Add the dye to test tube labeled 10^{-1}. Mix by vortexing gently. Be careful that the liquid doesn't come over the top of the tube.
4. The 10^{-2} tube is a 1 in 10 dilution of the 10^{-1} tube. The same volumes are used, only the 10^{-1} tube is used as the dye source. Mix gently by vortexing.
5. The 10^{-3} tube is a 1 in 10 dilution of the 10^{-2} tube. The same volumes are used, only the 10^{-2} tube is used as the dye source. Mix gently by vortexing.
6. Determine your pipetting precision by measuring the amount of dye in a spectrophotometer. Set the absorbance to **587 nm**. Blank with deionized water. Measure the absorbance of the 10^{-3} tube.

Expected value: 0.129
Your absorbance value: _____ (enter on worksheet)

To determine the percent error, divide the difference of your value and the expected value by the expected value, then multiply by 100%:

E.g. Your absorbance value was 0.8. The expected value was 0.75.[0.8-0.75] / 0.75 * 100% = 6.7%

If your value was less than the expected, you'd subtract it from the expected value in the numerator.

7. Clean up: empty the test tubes into the mixed inorganic waste then rinse in the sink. Invert on a tube rack to dry. Cuvettes can be rinsed and inverted to dry.

Pipetting Exercise

To be completed by each student: This exercise is based on the principle that 1 ml of water is equal to 1 g; 1 µl of water is equal to 1 mg.

8. On an analytical balance, weigh 3 microfuge tubes and record the values.
    a. I find it useful to record on the sides of the tubes.
9. Dispense approximately 100 ml of deionized water into a beaker. Pipette the following volumes into each microfuge tube:
    a. 800 µl into one tube TWICE
        i. Pipette used (e.g. P2, P20, etc.):_____
    b. 150 µl into one tube TWICE
        i. Pipette used (e.g. P2, P20, etc.):_____
    c. 20 µl into one tube THREE TIMES
        i. Pipette used (e.g. P2, P20, etc.):_____
10. Re-weigh each tube and determine the volume pipetted. Determine your precision and the percent error like above.
11. Microfuge tubes are disposed of in orange biohazard bags.

LAB 3: BACTERIAL GROWTH

Learning Objectives

The purpose of this lab is to introduce you to methods of measuring bacterial cell numbers and working with bacteria aseptically.

- Practice aseptic work on the benchtop.
- Use a Biological Safety Cabinet (BSC).
- Calculate the number of bacterial cells in a culture based on colonies in a dilution (colony-forming units on an agar plate).

Introduction

One of the tests done on bacteria is a growth curve. This allows you to determine how quickly the bacteria grow over time. You can also learn about the preferred environment of the bacteria by modifying the growth conditions and observing how growth changes. Bacteria have optimal temperatures, pH, salinity, oxygenation, and nutrient concentrations for growth.

To perform a growth curve, we must take an aliquot (a subsample) of the culture at defined times. This sample is measured in a spectrophotometer at 600 nm to determine the optical density, or relative number of cells in the sample is determined. At the same time, the sample is put on agar plates (called “plating”) to determine the number of colony forming units (CFU) per milliliter.

Typically, growth curves take many hours and up to several days to complete. You must continue to take readings until the bacteria enter stationary phase. Since our lab period is only three hours, we must perform this growth curve in a different way. In this lab, we will use a culture of exponentially growing *E. coli*. These bacteria were grown overnight in broth. This morning, that overnight culture was used to inoculate a new flask of broth. We will be hopefully sampling the bacteria while they are in exponential phase.

You will be measuring growth in your flask every 30 minutes for two hours. In total each group will take five optical density measurements.

Table 3.1. Sampling times for determination of *E. coli* growth

Sample name[a]	Reading 1 (min)	Reading 2 (min)	Reading 3 (min)	Reading 4 (min)	Reading 5 (min)
E. coli	0	30	60*	90	120*
Pre-grown *E. coli*	60	90	120	150	180

** The asterisk indicates the time points for which you will determine cell numbers by plating.*

Growth rate calculations

Indirect growth rate

Plot the data on a graph with OD on the y-axis and time on the x-axis. From the complete growth curve, we can determine the growth rate of the bacteria, and the generation time. **Generation time (g) is the time for one generation.**

The units of time are defined by values used in the equation. E.g. Generation time will be in minutes if you use minutes in the equation *or* it will be in hours if you use hours in the equation.

Calculating generation time from the growth curve:

Determine where exponential phase is on the graph. We have attempted to align the bacterial growth with your lab period so the bacteria will be in exponential phase during the two hours that you take measurements. During exponential phase, growth is steady, or occurring at a constant rate.

You can determine growth rate by two different methods using a graph.

Method 1 (less accurate)

1. Choose two optical densities in exponential phase, one that is double the value of the other.
2. Draw a horizontal line from the y-axis (optical density) to the growth curve. Now draw a vertical line from the growth curve to the x-axis (time).
3. Repeat this for both optical density lines.
4. Subtract the greater time from the lesser time to get the generation time (g).

Example: Growth Rate of *Salmonella typhimurium* (Method 1)

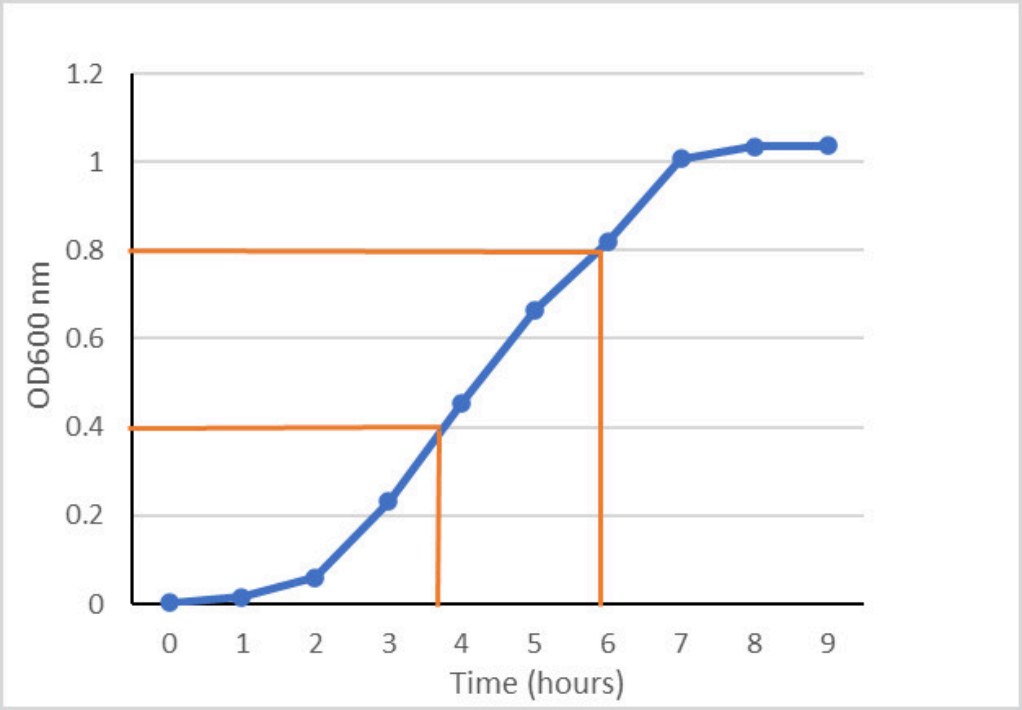

Figure 3.1. Growth curve of *Salmonella typhimurium* in NB. Lines have been drawn to determine the x-intercept at OD 0.4 and OD 0.8.

- The culture is at OD 0.4 around 3.5 hours and it's at OD 0.8 at about 6 hours.
- Since these optical densities are double, we can subtract the times to get an approximate doubling time: 6-3.5 = 2.5 hours

Method 2 (more accurate)

Use the equation of the line to calculate x intercepts. Determine the equation of the linear portion of the line, then calculate x for two y values that are double each other.

Example: Growth Rate of *Salmonella typhimurium* (Method 2)

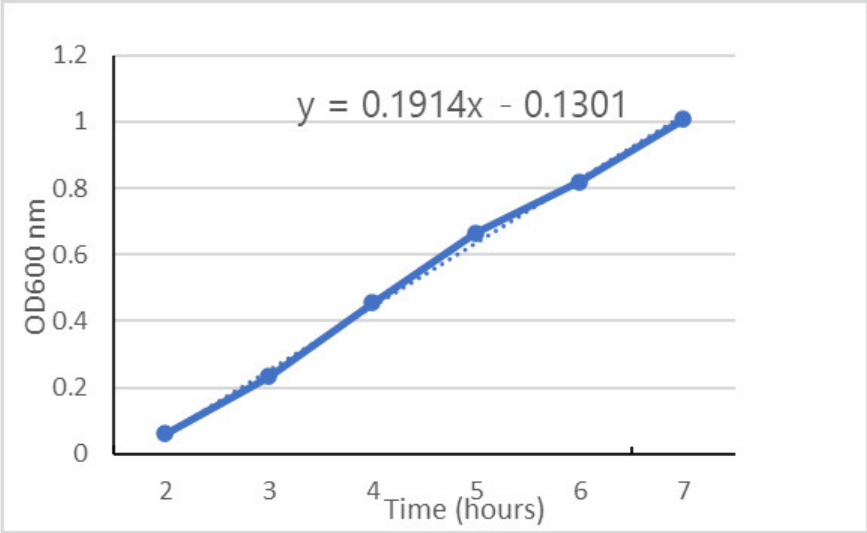

Figure 3.2. Exponential phase growth of Salmonella typhurium in NB.

In Figure 3.2, the lag and stationary phase points were eliminated from the graph, leaving only exponential growth.

The equation of the line for absorbance values over time is linear:

$$y = 0.1914x - 0.1301$$

Re-arranged, this is

$$x = \frac{(y + 0.1301)}{0.1914}$$

Calculate x for $y = 0.4$ OD:

$$x = \frac{(0.4 + 0.1301)}{0.1914}$$

$$x = 2.7$$

Since the x axis starts at 2 and not 1, we need to add 1:

$$2.7 + 1 = 3.7 \text{ hours}$$

Calculate x for $y = 0.8$ OD

$$x = \frac{(0.8 + 0.1301)}{0.1914}$$

$$x = 4.9$$

Since the x axis starts at 2 and not 1, we need to add 1:

$$4.9 + 1 = 5.9$$

Now subtract to determine the doubling time:

$$5.9 - 3.7 = 2.2 \text{ hours}$$

Direct growth rate

Generation time can also be calculated from the number of cells at two time points in exponential growth using the following formula:

$$g = \frac{t}{3.32\,(\log N_2 - \log N_1)}$$

Where:

- N_2 is the CFU/ml at time 2
- N_1 is the CFU/ml at time 1
- t is the time difference between N_2 and N_1

Cell density calculations

You will need to determine the number of cells in the flask for each time point. Since there can be billions of bacteria in a single test tube, we need to dilute the sample in order to count the bacteria. You will be performing serial 10-fold dilutions (you did last week with dye). The dilutions you perform depend on how long the sample has been growing (if the sample has been growing for a long time, you will need to perform higher dilutions).

Each series of dilutions will be plated onto the same agar plate. This technique is called drop plating. You will drop 20 ul of each dilution into its own spot on the plate. After incubation, count those drops that have over 8 colonies and clearly distinct colonies. The upper limit is what you can resolve with your eyes or a Quebec colony counter. You will likely only be able to count one or two dilutions per series.

If there are too many colonies to resolve, record the result as TNTC (too numerous to count).

- In figure 3.3, dilutions 10^{-1} to 10^{-4} are TNTC

If there are less than 8 colonies, record the result at TFTC (too few to count).

- In figure 3.3, the 10^{-6} dilution has only 2 colonies and is TFTC

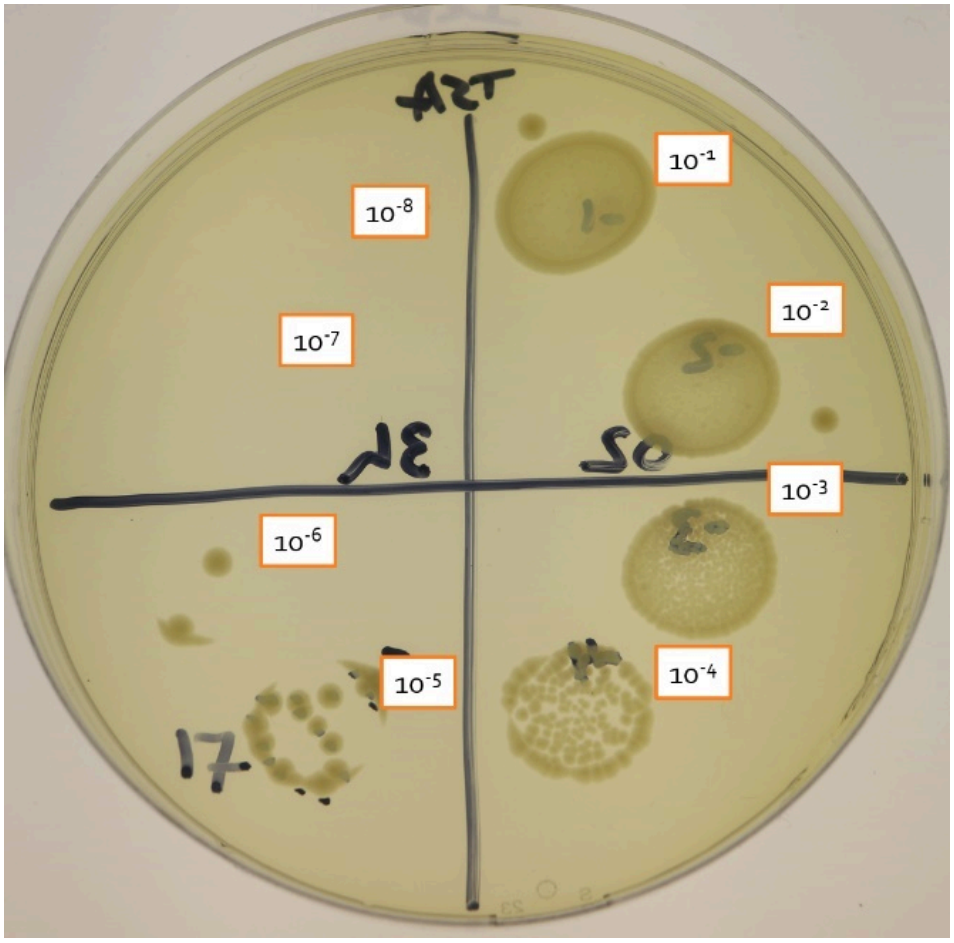

Figure 3.3. Drop plate of E. coli showing dilutions from 10-1 to 10-8. The only dilution that is in the countable range is 10-5, which has 17 colonies.

Determine the plating factor. Remember, we are determining CFU/ml. There are 1000 µl in 1 ml. If you plated 25 µl, therefore:

$$\text{Convert } 25\ \mu l \text{ to } ml = 0.025\ ml$$

1. You must divide the number of colonies you counted by the plating factor to calculate the CFU/ml of the dilution you counted the cells at. In this case, the plating factor is 0.025 ml.

$$17\ \text{CFU}/0.025ml = 680\ \text{CFU per}\ ml\ \text{of the}\ 10^{-}5\ \text{dilution}$$

2. Lastly, account for the dilution that the cells were counted at. This can be accomplished by multiplying by the reciprocal of the dilution (e.g. “per 10^{-5} dilution” becomes 10^5 in your equation):

$$680\ \text{CFU}/ml \times 10^5 = 6.8 \times 10^7\ \text{CFU}/ml$$

The general steps for calculating cfu/ml are:

1. Account for the plating factor.

    - cfu/plating factor = cfu/ml of the dilution plated

2. Account for the dilution factor.

    - cfu/ml x dilution factor

Aseptic Technique

We need to work in a manner to reduce contamination of our sample with organisms found on our bodies and in the lab environment. To create this environment, we use a practice called aseptic technique. There are four main requirements for performing aseptic technique.

Sterile work area

Before starting work in the lab, wipe down your bench with disinfectant. This kills organisms that have landed on the bench from dust in the air, and from previous students' work in that lab.

We will be using Bunsen burners to provide a sterile work area. The Bunsen burner is also used to sterilize metal utensils, and to allow sterile containers to be opened by passing the container through the flame.

Additionally, groups will take turns in the biosafety cabinets (BSCs). This is a work area that maintains sterility by the air flow and not through the use of a flame.

Personal hygiene

Although you wear gloves for all activities in this lab, it is good laboratory practice to wash your hands before and after each lab session. Wearing a lab coats prevents organisms on your clothes from contaminating the work area.

Sterile reagents and media

All bacterial media must be sterilized prior to its use to prevent contamination. Media is sterilized in an autoclave, a large unit that uses pressure and steam to achieve an internal temperature of 121 ˚C. Some reagents can be autoclaved, while heat-sensitive reagents must be sterilized by other means.

Sterile handling

When attempting to make a pure culture of bacteria, you must use a bacterial sample and sterile bacterial media. The media must be handled aseptically to prevent its contamination. Only open the media when it is near the Bunsen burner, and only when it is about to be used. If the media is in a glass container (usually a bottle or flask) follow these steps:

a) Pass the neck of the container (the part near the opening) through the flame.

- Do not hold it in the flame or it will heat up and prevent you from opening the container

b) With one hand holding the container on an angle, open the container with the other hand.

> Holding the container on an angle prevents airborne organisms from falling into the container.

c) Pass the neck of the now open container through the flame.

d) Perform the desired task: insert a sterile inoculating loop, pipette, etc.

e) Pass the neck of the open container through the flame.

f) Close the container.

> You have been holding the lid of the container and a utensil in your other hand since opening the container. Try holding the lid in your pinky and ring fingers, and the utensil with your thumb and index fingers. If the lid is too large, place on the bench near the flame, sterile side down.

Aseptic technique tips

Perform these tasks as rapidly as is possible to limit contamination. In the beginning it will take you longer than after some experience.

Avoid talking, singing and whistling while performing aseptic technique to limit contamination.

Never leave culture media open for even a short time, unless you intend to throw it out after (culture media examples: petri plate, test tube, flask of media).

For most inoculations, you will use a metal loop to transfer bacteria. This loop must be made red hot along the entire length of wire prior to opening the culture media. Cool the loop by touching to sterile media before transferring bacteria.

Keep flammables away from the flame. Frequently, 70% alcohol is used to sterilize glass utensils. Do not place hot utensils in the alcohol.

Using a Biological Safety Cabinet (BSC)

Our lab has two class II BSCs. These are work areas that have regulated air flow. The air inside the cabinet is filter-sterilized and under negative pressure. There is a wall of air preventing air from inside the cabinet from entering the room and from contaminated room air from entering the cabinet.

Procedure for using the BSC.

1. Turn on the blowers 5 minutes before running.

2. Spray the entire inside work surface with 70% alcohol and wipe down.

3. Assemble the equipment you will be taking into the BSC.

- Note: you cannot use a flame in the BSC, so no metal loops can be used. Instead, we can swab with sterile wooden applicators.
- Wipe all objects that are hard-surfaced with 70% alcohol.
- You will probably need pipettes, tips, a waste container.

4. Place the objects inside the BSC.

- Place in a logical order that allows you to work across the BSC from cleanest to dirtiest.
- Keep objects off the front grill and keep space between the objects and the walls of the BSC.

5. When working in the hood, minimize movements to prevent disturbing the airflow. This prevents outside air from mixing with BSC air.

- Especially limit lateral (side-to-side) movements and the number of times your arms enter/exit the BSC.

6. To prevent aerosols in the BSC, keep a lid over open containers of media and bacteria.

7. When you are done in the BSC, remove your items. Wipe down the inside with alcohol and allow the blowers to run for 5 minutes after you've exited before shutting it off.

Note: You can use either the Bunsen burner on your benchtop or the BSC to work aseptically. Please try to get BSC experience this lab or next week before being evaluated on this skill.

Growth Exercise

Objective

To determine the growth rate of *E. coli* in Luria Bertani broth.

Materials

- 16 microfuge tubes
- P100 pipette and yellow tips
- P1000 pipette and blue tips

- 2 older agar plates
- 6 Cuvettes
- Sterile water
- Sterile Luria-Bertani broth
- Flask of *E. coli* culture

Table 3.2. Division of work for the lab.

Reading	Spectrophotometer	Dilution and plating
1 (0 h)	Reading	
2 (0.5 h)	Reading	Prepare tubes for 1 h plating
3 (1 h)	Reading	Dilute and plate
4 (1.5 h)	Reading	Prepare tubes for 2 h plating
5 (2 h)	Reading	Dilute and plate

METHOD

1. Spectrophotometer readings: Take every 30 minutes for 5 in total.

a. Prepare the blank by putting 1 ml of liquid into a cuvette. Reserve this for all your future readings.

   - What will be the solution to zero the instrument? What are the cells suspended in?

b. Adjust the instrument to 600 nm then zero it with the blank.
c. Aseptically transfer 1 ml from the flask to a cuvette. Read immediately.

   - If the reading is out of range of the spectrophotometer (>1.0 absorbance), you will need to dilute the culture in the cuvette. If you have 1 ml of culture, add 1 ml broth. Then, you will need to multiply the absorbance value by 2.

d. Return the flask to the incubator.

Table 3.3. Spectrophotometer Reading Worksheet [download worksheet]

Time	Spectrophotometer reading
1 (0 h)	
2 (0.5 h)	
3 (1 h)	
4 (1.5 h)	
5 (2 h)	

2. Drop plates for cell enumeration: Done at reading 3 and 5 only.

0. Label 8 tubes with "10^{-1}" to "10^{-8}"
a. Add 900 ul sterile water to each tube
b. Add 100 ul culture to the 10^{-1} tube. Change your tip.
c. Vortex the 10^{-1} tube then transfer 100 ul from this tube to the 10^{-2} tube. Change your tip.
d. Vortex the 10^{-2} tube. Repeat the process until you have vortexed the 10^{-8} tube.

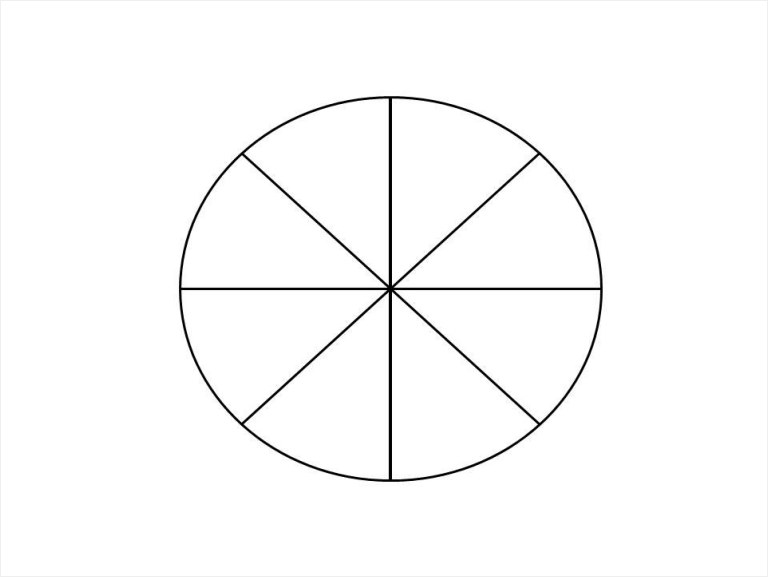

Figure 3.4. Petri plate divided into eight equal sections.

f. Divide a petri plate into 8 sections by drawing 4 lines across the plate (Figure 3.4). Label each section with a dilution name.
g. Starting at the most dilute sample (10^{-8}), place 20 µl onto the appropriate section.
h. Keeping the same tip, now drop the 10^{-7} dilution onto its section. Repeat until all dilutions have been plated.

i. Keep the plate open and near the flame until the drops have dried. Then invert the plate.
j. Incubate the plate at room temperature for 24 hours (or 37 C for 18 hours). Count colonies if the drop has 8 or more colonies. Determine CFU/ml. Use the Quebec colony counter. It has a magnifying glass to allow you to count smaller colonies within the drops you plated.

LAB 4: ISOLATION EXERCISES

Learning Objectives

- Culture bacteria from the environment.
- Use streak plating to obtain pure cultures of bacteria.
- Outline the components of a Bunsen burner.
- Practice lab techniques that limit bacterial contamination.

Introduction

Our world is full of bacteria. With few exceptions, every surface and every organism in nature is colonized by numerous species of bacteria. The challenge of the lab worker is to separate the species of interest from all the other species present in that environment. To accomplish this task, the lab worker must establish the species of interest in pure culture. Pure culture means only one species of bacteria is being grown in a population. If more than one species is present, the undesired species are contaminants.

To get a pure culture, the lab worker must isolate one bacterial cell from all the rest in an environment. When that one cell is placed in sterile growth medium, that cell gives rise to new cells as it divides. Growth medium is the substrate on which bacteria are grown; it meets all the nutritional requirements of the species it is meant to grow. In this lab, you will learn how to isolate bacteria and grow a pure culture.

Isolation Techniques on Petri Plates

There are many ways to isolate bacteria in pure culture. We will discuss techniques that involve agar plates. The goal of the below techniques is to isolate cells which will then grow into isolated colonies. The isolated colony can then be selected by the lab worker and inoculated into sterile media.

Streak plate

Bacteria are spread on an agar plate using an inoculation loop to get isolated cells. These cells give rise to a colony of clones after an incubation period. The bacteria are usually “picked” from another agar plate from a complex sample and non-isolated colonies.

Streak plates are used in conjunction with other methods of isolation. For example, an environment may be swabbed and put on an agar plate, then bacteria selected off the plate for isolation on a streak plate. This is what we will be doing.

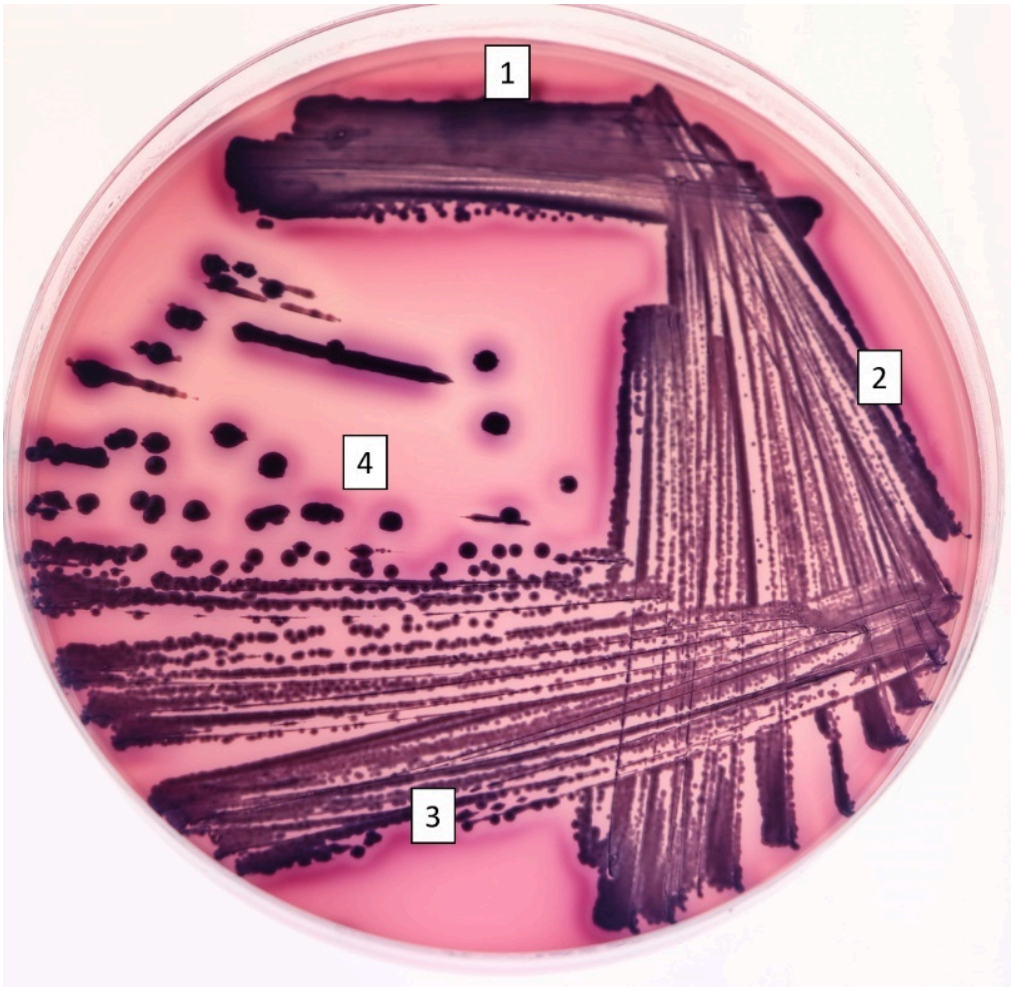

Figure 4.1.Pattern of streaks on a four-pull streak plate

Pour plate

The sample is added to melted agar then poured into a petri plate. Individual cells are isolated and grow into colonies in the agar matrix. The sample may need to be diluted to achieve isolated colonies. For example, if you have one billion bacteria per ml of saliva and you plate 1 ml of saliva on a petri plate, you will not be able to resolve one billion colonies on one petri plate with your eye. This sample would need to be diluted to achieve isolated colonies.

Swab culture

Bacteria in an environment can be cultured by removing them from the environment with a sterile swab. The swab is squiggled across a perti plate, then bacteria are isolated in pure culture using a streak plate. Figure 4.2 shows the pattern of swabbing an object: Swab is passed horizontally first (blue line), then vertically (red line) across the area being swabbed. Next, the swab is then squiggled across the entire width of the plate starting at the top and proceeding to the bottom.

After incubation, bacteria have grown all over the petri plate surface (Figure 4.2 right). Isolated colonies can

be selected and purified by streak plating to obtain a pure culture. The bacterial strain that you isolate will be characterized in your following labs.

We are going to use an agar growth medium that is selective for Gram negative bacteria because most of the tests we will perform in the coming weeks are for Gram negatives. The medium we will use for swabbing is Violet Red Bile Glucose (or VRBG for short). This medium has glucose and neutral red; fermentation of glucose creates acids which creates pink colonies in enteric bacteria. Gram positive bacteria and non enterics (bacteria that live in our intestines) are inhibited by bile salts and crystal violet. Some nonenteric Gram negatives may have grey colonies. The pink colonies you see should be Gram negative enteric bacteria.

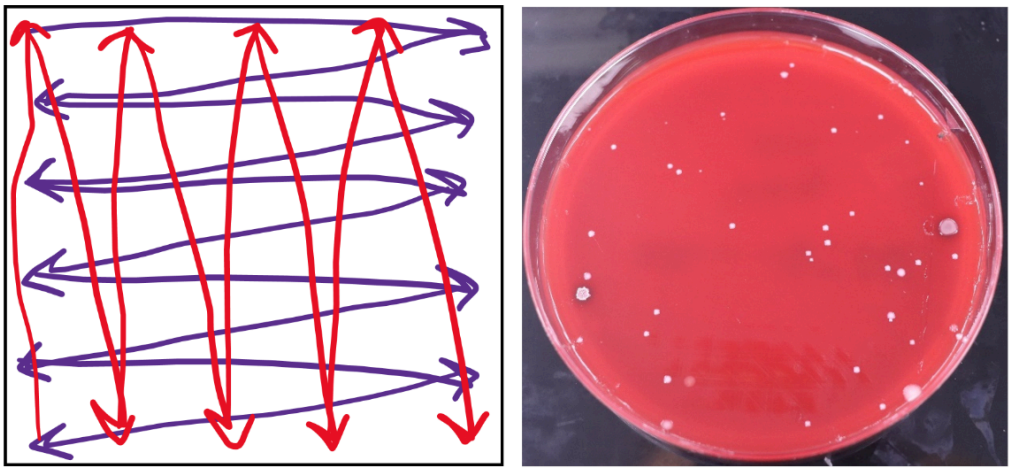

Figure 4.2. Swabbing technique. Left: Pattern of swabbing an object. Right: Swab plate results.

Taking Pictures in the Microbiology Lab

One of the best ways to record observations is through a picture. During this course, you may want to take pictures of plates and tubes. To take pictures safely:

- Remove your gloves and wash your hands then retrieve your phone.
- Your partner keeps their gloves on and manipulates the cultures.
- Place the cultures in the BSC for an image if you must remove the lid of the petri plate due to condensation
- As soon as you are done taking pictures, put your phone away so it doesn't become contaminated.
- When you are taking the picture, make sure the specimen is:
  - in focus
  - well-lit: you can use a lightbox that lights plates from the back for this purpose too.
  - most of the field of view

To use these images effectively in your lab reports

- re-size small images by dragging from the corner of the image to maintain the correct aspect ratio
- crop the image to remove background
- label the data in the image with a figure legend below the image

Bunsen Burner Exercise

Materials

Bunsen burner

Method

1. Close the collar of the Bunsen burner by turning it clockwise so there is no air gap at the base of the burner. Light the flame.

- What colour is the flame with the collar closed?

2. Slowly open the collar by turning it counterclockwise.

- What colour is the flame with the collar opened?

3. Turn the needle valve.

- What effect does the needle valve have on the flame?

Aseptic Technique Exercise

Materials

- Bunsen burner OR BSC
- Inoculating needle
- Inoculating loop
- Mixed culture of *Salmonella typhimurium* and *Staphylococcus aureus*
- Tube of nutrient broth
- Slant of nutrient agar

Methods

1. Set up the Bunsen burner and arrange your lab bench so you can work quickly and efficiently once performing aseptic technique.

- Or alternatively, set up the BSC to prevent air flow disruptions.

2. *Tube to tube transfer*. Using the inoculating loop, transfer a loopful of bacteria from the culture to a tube of sterile media. Label your tube with the name of your lab group, the date, and the inoculum.

- Test tubes are used for routine growth of bacteria.

- If you are in the BSC, use a sterile wooden applicator instead of the loop

3. *Tube to slant transfer*: Using the inoculating loop transfer bacteria from the culture to the slant. Streak the loop across the surface of the slant. Label your slant with the name of your lab group, the date, and the inoculum.

- Slants are used for longer term storage of bacteria (weeks or months when stored in the refrigerator)

4. Place all freshly inoculated media in a 37 °C incubator.

5. **Check the tube and slant after 24 hours. Check FOL for the times you are designated to come in.** *S. aureus* is non-motile and yellowish. *S. typhimurium* is flesh-coloured and motile. In the test tube, note if bacteria are on the bottom of the tube or throughout the broth. In the slant, note the colour of the colonies. Take a picture to add to your data in the lab report.

- If not in the incubator, you can find your plates on the bench in front of the windows, beside the incubators.
- Make observations of the slant, broth and streak plates
- take the plates to the biological safety cabinet (BSC) and take pictures of the plates if desired
- discard plates in the orange biohazard safety bags on the benchtops

Isolation Technique Exercise

Materials

- Inoculating loop
- *Staphylococcus* and *Salmonella* mixed culture
- One nutrient agar plate per person
- Sterile cotton swab
- Tube of dilute glycerol
- agar plate of culture media selective for gram negatives (VRBG)
- For 48 hour streak plate: one nutrient agar plate

Methods

Streak plate technique: Using NA (nutrient agar), make a three or four part streak plate using the mixed culture as your inoculum. Each lab partner should make a streak plate. Label the plate with your lab group name, the date and the inoculum.

1. The inoculating loop is made red hot by placing in the inner blue flame, holding it at an angle.

2. Cool the loop by touching to a sterile part of the agar.

2. The loop is touched to a bacterial colony then streaked on a new petri plate (Figure 4.1).

3. The loop is sterilized as previously, cooled on the agar, then used to spread the bacteria streaked in section 1 into section 2. Return to the previous streaked area only three or four times.

4. Step 3 is repeated once or twice more, returning to the previously streaked section depending on if a three-part or four-part streak plate is made. A successful streak plate has isolated colonies that can be selected for use in future studies.

5. Incubate the plate at 37 °C.

6. **Check the plate after 24 hours.** Make observations on the bacterial growth and if the plate was a success, based on the goal of a streak plate.

Swab culture technique: Using agar selective for gram negatives, you can choose an object to swab.

- Examples: cell phone screen, door handles in the college, lab bench chairs
- label your plate clearly with your names.
- Examine the bacterial diversity that grows. Bacterial diversity is represented here by the number of different types of bacteria present.

1. Aseptically remove a sterile swab by opening the paper at the handle end of the swab.

2. Wearing gloves, remove the swab.

3. Dip the swab in sterile dilute glycerol. Remove excess glycerol by turning the swab against the inside of the tube.

The dilute glycerol helps to lift bacteria off surfaces.

4. Swab a defined area of 50 cm^2: 10 cm wide by 5 cm long. Swab in one direction then in the direction perpendicular to the first direction. (Figure 4.2, left image)

- Hold the swab at 30_0 to the surface.
- Rub the surface slowly and thoroughly
- Go over the surface three times, reversing direction between the strokes

5. Place the swab in the sterile tube to transfer it back to the lab.

6. Aseptically pass the swab across the surface of a blood agar plate in a squiggling pattern.

- Write the object on your plate, along with your group's name and the date.

7. Incubate at 37 $_0$C for 24 hours.

- Make observations on bacterial diversity (number of different bacterial colonies present).

8. **After 24 hours,** make a streak plate in the BSC using tryptic soy agar (TSA) or nutrient agar (NA) to isolate one bacterial colony from your swab culture.

- Pick a colony to streak
- Make observations of the type of colony you are trying to isolate and how abundant it is. This way, you will know if you were successful in your isolation.
- If your plate doesn't have a colony, pick a colony off another group's plate
- See the box below for streaking in the BSC.

9. **At 48 hours**, observe the streak plate. Do you have a pure culture? Is the colony the same as the one you tried to isolate? Are the colonies isolated?

- Wrap in parafilm then store this plate in the prep area's fridge A. We will be using it in the coming weeks.

Streak Method in BSC

a. In the BSC, use a sterile wooden applicator to pick a colony off the plate and streak it onto 1/3 of the new plate. Discard the applicator into the 'used' container.
b. Pick a new sterile applicator and streak from the first 1/3 to the second 1/3 of the plate. Discard as before.
c. Pick a new sterile applicator and streak from the second 2/3 to the last 1/3
d. Put the plate in the 37 °C incubator on the tray labeled '24 h streak plates'
e. Label your plate with your name and section number

LAB 5: GRAM STAIN AND POTASSIUM HYDROXIDE STRING TEST

Learning Objectives

- Perform the Gram stain on bacterial cultures and determine cell morphology.
- Corroborate Gram stain results by performing KOH string test on bacterial cultures.
- Describe bacteria based on cultural morphology.

Introduction

In previous labs, we have been using simple staining. Simple staining uses only one dye and all cells appear the same. It is done to improve visualization of otherwise transparent bacterial cells under the microscope. Today, we will perform differential staining. Differential staining uses more than one dye and is done to differentiate some bacteria from others, based on characteristics of the bacteria.

The Gram stain was introduced in 1884 by Danish pathologist, Hans Christian Gram. Gram staining is often the first step performed in characterizing an unknown culture of bacteria. It allows bacteria to be divided into one of two groups: Gram positive or Gram negative, based on the thickness of the cell wall. There are four steps of a Gram stain. All Gram stains start with a smear.

Making Smears

Making a smear from broth:

- Draw a circle on the slide with a marker. (This gives you a target for searching under the microscope)
- Inside the circle, place two loopfuls of bacteria.
- Allow to air dry.
- Fix with 95% methanol for 2 minutes.
- Flood slide with methanol. Let sit 2 minutes
- Decant excess methanol.
- Air dry slide
- Gently blot the slide dry on bibulous paper.
- Paper towel is not used because it can leave fibers on the slide which can be confusing when looking under the microscope.
- Stain and view under the microscope, starting at 4X objective and working up to at least 40X objective.

Making a smear from agar:

- Draw a circle on the slide with a marker.
- Inside the circle, place two loopfuls of distilled water on the slide.
- Sterilize the loop then use it to pick up a small amount of bacteria off the plate. (If too many bacteria are placed on the smear, it will be too thick to see through under the microscope.)
- Swirl the loop in the water until the water is cloudy.
- Allow to air dry and proceed as above.

Gram Staining

a) **Crystal violet (purple)** is the primary stain. All cells are stained by crystal violet, as we have seen in previous labs.

- All bacteria that we work with in this lab have a cell wall. The cell wall is made of a mesh work of peptidoglycan (PG).
- Gram positive bacteria have a thick layer of PG, while Gram negative bacteria have a thin layer of PG.

b) **Iodine** is a mordant; it increases the binding of crystal violet to the cells.

c) ***Decolourizer*** *contains alcohol*, or acetone or mixture of the two, which solubilizes crystal violet and helps to wash it away.

- The crystal violet in Gram positives is not removed as easily as Gram negatives due to the thick cell wall.
- The goal of this step is to decolourize Gram negatives while allowing Gram positives to remain stained with crystal violet.

d) **Safranin (pink)** is the counter stain. All cells are stained by safranin, but it will only be visible in cells that were completely decolorized by the previous step.

- Gram negatives will show the safranin stain, while Gram positives will show the crystal violet stain when viewed under the microscope.
- Notes: A young culture is used for Gram staining. In older cultures, cells can start to break down their cell walls. This can give misleading results.

e) View the slide under the microscope to determine the Gram reaction.

- If you see very long, thin “bacteria” with pointed ends under the microscope, you are probably looking at stain crystals.
- You will be able to describe cell shape and arrangement.

Using the Microscope

Before you start using the microscope for each lab you should clean it. You cannot be sure that the previous person cleaned the microscope.

Clean the ocular lens, condensor and objective lenses with lens paper only. Do not use other materials as these may scratch the lenses.

When you are done:

a) Remove the last slide being viewed.

b) Clean the lenses and clean the oil from the 100X objective lens. Wipe the stage as it may have oil or other debris on it.

c) Turn the nosepiece so that no objective lens is in position.

d) Lower the stage to the lowest position.

Controls

Last week, you isolated bacteria from an environmental swab. This week, you will determine the gram reaction for the isolate. In order to interpret the results, you need to use a known gram negative culture and a known gram positive culture. The result of test isolate can only be interpreted if the gram positive and negative controls are the correct colours. You will be picking the controls for the experiment.

Cultural and Cellular Morphology

We know that different species of animals appear differently. The same is true of bacteria. In this lab, we will be investigating:

cell morphology– how individual cells appear and how cells are arranged relative to each other

cultural morphology– how groups of cells in pure culture appear in a test tube, on a slant, and on agar plates

By investigating cellular and cultural morphology, we can gain important information about bacteria we are working with in the lab. By being familiar with the morphology of a species you are working with, you can quickly notice if the culture has become contaminated and take steps to obtain a pure culture again.

Cellular Morphology

To examine cellular morphology, you must look at cells through the microscope. Since bacteria are so small, you will need to be using the 40X objective lens at least, and ideally you will use the 100X objective lens. When describing bacterial cells, there are two questions that you are answering:

What is the shape of the cell?

> Common shapes are: rod (or bacillus), coccus (pl. cocci), coccobacillus (a short rod, looks like a jelly bean), vibrio (comma-shaped), spirochete(flexible spiral), spirillium (rigid spiral)

How are the cells arranged relative to each other?

> Common arrangements are: single, diplo- (two), strepto- (chain), tetrad (four cocci in a square), sarcinae (eight cocci in a cube), staphylococcus (random cluster), palisades (rods aligned along long axis)

Determining the arrangement can be difficult with a smear because cells can appear artificially close together. This is just due to cell density on the smear and has no biological relevance. Look to the edges of the smear, where there are fewer cells and they are well spread out. If you think you see an arrangement, all cells should be in that arrangement. For example, if you see two cells next to each other and you think it is the diplo- arrangement, all cells should be in pairs. Refer to figure 5.1 for illustrations of the common cell morphologies we will encounter in this lab.

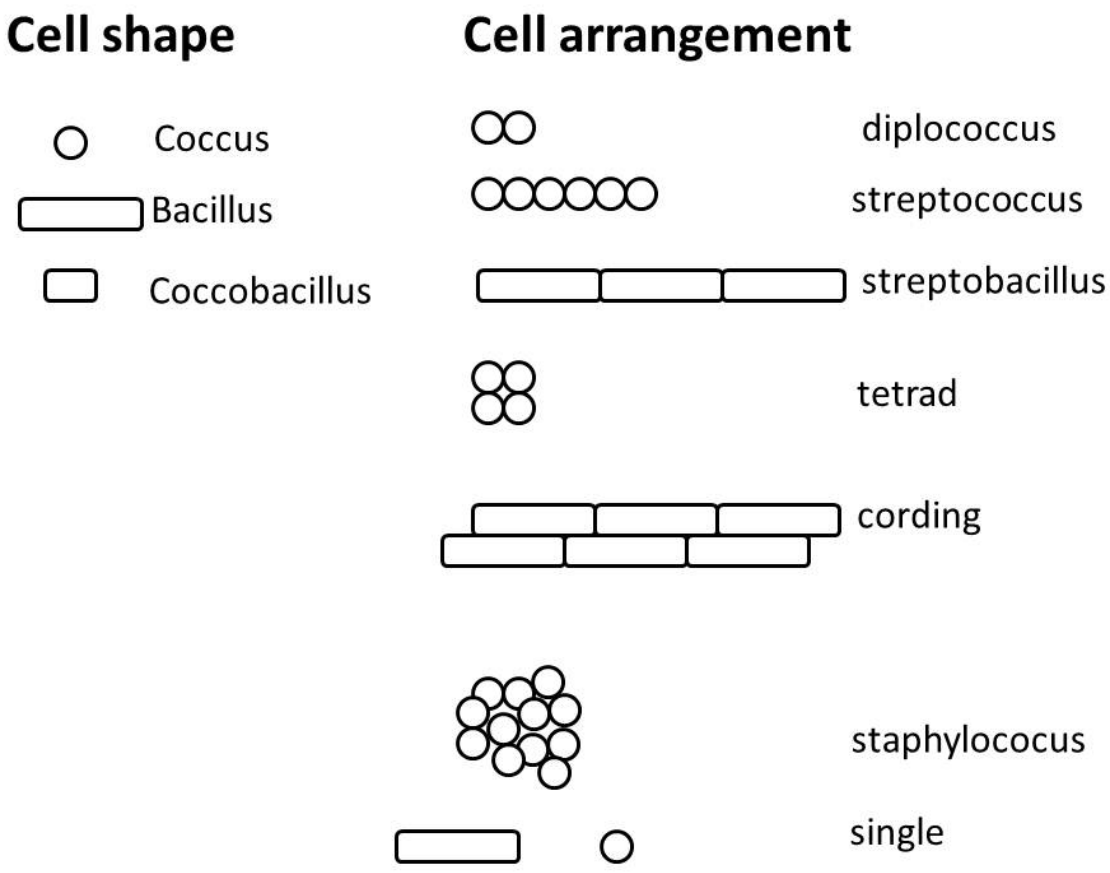

Figure 5.1. Cell morphological characteristics.

Cultural Morphology

Cultural morphology can be determined in any growth medium. How the bacteria grow as a group are examined. To determine cultural morphology in broth, the bacteria are grown without shaking the test tubes. When the tubes are examined, care is taken not to disturb the cells as this will destroy the cultural features you are trying to observe.

Table 5.1. Colony morphology in broth test tubes.

Broth growth habit	Description
Turbid	Uniform growth throughout the broth. Tubes appear cloudy compared to uninoculated media.
Precipitation	Cells are concentrated in the bottom of the tube
Flocculation	Cells are aggregated into large flocs (or chunks) throughout the medium. The flocs may settle on the bottom of the tube and can be differentiated from precipitation by the presence of chunks.
Pellicle	A skin forms at the air-liquid interface. This is independent of growth characteristics in the tube. E.g. A turbid tube can have a pellicle.
Ring	Bacteria aggregate on the glass of the tube where the liquid contacts the glass. This occurs independently of growth characteristics in the tube.

On **petri plates**, each colony on a plate represents a group of related bacteria, all arising from an individual cell. Refer to your lecture notes for descriptions of colony morphology.

Table 5.2. Surface and Texture of colonies on agar plates

Surface characteristics: the appearance of the colony surface viewed from the top	
Colony surface characteristic	**Description**
Concentric	Concentric rings of growth
Smooth	No visible surface features
Contoured	Random elevation changes in colony
Radiated	Lines radiating out from centre of colony
Wrinkled	Surface is deeply wrinkled and looks dry
Texture of colony when probed with a toothpick	
Colony texture characteristic	**Description**
Butyrous	Buttery
Viscous	Gummy- cells stick to themselves
Dry	Cells crumble apart
Mucoid	Slimy

Before your lab, fill out the table below using your lecture notes.

Table 5.3: Colony morphology on agar plates

Colony characteristic	Description (can include pictures)
Colony shape (form): the overall shape of the colony	
Circular	
Filamentous	
Irregular	
Rhizoid	
Colony elevation: observed by looking at the colony from the side	
Flat	
Raised	
Convex	
Crateriform	
Umbonate	
Margin: the shape of the edge of the colony	
Entire	
Undulate	
Filamentous	
Lobate	
Curled	

A note on the strains: Since bacterial strains within a species are heterogenous, often, the results you might find from an internet search do not align with our lab strain's results. Make observations on your strains in lab.

Experimental Overview

We are doing two labs in one today: Gram staining and morphology of cells and cultures.

Bacterial Isolates

Choose one agar plate culture and a matching tube culture from Gram negatives (column A) and Gram positives (column B), and your streak plate isolate from last week.

- You will have three bacterial isolates: a plate and tube of one Gram negative, a plate and tube from one Gram positive and a plate of your unknown.

A: Gram negatives	**B: Gram positives**	**Unknown**
E. coli (Ec)	*Staphylococcus aureus (Sa)*	Your isolate from last week
Pseudomonas aeruginosa (Pa)	*Micrococcus luteus (Ml)*	
Salmonella typhimurium (St)	*Staphylococcus epidermidis (Se)*	
Proteus vulgaris (Pv)	*Enterococcus faecalis*	

You will do three smears (one for each isolate) and three Gram stains. Examine the cell morphology during the gram stain. Observe the colony morphology on agar plates, and describe the broth growth characteristics.

Taking pictures on the microscope

1. Using the microscope, centre your sepcimen.
2. Increase the light source to the maximum intensity.
3. Hold the camera lens against the microscope eyepiece. A small circle of light can be seen on the phone's screen.
4. Use the camera's zoom function to increase the size of the cells.
5. Move the camera lens small distances across the eyepiece to centre the circle. The auto-focus should then self-adjust to give a clear image.
    - This is the most difficult step.
6. Adjust the fine focus of the microscope to maximize image clarity.
7. If the image is too dark or grainy, select the "darkness" or "nighttime" setting on your phone camera.
8. While holding the camera very still, take a photograph. Examine the image to see if it is satisfactory.

Gram Stain and Cell Morphology Exercise

Materials

- Staining trays
- Slides
- Marker
- 95% methanol
- Gram staining reagents
- Isolates from above

Method

Refer to the beginning of this lab for a detailed procedure on making a smear.

1. On one slide, draw three circles with the marker. You will be testing your unknown strain for its gram reaction. One circle is for the positive control, one for the negative control, one for your test strain.

    What are the controls that will go in the other two circles?

    Positive control:

    Negative control:

2. Over the staining tray, flood the slide with crystal violet. Let sit 1 minute. Gently wash the slide with distilled water by holding the slide on an angle and allowing the water to wash over the smears.

3. Flood with iodine and let sit 1 minute. Wash as above.

4. Destain with decolourizer, holding the slide on an angle and allowing the decolourizer to wash over the slide. **Stop this step as soon as no more purple colour washes off the slide** (the drops of decolourizer coming off the slide will be clear). Wash with distilled water as above.

5. Counter stain with safranin for 30 seconds. Wash with distilled water and gently blot the bottom of the slide dry (this prevents the slide from sticking to the microscope stage). Examine under oil immersion (you may need to adjust the light and contrast to see the colours well). Record your results below

- If either of the controls are not the correct colours, repeat the process, starting with new smears. These must be the correct colours to determine the Gram reaction for the unknown culture
- 6. View under the microscope.

Isolate	Colour of cells	Gram reaction	Cell morphology (shape and arrangement)	Drawing or image
Control 1:_____				
Control 2:_____				
Environmental isolate				

7. Put the microscope away, referring to the steps outlined above in "when you are done".

KOH String Test

KOH String test is an alternative test that may be used as a confirmatory test for the Gram stain. Gram negative bacteria cell walls dissolve with 3% KOH while gram positive bacteria cell walls are not disrupted. When the cell walls of gram negative bacteria are lysed, cellular DNA is released which makes the mixture viscous (stringy). The formation of a string in 3% KOH within 60 seconds is a positive test result and an indication that an isolate is a gram negative organism.

Materials

- 3% KOH
- Glass slide
- Culture loop
- Agar plates: Use the same cultures you used in the gram stain above.

Method

1. Place a drop of 3% KOH on a glass slide

2. Mix a loopful of bacteria into the KOH and continue to mix the suspension for about 30 seconds.

- Take bacteria from the first and second streaking area of an agar plate. You cannot take from broth as there will be too few bacteria to give a reliable result.

3. Slowly lift the loop to observe for formation of string. Indicate if the test for each organism is positive (formation of a string) or negative (absence of a string) and interpret your results.

4. Repeat for each culture.

5. Disposal: Glass slides go in the glass tray. Staining trays are dumped in the mixed inorganic waste then returned to the cupboard. Rails go back in the toolbox.

Precaution: False negatives may occur if too few cells are taken and false positive if too many cells are used if bacteria form mucoid colonies.

Isolate	KOH test observation	Interpretation (Gram positive or negative?)
Control 1:_____		
Control 2:_____		
Environmental isolate		

Cultural Morphology Exercise

Materials

Cultures from above experiment: gram negative plate and tube, gram positive plate and tube, your unknown

- Ruler
- wooden applicator
- magnifying glass

Methods

1. Broth culture: Observe the growth in the test tube and describe using the terms outlined above. Be careful not to disturb the tubes for other groups.

Isolate	Growth in broth
Gram negative: _____	
Gram positive: _____	

2. Agar plates**:**

a. Measure the diameter of a typical colony in millimeters.
b. Note colony opacity (how well light goes through it): transparent, translucent, opaque.
c. Note pigmentation presence: are the cells pigmented or is pigment being secreted into the agar? What colour is the pigment?
d. Record the four characteristics described in the table (margin, shape, elevation, surface).
e. Using a wooden applicator, determine the colony texture.
f. Take an image of your environmental isolate's colonies on agar.

Document your results in the Lab 5 Worksheet (download links at top of page)

Species	Diameter (mm)	Opacity	Pigmentation	Colony description (4 terms)	Texture
Gram negative: _____					
Gram positive: _____					
Your isolate					

Making a Figure

To document your observations, you will be taking pictures and making figures for lab reports. Here is how to make them.

The specimen (plate, tube, colony) should be in focus and well-lit. Use a light box or photograph against a white sheet of paper.

Crop out the background if it isn't conveying information.

I like to make figures in PowerPoint to easily align different images, then put the slide in presentation mode and take a screenshot of the slide. Then I crop out the background and paste the single image into Word. The image below is a composite of four images. You can also label the images with text boxes.

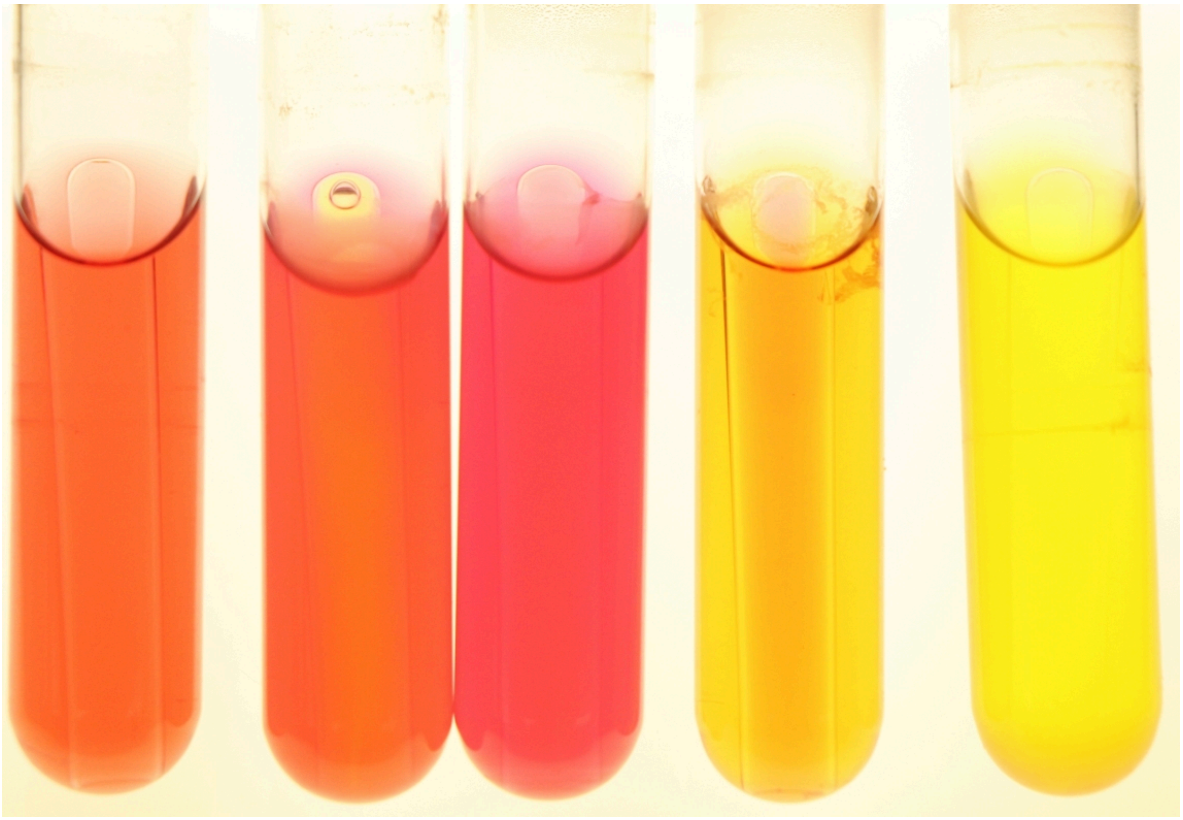

Figure 5.2: Bacteria growing 24 hours in Phenol Red Sucrose broth. Each tube from left to right: *Serratia marcescens*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Escherichia coli*.

(generic) **Figure number**: Title of figure. Description of items within the figure to guide the reader.

LAB 6: BACTERIAL MEDIA

Learning Objectives

- Test bacterial reactions on selective and differential media.
- Use controls in quality control of culture media.

Introduction

In the labs so far, we have been using general, nonselective media (Luria Bertani broth, LB; trypticase soy broth, TSB; nutrient agar, NA). The growth medium you use depends on the organism you are trying to grow; the organisms we have been growing in the lab grow well on these rich, complex media.

What would happen if you were given a sample of pond water and asked to isolate one genus of bacteria? If you grew this sample on rich, complex media, such as LB, you would grow many species from many genera. One way to narrow the types of bacteria you culture to target one species or genus of interest is to use selective and differential media.

We will be these three types of media in this lab:

Selective media allows the target organism to grow while preventing the growth of other organisms that may be present in the sample.

Differential media allow visual distinctions to be made between growth of different types of organisms.

Multitest media possesses multiple tests within one medium (Figure 6.1).

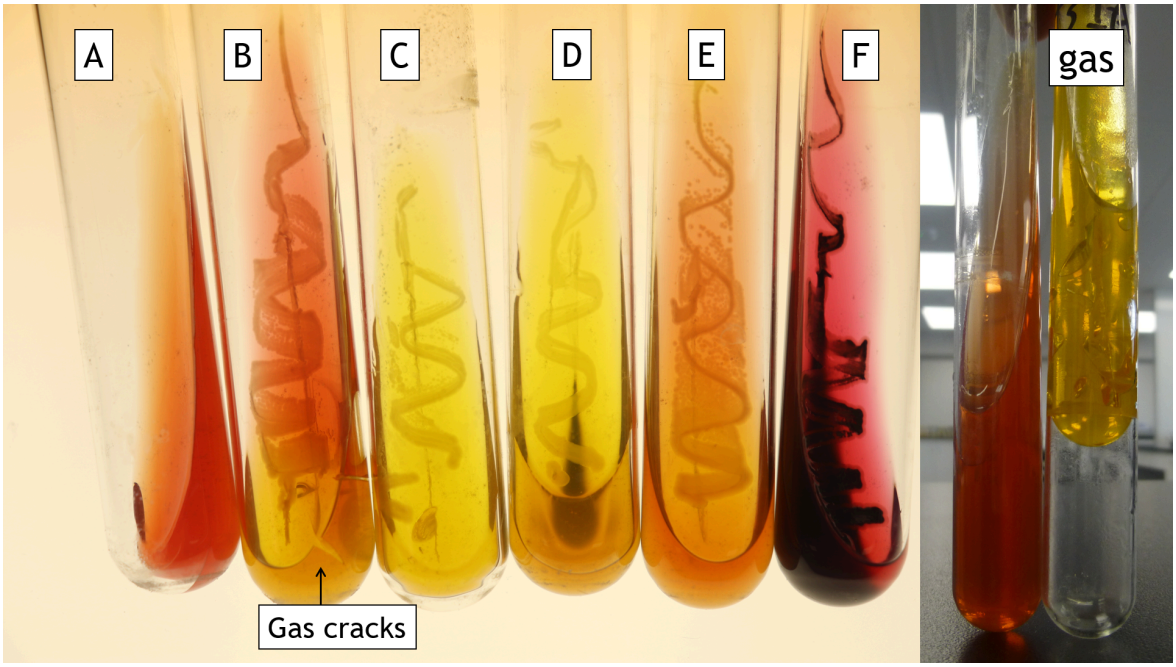

Figure 6.1. Triple sugar iron agar (TSIA) is a multitest media. Uninoculated (A) is orange. Glucose-fermenter has acid butt and alkaline slant (B). Sucrose/lactose fermenter (C and D) have an acidic butt and slant. Non-fermenter (E) does not change the media colour. Hydrogen sulphide producers (D and F) are black. Gas can appear as cracks (B) or it can push the culture media up (right).

Let's consider components of media to help you understand differences between media.

Carbon and Nitrogen Sources

All media needs to provide the macronutrients needed for cell growth. Carbon and nitrogen are required in the greatest amounts. In complex media, these are added as complex biomolecules. This approach also ensures hydrogen, phosphorus and sulfur are also added as part of the biomolecules. Common carbon and nitrogen sources are a proteinaceous source that has been partially degraded by enzyme or acid digestion. In a media recipe, these ingredients can be found below.

Carbon and nitrogen sources

Peptones, enzymatic digest of casein (e.g. pancreatic digest), enzymatic digest of soy meal, proteose peptone, tryptone

- These are all found in complex media since their exact chemical composition isn't known.

Carbon source only

Sugars: lactose, sucrose, glucose, mannitol (Figure 6.2)

Nutrient Extracts

These can be found in some, but not all media. They are a good general nutrient base, including vitamins and minerals with some carbon and nitrogen. Extracts can be made from almost anything, by soaking a substance in water. These can be tailored to the type of organism you are trying to isolate. For example, if you are trying to isolate soil bacteria, you could add a soil extract to your media to mimic the soil environment.

Typical extracts include beef and yeast.

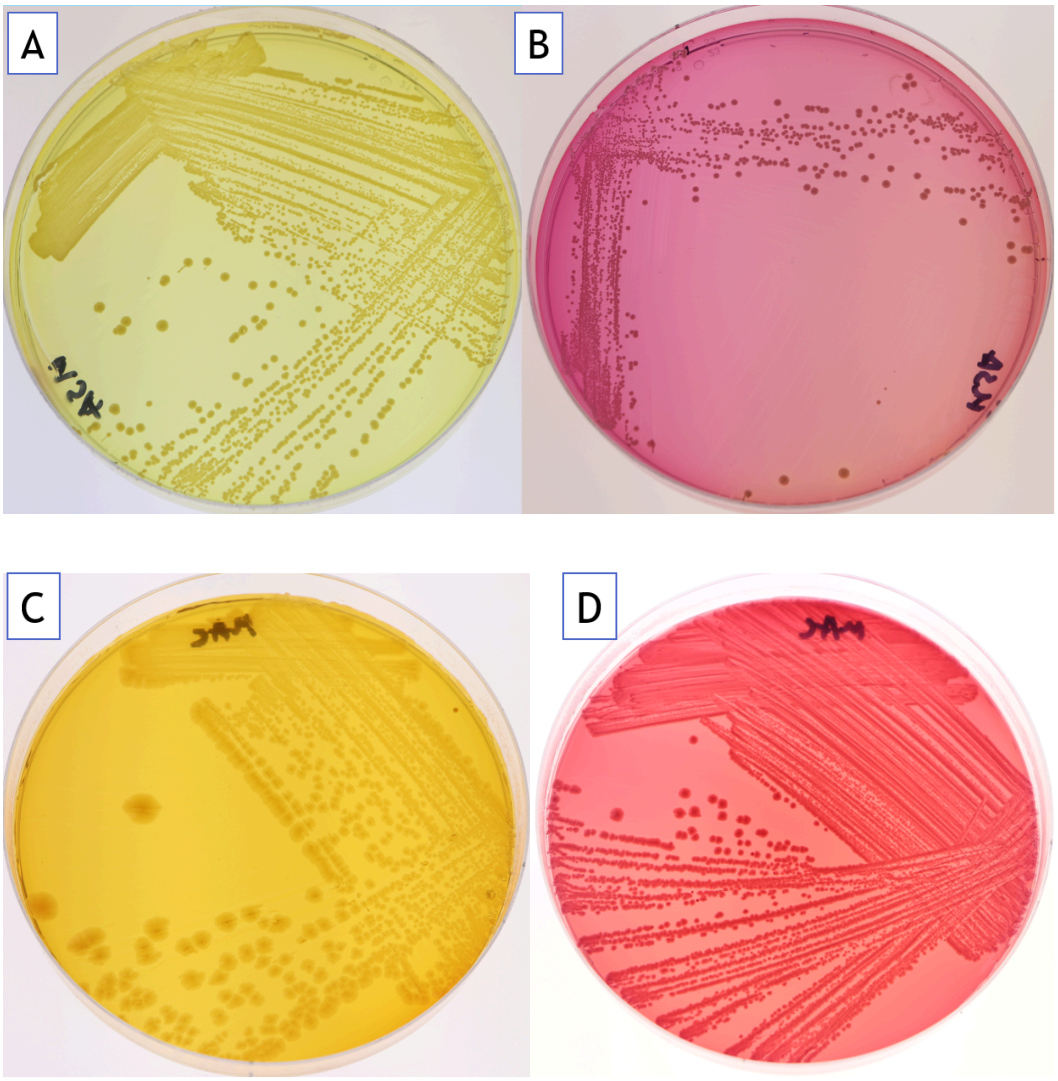

Figure 6.2. Top: Mannitol salt agar(MSA) and, bottom: MacConkey Agar (MAC) are selective and differential. (A) Mannitol fermenting *Staphylococcus aureus* turns MSA yellow. (B) Non-mannitol fermenting *S. epidermidis* does not change the colour of MSA. Not shown: *E. coli*, which is inhibited by the NaCl concentration. (C) On MAC, non-lactose fermenting *Salmonella typhimurium* is yellow. (D) Lactose-fermenting *E. coli* on MAC forms brick-red colonies and the agar turns red.

Solidifying Agent

If a solid or semi-solid medium is being made, the liquid is solidified. Typically, agar is used although other gelling

agents exist and are used when agar is toxic to the organism of interest. In the petri plates we have been using, 15 g/L of agar is in the medium. By altering the amount of agar in the medium, bacterial motility (the ability of the bacteria to propel themselves) can be tested.

- By reducing the agar to 3 g/L, the medium becomes semi-solid; such plates are termed “swim plates” because they allow bacterial swimming to be examined.
- At 10 g/L agar in the medium, the ability of bacteria to swarm or glide can be tested

Inhibitors in Selective Media

These are found in selective media and they act by inhibiting the growth of certain bacteria. Inhibitors can be bactericidal (kill bacteria) or bacteriostatic (prevent the growth of bacteria).

- If the inhibitor is bacteriostatic, when you pick a colony off the plate, you may have passenger cells of the undesired group, which can resume growth when placed in nonselective media. Thus, it is always good practice to streak for purity after selecting colonies from selective and differential media.
- When a medium is selective for a certain group of bacteria, this does not mean that only this group will grow. Media vary in how selective they are (based on the type of inhibitor and its concentration)

Typical inhibitors include bile salts, alcohol, high concentrations of some compounds (e.g. NaCl), some dyes (e.g. methylene blue).

Inhibitory conditions can also be used. These include pH, incubation temperature, oxygen concentration.

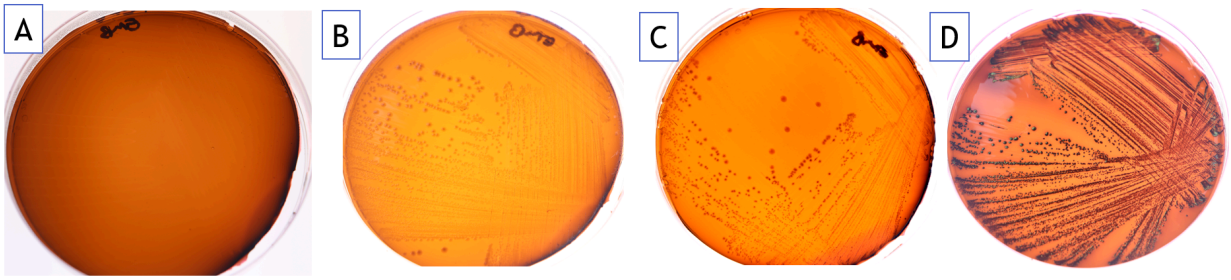

Figure 6.3. EMB agar is selective and differential. (A) Gram positive strain is inhibited by methylene blue. (B) Non-lactose fermenter is colourless. (C) Lactose-fermenting *Enterococcus aerogenes* is red. (D) Vigorous lactose fermenter *E. coli* is metallic green.

Special Substrates in Differential Media

Differential media rely on a visual change between growth characteristics. Often, the visual change is due to a colour change in pH indicating dye. The pH change is due to carbohydrate utilization. The principle is that only some bacteria will use a carbohydrate and thus, alter the pH, changing the colour.

With blood agar plates, the appearance of growth resulting from red blood cell hemolysis (lysing of red blood cells) depends on the bacteria present (Figure 6.4).

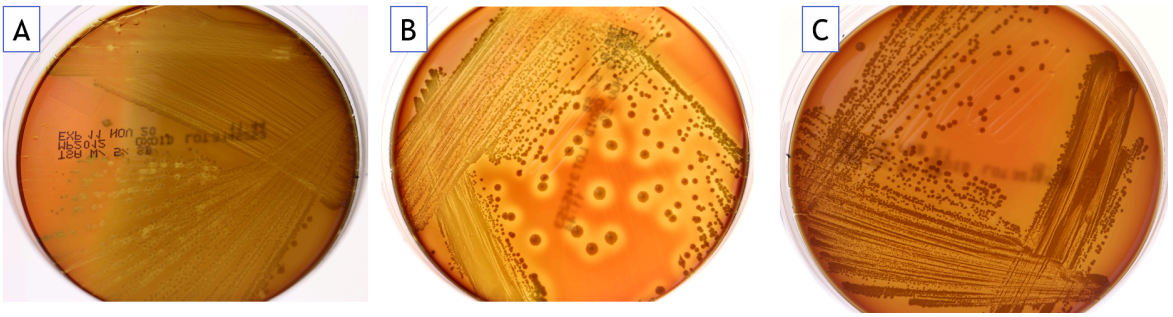

Figure 6.4. Blood agar is differential. Alpha or partial hemolytic bacteria turn the red blood cells greenish (A). Beta or total hemolytic bacteria cause clearing in the agar (B). Gamma or non-hemolytic bacteria make no change in the agar (C). When phenylethyl alcohol is added (PEA) then the plates are selective for gram positives.

Using Controls

When using media, we need to have controls to ensure the media is made properly and the growth conditions give the expected result.

- The **positive control** is an organism that performs the reaction that the media is testing for.
- The **negative control** is an organism that does not perform the reaction that the media is testing.

In the media today, one strain will be the positive control, one will be the negative control, and your environmental isolate is the test.

- If the control doesn't give the expected result, note this. It means either the strain didn't grow properly (due to contamination most likely) or the media was made improperly. This limits your ability to interpret the test strain.

Media Exercise

Materials

- 1 plate of each: PEA, MSA, MAC, EMB, Blood
- 1 general purpose media (NA, LA, TSA)
- 4 tubes: TSIA
- Strains: *Escherichia coli, Proteus vulgaris, Serratia marcesscens, Staphylococcus aureus, S. epidermidis, Bacillus subtilis, Salmonella typhimurium*
- Your environmental Isolate

Method

1. With a marker, divide the PEA, MSA, MAC, EMB and Blood plates into three.
2. **Pick a single colony of the stock plate,** then using the streak plate technique, label then inoculate the following strains onto each medium (controls are indicated with pos or neg):

    a. PEA: *S. aureus* (pos), *E. coli* (neg), your isolate
    b. MSA: *S. aureus* (pos), *S. epidermidis* (neg), your isolate
    c. MAC: *E. coli* (pos), *S. typhimurium* (neg), your isolate
    d. EMB: *E. coli* (pos), *S. typhimurium* (neg), your isolate
    e. Blood: *B. subtilis* (neg), *S. aureus* (pos), your isolate
    f. Make a fresh streak plate for your isolate.

3. Label the TSIA, then inoculate using the inoculating needle by stabbing into the butt of the tube then streaking across the surface of the slant: *E. coli, S. marcescens, P. vulgaris* and your isolate.
4. Incubate the plates for 24 hours at 37 C.
5. Read the test results, noting the colour of the colonies and media in your lab book.

    - Take a picture of your plates (use the BSC if you are removing lids), using an ungloved hand on the phone.
    - Interpret results using this lab and FOL

6. Put your environmental isolate in the fridge to use next week. Either wrap it in parafilm or put it in a Petri plate bag to prevent evaporation.

LAB 7: UNKNOWN MOLECULAR LAB PART I

Learning Objectives

- Isolate the genomic DNA from an unknown species of bacteria.
- Perform 16S rDNA PCR on an unknown species of bacteria.

Introduction

We know how to isolate bacteria from the environment and how to select out certain species while not growing others (using selective and differential media). Bacteria are divided into species just like higher organisms. Members of the same species can still vary considerably; these are called strains. Consider the case of *E. coli*: some strains of *E. coli* may cause hemolytic uremia (destruction of red blood cells), some cause urinary tract infections, while another strain may be a harmless resident of your intestines. If these strains were on petri plates, they would all look similar. How would you tell these strains apart in the lab?

You will be determining the species of an unknown culture by two methods. This week and next, you will determine the species using molecular testing. In the final lab of the course, you will determine the species using culture. Then you can compare the two methods.

One way to determine the species and strains of bacteria is by isolating and testing their DNA. This is the most reliable way to determine bacterial species and is considered the gold standard. Molecular testing is also more rapid than culture, although it requires more technical skill. The results are a DNA sequence that is compared to other DNA sequences of the same gene in a database. This is much less arbitrary than determining results of bacteria cultured on differential media.

Depending on the bacterial species being identified, Public Health Ontario may have a culture-based test or a molecular test.

Let's examine the process of identifying bacterial based on their DNA.

DNA Extraction

The first step requires you to isolate bacterial DNA from the rest of the biomolecules in the cell. The other biomolecules will inhibit the downstream reactions you will perform.

The bacteria need to be lysed to release the DNA. There are many ways to lyse bacterial cells: sonication, heat, chemicals, enzymes. These methods all work to degrade the lipid membrane and cell wall. We will be doing a

boiling lysis preparation. The solution the bacteria are suspended in will being to inhibitors of the downstream reaction (these are the proteins and other small molecules in the cytoplasm). The bacteria are boiled in this solution to break open the cells. At the end of the procedure, pure DNA remains in the solution while other cellular components are bound to a particle (called Chelex) and form a precipitate at the bottom of the tube. We will use the genomic DNA-containing supernatant in the next step.

Polymerase Chain Reaction

Now, you are going to amplify one gene in the genomic DNA. This gene, 16S rDNA gene, is present and conserved (has a similar sequence) in all bacteria. Remember, the 16S rDNA gene is transcribed into 16S rRNA, which becomes part of the small subunit of the ribosome. This gene is used for molecular testing because it is present and has a conserved sequence. We will amplify (increase the number of copies) of this gene so its sequence can be determined. If the 16S rDNA gene wasn't amplified, it would exist at the same concentration as the rest of the genes on the genome and it would be difficult to determine the sequence of this gene.

The gene will be amplified using polymerase chain reaction (PCR). This is essentially DNA replication in a tube. You will mix genomic DNA with DNA polymerase (called Taq polymerase), nucleotides (adenine, guanosine, cytosine, thymidine), and short pieces of DNA called primers (ours are called 341F and 805R) that will ensure only the 16S rDNA gene is amplified.

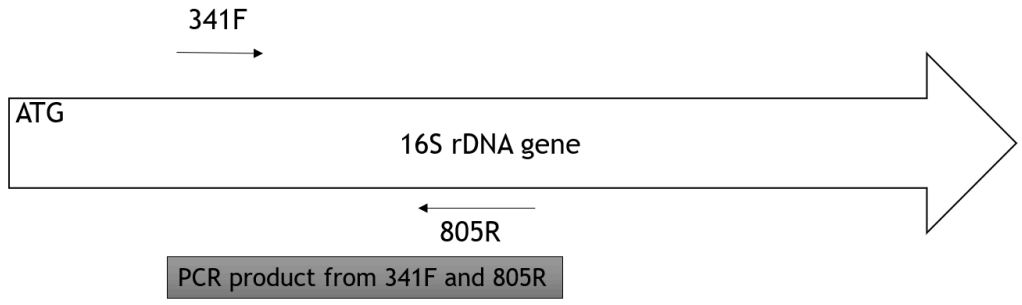

Figure 7.1. PCR amplification of 16s ribosomal subunit gene using primers 341F and 805R results in a DNA products that is 454 nucleotides long.

Using a Microcentrifuge

Centrifuges spin samples inside tube at high speeds to separate cellular components. Larger particles will travel faster to the bottom of the tube than smaller particles. We will use a centrifuge today to separate bacterial cells from the solution they are suspended in. Later, we will centrifuge the lysed cells to separate the DNA in solution from the lysis matrix and large cellular debris.

Supernatant is the liquid fraction after centrifugation.

Pellet is the solid fraction at the bottom of the tube after centrifugation.

Pay attention to the lab protocol! We keep the pellet after the first centrifugation today, but we use the supernatant after the second centrifugation!

When using a centrifuge, we need to make sure we:

balance the tubes so that your tube is opposite from another tube of equal mass.

close tubes to prevent

DNA Extraction Exercise

Materials

- BioRad InstaGene Matrix
- Bacterial culture: your environmental isolate
- Micropipettes and tips
- Sterile 2 ml tubes
- Sterile water

Method

1. Select bacteria to lyse
2. From agar plate: if the culture looks pure, scrape up several colonies with a sterile pipette tip and resuspend in 500 µl sterile water in 2 ml tube.
3. From test tube: Resuspend bacteria using gentle mixing of the tube if the bacteria have settled to the bottom. Transfer 500 µl broth to 2 m tube.
4. Centrifuge at 13,000 rpm for 1 minute.
5. Balance your tube with another group's tube.
6. Gently remove the supernatant by pipetting. A small amount of residual liquid will remain in the tube in either case.
    - The cells are in the pellet. We are extracting the DNA from these cells.
7. Using P1000 pipette (blue), add 200 µl InstaGene Matrix to the tube.
8. The matrix will be stirred to keep the matrix particles in suspension while you withdraw your sample.
9. In block heater, incubate at 56 ºC for 15 minutes.
10. Vortex at high speed for 10 seconds.
11. Place in heating block at 100 ºC for 30 minutes.
12. Vortex at high speed for 10 seconds.
13. Centrifuge at 13,000 rpm for 3 minutes.
    - Remember to balance your tube with another group's tube.
    - DO NOT DISCARD THE SUPERNATANT THIS TIME! This is where the DNA is.
14. Determine the concentration of DNA using a UV spectrophotometer (Qubit 4): In a 0.5 ml tube, mix 180 µl of Qbit reagent and 20 µl of supernatant. Measure the concentration of this.
    - The Qbit will calculate the ng/µl of DNA in your original sample if it is told the volume of the sample you

measured.

15. You may need to dilute your DNA if the concentration is more than 5 ng/µl. We can use a maximum of 20 µl template in the PCR. 20 µl x 5 ng/µl = 100 ng
    - $c_1v_1 = c_2v_2$
    - C_1 = qubit result in ng/µl
    - C_2 = 2 ng/µl
16. Carefully transfer the tube to ice while you prepare the PCR.

PCR Exercise

Materials

- The reagents for PCR will be stored on ice.
- PCR mastermix
- Template DNA (from above)
- Forward and reverse primers
- PCR tube

Method

1. Warm up the thermocycler to 95 C.

    a. This allows you to immediately start the PCR in the thermocycler.

2. Complete the following table:

Reagent	Stock concentration	Final concentration	1 reaction volume (ul)
Template		100 ng	Up to 20 ul
Mastermix			25
16S fwd primer: 341f	100 µM	1 µM	
16S rev primer: 805R	100 µM	1 µM	
DNA-free water			
Final volume			*50*

Note: The final volume of the PCR is 50 µl. Use this value when determining the concentration of primers and volume of water in the final solution.

Primers: forward primer is 341F; reverse primer is 805R; product is 464 bp

The mastermix has the Taq DNA polymerase, dNTPs and $MgCl_2$ (required for DNA polymerase to function).

3. Mix the reagents on ice in a PCR tube.

    a. The professor will mix the **negative control**. This is the same as your reaction, only without template DNA.

    b. The professor will mix the **positive control**. This is a strain of bacteria known to produce a product with the primers: *E. coli*

4. Place the tubes in the thermocycler and run on a program with the following parameters:

- 3 min at 94° C.
- 5 cycles of [30 seconds at 94° C, 20 seconds at 45° C, 30 sec at 65° C]
- 35 cycles of [20 seconds at 94° C, 20 seconds at 55° C, 30 sec at 72° C]
- 5 min at 72° C,
- 4° C hold

Note the placement of your tubes in the thermocycler. Sometimes the markings are removed during the reaction.

Once the PCR is complete, put your tubes in the freezer for next week.

LAB 8: UNKNOWN MOLECULAR PART II: AGAROSE GEL AND PCR PURIFICATION

Learning Objectives

- Assess DNA quality and PCR results using agarose gel electrophoresis.
- Purify DNA of chemical contaminants.
- Determine the quantity and purity of DNA using spectrophotometry.

Introduction

Last week, you extracted DNA from an unknown bacterial species. Then you set up a PCR to amplify the 16S rDNA gene. This week, you will check if the PCR was successful. If it was, you will prepare your sample for sequencing to determine the nucleotide sequence in the 16S rDNA gene. The resulting DNA sequence can be searched in a database to identify the unknown species.

Agarose Gel

We need to determine if your PCR of the 16S rDNA gene was successful. By running a small volume of your sample on an agarose gel, we can determine if any DNA was amplified in the PCR and if so, if it is the correct size.

On an agarose gel, smaller fragments of DNA travel faster through the gel than larger. By loading a sample consisting of standard sizes (called a molecular weight ladder), you can compare the size of products in your sample to known sizes of DNA. This will allow you to determine the size of your PCR products (Table 8.1). Depending on your primers, the DNA product will be 1465 nucleotides long (27F and 1492R primers) or 464 nucleotides long (341F and 805R primers).

Table 8.1. Most likely PCR and agarose gel results.

PCR	Appearance on Gel	Likely Reason
No amplification	No bands	Incorrect annealing temperature, reagent absent
Amplification of several DNA regions	Several bands	Annealing temperature too low
Amplification of target DNA only	One band of the correct size	Correct physical and chemical conditions met

You added genomic DNA to your PCR, but it will likely not be visible on the agarose gel. Genomic DNA is large (millions of nucleotides) and won't travel very far on the gel.

The loading dye consists of three dyes. These dyes give you an indication of the location of DNA on the gel. If you run the gel for too long, your sample may run off the end of the gel and be lost in the buffer. Conversely, if you run the gel for too little time, your sample won't separate well and it will be difficult to determine the size of any PCR products. By watching the location of the bromophenol blue band, you can ensure your sample is run the correct amount of time.

Table 8.2. Loading dyes and their approximate size in 1% agarose TAE gel.

Loading Dye	Approximate Migration (size in nucleotides)
Xylene cyanol	4000
Bromophenol blue	300
Orange G	50

PCR Purification

Remaining in your PCR from last week, there are unused primers and DNA polymerase enzyme. These can interfere with the sequencing reaction. We need to remove everything except the DNA amplified by PCR.

To purify the DNA, your sample will be loaded onto a column. The column contains a silica resin that binds DNA under high salt conditions, while proteins (like the DNA polymerase enzyme) do not bind in these conditions. Then, the DNA is eluted (released) from the column under low salt conditions. The high and low salt conditions are created by passing different buffers (solutions of chemicals) through the column. The end result of PCR purification is 16S rDNA gene product in elution buffer or water.

DNA Quantification

The DNA sequencing process is simply another PCR. This time, it will be done using the 16S rDNA gene product

as template with a primer that will bind to your PCR product. Because you have purified this PCR product, you may have a very high concentration of DNA. A PCR can be inhibited if there is too much template. You need to ensure this template DNA is in the optimal range for the sequencing reaction. To do this, you need to quantify the amount of DNA in your sample.

Double-stranded DNA absorbs at 260 nm. When this wavelength is passed through a sample with DNA, the amount of absorption corresponds to the amount of DNA present. The instrument is zeroed with the solution the DNA is suspended in, typically water or elution buffer.

After determining the result of DNA concentration, you will adjust it so that you have 5-20 ng/ul.

DNA Sequencing

This is performed by an organization outside the college. We are using the Sanger or dideoxy method of sequencing. Read about it here.

Agarose Gel

Materials

- 50X TAE
- Agarose powder
- Loading buffer
- Molecular weight ladder
- PCR from last week
- 6x loading buffer (contains dye)
- Red Safe dye

Method

Only one gel needs to be made for every five groups.

1. Make 200 ml of 1X TAE in distilled water, using the 50X stock.
    - Use the $c_1v_1=c_2v_2$ formula and solve for v_1.
2. Using 50 ml of 1X TAE, make a 1% agarose gel (w/v).
    - The agarose mass is calculated based on 1% of the volume of the gel. In other words, 1% of 50 ml is the mass in grams of agarose required.
3. Add **2.5 µl RedSafe** dye per 50 ml of gel solution.
    - We need to use this dye to see the DNA after running the gel.
4. Microwave in short bursts of 20 seconds to melt the agarose into the buffer.

- You should see no flecks of agarose in the solution.

5. Handle the flask with hot hands and keep the opening pointed away from everyone in case it boils over.
6. Cast the gel using the 6-well combs.
7. Once the gel is solidified, remove the comb, and pour in the buffer.
8. Load the gel: Mix 5 µl of sample with 1 µl of **6x loading dye** on a piece of Parafilm.
    - Load the MW ladder in the middle lane.
    - Load 5 µl of your sample in another lane.
9. Record the lane that you loaded your sample in.

Note: There are two types of dyes used in this gel.

- **RedSafe is for staining DNA to allow it to be seen under UV light.** If you don't add this, you won't be able to see the DNA on the gel to know if your PCR was successful.
- **Loading dye is for marking where the DNA is on the gel while running.** If you don't add this, you won't know when to stop running the gel, because you won't know where the DNA is.

17. Run the gel at 100 V approximately 45 minutes while you complete the next steps. Don't let the orange dye run off the gel.
18. Using the gel imager, take a picture of the gel. Determine the size of your PCR product by comparing to the MW ladder.
19. Designate one person to handle the gel. Discard gloves immediately after.
20. Dispose of the gel in marked, white pail.

PCR Purification

This is usually done only if you have one PCR product of the correct size, visualized on the agarose gel. We will complete these steps while the gel runs, so everyone can try this protocol.

Materials

- Remaining PCR sample (approximately 45 µl)
- QiaQuick Spin column
- Buffers PB and PE
- Nuclease-free water or buffer EB

Method

1. Transfer the PCR product to a 2 ml tube. Label the tube with your initials.
2. Add 5 volumes of Buffer PB to 1 volume of PCR sample and mix.
    - E.g. If the PCR volume is 50 µl, add 250 µl Buffer PB.
3. Label a column on the side and top with your initials.
    - Handle the columns with great care, wearing clean gloves. Do not touch the base of the column to anything once removed from the bag. Place inside the collection tube immediately.
4. Using a micropipette, apply the PCR sample to the column.
5. Centrifuge the column at 13,000 rpm for 1 minute.
6. Discard the flow-through into a small waste container.
    - Flow-through is the solution that is now in the collection tube.
7. Add 750 µl Buffer PE to the column.
8. Centrifuge at 13,000 rpm for 1 minute. Discard the flow through.
9. Centrifuge the column at 13,000 for 1 minute to remove residual Buffer PE.
10. Label a new microcentrifuge tube with your initials and unknown sample name.
11. Place the column in the microcentrifuge tube.
12. Place 50 ul Buffer EB directly onto the membrane, without touching the membrane with the tip (changing your tip between each sample). Incubate the column 1 minute at room temperature.
    - When you open the cap of the membrane and look down the column, you will see the white membrane. Aim for this as you drop on the EB.
13. Centrifuge 1 minute at 13,000 rpm.
    - Record the location of each column in the centrifuge. Occasionally, the tops of the tubes snap off. If the location isn't noted and both lids snap off, there is no way of knowing which sample is in which tube.
14. Remove the column and store your tube on ice.

DNA Quantification

If you had DNA to purify, use the Qubit fluorimeter to quantify the DNA using the dsDNA high-specificity assay.

- Select dsDNA as the assay on the touchscreen.
- In a small tube, mix 2 µl PCR mix with 199 ul Qubit working solution. Incubate 2 minutes then read.
- If this is too much, repeat with 1 µl PCR mix.
- If this is too little, repeat with 10 µl PCR mix and 190 µl Qubit working solution.

Using $c_1v_1=c_2v_2$, you will adjust the DNA concentration of your sample using nuclease-free water.

Target C_2 = 10 ng/µl

C_1 = result from Qubit Fluorometer.

V_1 = *x* µl

V_2 = 10 µl

Amount of nuclease-free water to add:

$10 – v_1$ = µl H_2O to add

In a 1.5 ml tube, mix the volume of sample (v_1) and nuclease free water.

341F will be used as the sequencing primer. The primer will be added at 2 µM concentration and 5 µl volume.

LAB 9: BACTERIOLOGICAL ANALYSIS OF WATER

Learning Objectives

- Detect and enumerate total coliforms and *E. coli* in a water sample.

Introduction

Many illnesses are caused by waterborne bacteria. Testing water for the quantity and types of bacteria is essential to preventing these illnesses. In Canada, a lab performing these tests must be accredited by a governing body, for example, the Standards Council of Canada (SCC). We will be performing the same techniques in our lab, however since we are not accredited, the results are not valid.

If you can safely sample outside water (pond, stream, rain barrel), or if you live in a rural area with well water, please feel free to bring in a water sample. A sample of 500 ml will be more than enough for this lab. If you cannot, we will have water samples in the lab.

In Ontario, drinking water is regulated provincially by the Ministry of the Environment and Climate Change (MoECC), while bottled water is regulated federally by the Food and Drug Act. See page 23 of the Public Health Ontario document posted on FOL to read about drinking water tests done in the province.

Water quality testing uses the principle of indicator organisms. The presence of certain groups of bacteria suggest the water may be contaminated with pathogens and should not be consumed.

Coliforms are Gram negative, non-spore forming, facultatively anaerobic rods that ferment lactose to acid and gas in 48 hours. Coliforms are members of the family *Enterobacteriaceaae* and include *Escherichia*, *Serratia*, *Proteus*, *Enterobacter*, *Klebsiella* and *Citrobacter*.

The presence of coliforms is common in soil and plants, and do not necessarily indicate unsafe water.

Fecal coliforms (also known as Thermotolerant coliforms) are bacteria associated only with the intestines of mammals. They can grow in the presence of bile salts, a common molecule in the intestine. It is added to selective media to isolate fecal coliforms. These bacteria can produce acid and gas at 44 ^{0}C within 48 hours. The presence of these bacteria in water suggests a contamination event, and the water should not be consumed.

E. coli is a fecal coliform. Its presence is detected using selective and differential media to indicate potential fecal contamination.

The allowable limit of total coliforms is 0 CFU/100 ml and for *E. coli* it is 0 CFU/100 ml. In this lab, we will use two common techniques to detect and enumerate coliforms and fecal coliforms in water samples: most probable number and membrane filtration.

Most Probable Number

This is a statistical test based on dilution of a water sample until there are no bacteria present in the sub-sample used to inoculate media. The medium is lactose broth (LB, Figure 1). If coliforms are present, they consume the lactose and make gas, which is trapped in the inverted Durham tube. Three ten-fold dilutions are made (not in series) and each dilution is inoculated in three to five replicates.

- **Gas production in LB is a presumptive positive result for coliforms**
- The number of positive tubes is recorded and this number is used to refer to a table, which gives a probable range of CFU/100 ml. The number of positive tubes is written by descending volume: 10 ml – 1 ml – 100 ul.

Example: There are 3 positive 10 ml tubes, 2 positive 1 ml tubes, and 5 positive 100 ul tubes. The number used to determine the MPN on the reference table is 3-2-5.

To confirm fecal coliform presence, a sample is taken from LB and used to inoculate brilliant green lactose bile (BGLB). This medium contains inhibitors of Gram positive (brilliant green) and bile which selects for coliforms.

Gas production in BGLB is a confirmed positive result for fecal coliforms

To test for fecal coliform presence, we can also detect E. coli. A positive BGLB tube is used as inoculum for an EMB streak plate.

Presence of *E. coli* on EMB is a completed *E. coli* test result

There are other methods for detecting *E. coli* specifically, such as the use of substrates that become fluorescent after enzymatic activity.

Membrane Filtration

This method is rapid and allows precise quantification of total coliforms and *E. coli* in water samples. A vacuum is fitted with a 0.45 um membrane. The water sample passes through the membrane while bacteria remain trapped on top. The membrane is placed on an agar plate, bacteria-side facing upwards. The medium is selective and differential. It contains bile salts and dyes, which inhibit Gram positive bacteria, and lactose as the main carbon source, which selects for coliforms. When bacteria consume lactose, aldehyde is made, which reacts with dyes to produce a red colour. If the reaction is intense (as for *E. coli*), the dye crystallizes and creates a metallic sheen. We will use mENDO agar:

- Culture media selects for gram negative bacteria able to grow on bile salts
- coliforms will have a green metallic sheen
- atypical coliforms with less vigorous lactose metabolism may appear red
- Non-coliforms will have colourless, white, red or pink colonies

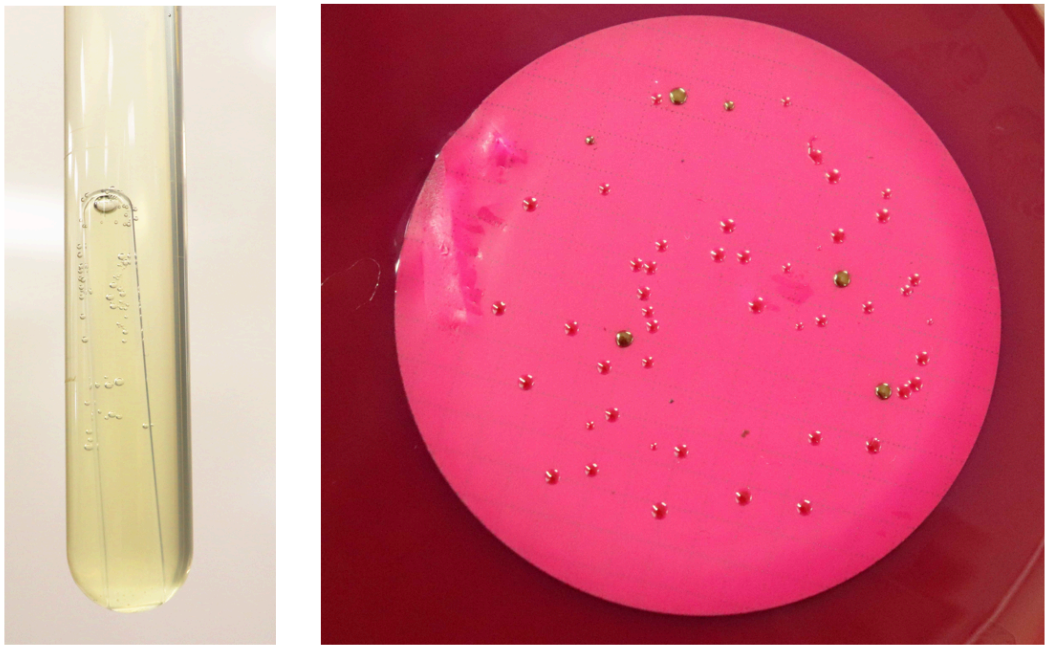

Figure 9.1. Water test media detects coliforms based on lactose fermentation. Coliforms on lactose broth and BGLB make gas (see in the tube on the left). E. coli on differential coliform agar (DC) are metallic green or gold (see the plate on the right).

Water Exercise

Materials

Day 1

- Water sample
- 3 double strength LB (dsLB)
- 6 single-strength LB (ssLB)
- 10 ml pipette and pipette bulb
- P1000 pipette and blue tips
- 2 agar plates
- Inoculating loop
- Vacuum pump
- Filter apparatus
- 2 membrane filters, 0.45 um pore size
- 1 bottle of 90 ml sterile water
- Forceps in 70% ethanol

Day 2

- 1 EMB plate
- 1 BGLB tube
- Inoculating loop

Method

1. **Most probable number:** Label 3 dsLB tubes “10 ml”, 3 ssLB tubes “1 ml” and 3 ssLB tubes “100 ul”.

    a. Aseptically transfer 10 ml of the water sample into each of 3 dsLB tubes.
    b. Aseptically transfer 1 ml into 3 ssLB tubes.
    c. Aseptically transfer 100 ul into 3 ssLB tubes.
    d. Incubate 24±2 hours at 37 C.

Day 2

    e. Record the number of tubes showing gas and turbidity. Use this MPN table to determine the CFU/100 ml.
    f. Select one tube with gas use this as inoculum for BGLB. Use a loop to transfer from the LB tube to BGLB. Incubate at 37 C for 24 hours.

Day 3

    g. If the BGLB tube has gas and is turbid, use a loop to streak an EMB plate from the BGLB tube. Incubate the

EMB plate at 37 C for 18-24 hours.

- If the tube has no gas but is turbid, streak an EMB plate to confirm that E. coli is not present
- Every group will streak an EMB plate

Day 4

h. Record the EMB result.

2. **Membrane filtration:** on two agar plates and, write "10 ml" and "100 ml" and your group name.

   a. Dilute the water sample: 10 ml water into 90 ml sterile water. Shake well by inverting the bottle at least 25 times. This is bottle 1. Bottle 2 is 100 ml of the original water sample.
   b. Soak the forceps for two minutes in alcohol. Set up the vacuum pump and filter apparatus, flame the alcohol off the forceps then aseptically place the membrane filter on the apparatus. Secure the funnel on the base.

      - While flaming, keep forceps angled downwards to prevent flaming alcohol from dripping on your gloved hands.

   c. Aseptically transfer the **white** membrane from the package to the filter apparatus.

      - The blue disc is the backing; it is resistant to water. Discard this.

   d. Add the entire volume of bottle 1 into the funnel and membrane filter. Remove the membrane filter and place on the DC plate labeled with the sample name (10 ml).

      - By starting with the most dilute sample, there is no risk of bacterial carry-over between samples, and you don't need to disinfect the filter apparatus between samples.
      - Make sure you place the membrane filter '**bacteria side up**' so the colonies can form properly.

   e. Using a graduated cylinder, add 100 ml of your water sample. Filter as above. Place the membrane filter on the mENDO plate labelled with the sample name (100 ml).
   f. Incubate for 24 hours at 37 C. Count the number of total coliforms (metallic sheen) and atypical coliforms (red colonies). When recording your results, use 'TFTC' for membranes with less than 20 colonies and 'TNTC' for membranes with over 150 colonies.

LAB 10: FOOD LAB

Learning Objectives

- Determine the number of aerobic bacteria, select pathogs, yeast, and mould in a food sample.

Introduction

In Canada, testing food for the presence of bacteria is regulated by the federal government. Health Canada sets out protocols for testing all bacteria (either aerobic or anaerobic) or specific pathogens.

In this lab, we will be following the protocol:

- **MFHPB-18 Determination of the aerobic colony count in foods**

Aerobic Colony Count

The food sample is homogenized then diluted water. Bacterial cells will recover better and grow on plates more often if they were diluted in peptone rather than water, but we are using water due to contamination issues with peptone. The bacteria on the food sample may be stressed and not in their optimal conditions, thus, if we are trying to count them in the lab, we will treat them as gently as possible to prevent killing these already stressed bacteria.

This protocol does not determine if the bacteria are pathogenic or merely normal flora on the food. The presence and number of spoilage bacteria depends on factors related to the food itself, called intrinsic factors, and factors related to the handling and storage of the food, called extrinsic factors.

- Refer to chapter 16 of the text for more information on food spoilage.

The peptone is then plated onto a non-selective rich medium for bacteria. The plates are incubated for 24 hours at 37 °C then counted.

The goal of MFHPB-18 is to ensure compliance with sections 4 and 7 of the Food and Drugs Act:

4. (1) No person shall sell an article of food that

(a) has in or on it any poisonous or harmful substance;
(b) is unfit for human consumption;
(c) consists in whole or in part of any filthy, putrid, disgusting, rotten, decomposed or diseased animal or vegetable substance;
(d) is adulterated; or
(e) was manufactured, prepared, preserved, packaged or stored under unsanitary conditions.

7. No person shall manufacture, prepare, preserve, package or store for sale any food under unsanitary conditions.

Xylose Lysine Desoxycholate Agar (XLD)

The previous method will determine total aerobic bacteria. Food manufactures are also required to test for specific pathogens. Selective and differential media is a common culture-based method to detect pathogens.

XLD is used for the detection of Salmonella and Shigella. The selective agent in XLD, desoxycholate, inhibits the growth of Gram positive bacteria. Xylose is the carbohydrate source that will turn the colonies red if it is fermented. Xylose fermentation is universal among enteric bacteria, except Shigella. The pH indicator is phenol red, which turns colonies yellow if the pH decreases due to xylose fermentation. A second differential agent, lysine, is used to differentiate Salmonella. When lysine is decarboxylated by Salmonella, the pH of the medium increases, causing the colonies to appear red (after the pH was acidic due to xylose fermentation). Sulphur source thiosulphate allows hydrogen sulphide formation under alkaline conditions, resulting in colonies with black centres. Typical colonies you may see on XLD are shown in Figure 10.1

Enterics such as Citrobacter, Proteus, E. coli: yellow colonies

- xylose fermented, lys not decarboxylated, hydrogen sulphide not produced

Salmonella: red colonies with black centres

- xylose fermentated, lys decarboxylated, hydrogen sulphide produced
  Shigella: red colonies
- xylose not fermented, lys not decarboxylated, hydrogen sulphide not produced

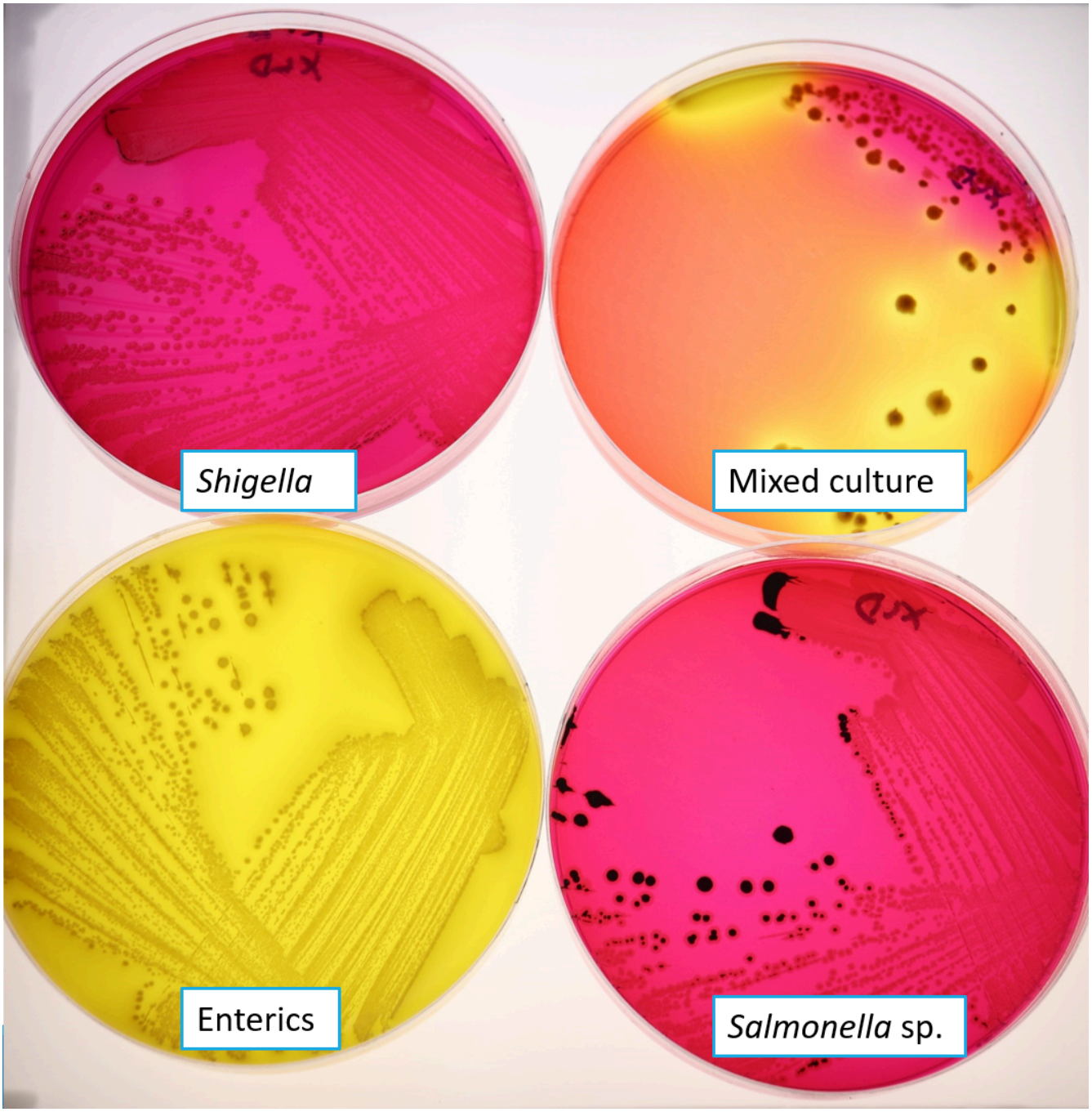

Figure 10.1 Bacteria on XLD agar. An environmental sample (top right) will have colonies of several colours.

Yeast and Mould are often detected in food in addition to bacteria. The growth conditions for yeast and mould are often lower temperature than bacterial pathogens and longer incubations. We will use petri film and agar plates for the yeast and mould enumeration. Petrifilms are easy to use and less waste than a petri plate. These are often used in industry. Yeast and mould agar that we are using is potato dextrose agar (PDA). Chloramphenicol is added to inhibit bacterial growth, otherwise bacteria would overgrow the plate. The medium is named PDA-c to indicate the presence of chloramphenicol.

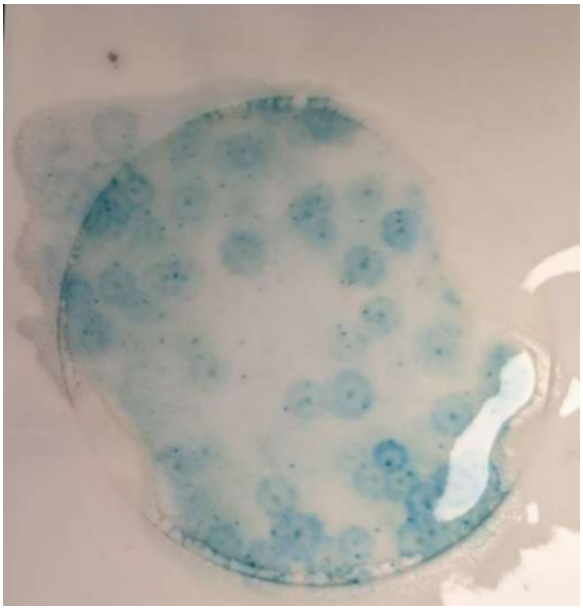

Figure 10.2 Yeast and mould Petrifilm. Mould appears as larger, blue colonies with a fuzzy edge. Yeast appears as small, pin-prick blue colonies.

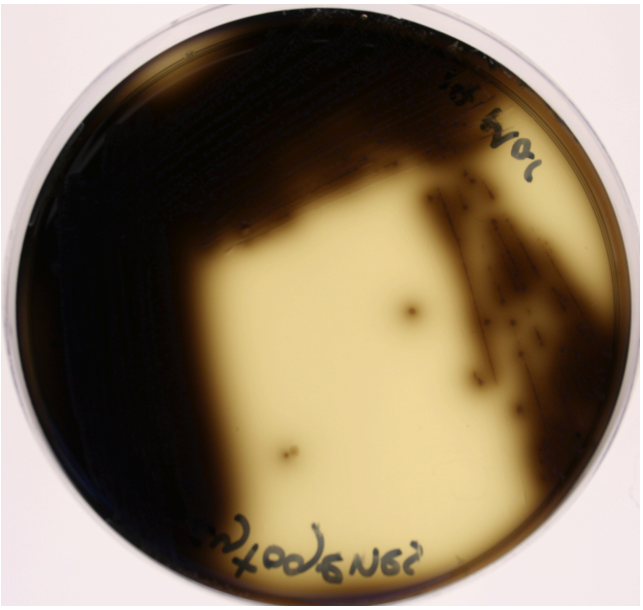

Figure 10.3 Listeria monocytogenes turns the agar black on Oxford agar.

Listeria is found in the soil and untreated water. It contaminates meat, dairy products, fruits and vegetables. It also can contaminate processed foods if the processing facility is contaminated. Since listeriosis poses a significant risk to the elderly, pregnant people and those with weakened immune systems, Listeria are monitored in food processing facilities. We will be following a modified MFHPB-30 from Health Canada. This protocol involves enriching (increasing bacterial numbers) the sample, then plating on selective media. Listeria enrichment broth (LEB for short) allows *Listeria spp*. To multiply while inhibiting the growth of other common organisms through the action of antibiotics in the medium (cycloheximide inhibits fungi, nalidixic acid and acriflavine inhibit bacteria). The broth is then streaked onto Oxford Agar after enrichment. This agar is inhibitory to gram negatives and most gram positives using the selective agents lithium chloride, colisitin, cycloheximide, acriflavine and Fosfomycin. *Listeria* produces a black precipitate in the agar surrounding the coloines. Soil frequently contains *Listeria*, however it is often non-pathogenic *L. innocua*.

Staphylococcus aureus is a common source of food poisoning. There are many strains of this species, but the strains responsible for food poisoning produce enterotoxin. These strains are typically also coagulase positive. *S. aureus* the produce the coagulase enzyme are able to form a clot in plasma. Detecting the production of coagulase is a way to differentiate strains causing food poisoning from other strains.

We will be using Mannitol Salt Agar (MSA) as before. Coagulase-positive *S. aureus* produce yellow colonies with bright yellow zones. Coagulase-negative strains produce small red colonies with no change in colour to the agar surrounding them (recall the appearance of *S. epidermidis* on MSA). We are following Health Canada's protocol MFHPB-21.

Bacillus cereus is a common contaminant in foods. When it grows to high densities, enough enterotoxin can be produced to cause food poisoning. Bacillus species make the antibiotic polymyxin and are resistant to it. We can select for Bacillus by growing in media with polymyxin. We will be using FDA protocol from the Bacteriological

Analytical Manual Chapter 14, which is followed by Public Health Ontario. This method involves MPN tubes with selective media containing polymyxin B at 0.15% in trypticase soy broth. Turbidity is a positive result and the number of positive tubes is used to approximate the inoculum in the food source. You will do a confirmatory test by making a smear from the broth and Gram staining the culture. Bacillus cereus are Gram positive rods in short to long chains. Spores, if present, are ellipsoid-shaped and central to subterminal (around the middle of the cell).

Food Exercise

Materials

- 2 nutrient agar (NA or TSA) plates
- 2 petri film plate for yeast and mould
- 2 MSA plates
- 3 double strength TSB+polymyxin
- 6 single strength TSB+polymyxin
- 1 Listeria Enrichment Broth tube
- 1 10 ml pipette (2 if you have a liquid food sample)
- 6 microfuge tubes
- P100 pipette and yellow tips
- P1000 pipette and blue tips
- Sterile water
- Empty petri plate (if you have a solid sample)
- Scalpel (if you have a solid sample)
- Vortex

Method

Aerobic Colony Count

1. **Solid food:** Remove several representative pieces off solid from different locations to get 10 g. Cut into small, uniform pieces in a sterile petri plate using a sterile scalpel.
   Liquid food: Shake food to mix. Remove 10 ml using a pipette.
2. Add the sample to 90 ml 0.1% peptone. Shake vigorously for 30 seconds to mix.
3. With a pipette, transfer 10 ml from the first bottle to the second bottle. Make sure both bottles were previously labeled with the dilution. Shake the second bottle as above.
   - Your professor will instruct which dilutions to plate. For samples with more bacteria, you will need to do extra dilutions.
   - **Refer to the growth lab for the procedure on serial dilutions. An outline is below**.
4. DROP PLATING: We are going to perform the remaining 6 dilutions in microfuge tubes and put all 8 dilutions onto the same Petri plate. These are 10-fold serial dilutions like you did in the growth lab. Refer to the drop plating protocol in the growth lab.

Note: If there is no growth on the lowest dilution, represent the value as "<".

Remember, there was a plating factor because 0.02 ml was plated for each dilution (instead of 1 ml being plated). Take this into account when calculating the dilution factor. Results are reported in cfu/ml.

XLD and MSA for pathogen detection

1. From previously prepared dilutions (technique MFHPB-18), plate 100 µl onto each XLD and MSA. Do not spread right to the edge of the plate as confluent growth can occur along the edge of the plate, which is difficult to count.
    - Bottle 1 (10^{-1} dilution) à two plates: one XLD, one MSA
    - Bottle 2 (10^{-2} dilution) à one plate: one XLD, one
2. Incubate at 37° C for 18-24 hours for XLD and MSA. Colony counts can be verified at 48 hours if required.
3. On XLD: Count **any** *Salmonella, Shigella* and other enterics. If there are more than 150 colonies on the plate, report it as TNTC.
4. On Baird-Parker, count any colonies that are 2-3 mm on an uncrowded plate, grey to jet black with an off-white margin.

Petri Film for yeast and mould

1. Lift the top film and place 1 ml of 10^{-2} dilution onto petrifilm rapid yeast and mould count plate. Repeat using 1 ml of 10^{-3} dilution.
2. Roll the top film down.
3. Place a flat object on the film to spread the sample volume across the film (an Erlenmeyer flask works well for this).
4. Incubate at 25 C for 48 hours.
5. Count the number of yeast and mould colonies that form. Yeast are small with a defined edge. Mould are large, flat with a diffuse edge.

MPN for Bacillus cereus enumeration

1. Perform MPN test as was done in the water lab.
2. Incubate tubes 48 hours at 30 C.
3. Positive tubes are turbid. Consult the table in BAM Appendix 2: Most Probable Number from Serial Dilutions for determining the most probable number.
4. Make a smear from one of the positive tubes.
5. Perform a Gram stain on the smear. Take pictures to identify if the cell morphology matches B. cereus.

Listeria monocytogenes detection

1. Cut sample into small pieces with sterile scalpel.
2. Transfer 2 g of sample into 10 ml Listeria Enrichment Broth.
3. Incubate at 37 C for 24 hours.
4. Transfer 10 ul of broth to Oxford Agar plate.
5. Streak plate as normal.
6. Incubate at 37 C for 24 hours.

- *Listeria* colonies are 1 mm with a black halo in the agar. Colonies can also appear green-black or brown-black.

Staphylococcus aureus enumeration in food

LAB 11: UNKNOWN LAB

Learning Objectives

- Identify the species of an unknown bacterial isolate.

Your unknown strain can be your environmental isolate if it is Gram negative. If not, you can use a lab unknown.

Introduction

We know how to isolate bacteria from the environment and how to select out certain species while not growing others (using selective and differential media). Bacteria are divided into species just like higher organisms. Members of the same species can still vary considerably; these are called strains. Consider the case of *E. coli*: some strains of *E. coli* may cause hemolytic uremia (destruction of red blood cells), some cause urinary tract infections, while another strain may be a harmless resident of your intestines. If these strains were on petri plates, they would all look similar. How would you tell these strains apart in the lab?

One way to determine the similarity of strains (called typing) is to test the biochemical reactions the bacteria can perform. Most of the biochemical tests are similar to those in differential media: consumption of a molecule changes the pH, leading to a colour change from a pH indicator dye.

When reading the tests, remember to distinguish between no growth and no reaction. A strain that cannot grow in a medium because it lacks an essential nutrient is much different from a strain that can grow but produces a negative reaction for the biochemical test.

In the microbiology laboratory, samples are often received and it the job of the technologist to identify the organisms in the sample. Typically, bacteria are identified by culture-based approaches (using selective and differential media), microscopy (e.g. Gram staining), and molecular approaches. In this lab, you will identify an unknown organism using microscopy and culture.

Many important human and plant pathogens are in the *Enterobacteriaceae* family. This family has four main traits:

- Gram negative
- Ferment glucose
- Test negative for oxidase
- Possess the enzyme catalase

Your Gram negative unknown may belong to the *Enterobacteriaceae* or be a non-Enterobacteriaceae. The non-Enterobacteriaceae group has three main traits:

- Gram negative
- Do not ferment glucose, but may oxidize it
- Many are oxidase positive

This lab is a summary of all the techniques you have learned to date. In addition, we will use a few new tests:

Gelatin hydrolysis

This medium is broth solidified with gelatin instead of agar. If the bacteria possess the enzyme gelatinase, they will liquefy the medium. Gelatinases are secreted proteases that degrade the protein gelatin. Since only some bacteria possess this enzyme, it is a useful test to differentiate closely related species; *Serratia*, *Pseudomonas*, *Bacillus*, and **Proteus** are positive for gelatinase production.

SIM medium (Sulfide-indole-motility)

This is a multi-test medium. It is a semi-solid agar in a test tube and it is inoculated by stabbing into the agar. Sulfide production is detected by the presence of a black precipitate (Fig. 11.1 Tube C), indole is detected using TSB and Kovac's reagent (Fig 11.1 Tube D), and motility is detected as haziness emerging from the stab line (Fig. 11.1 Tube A is motile and Tube B is non-motile).

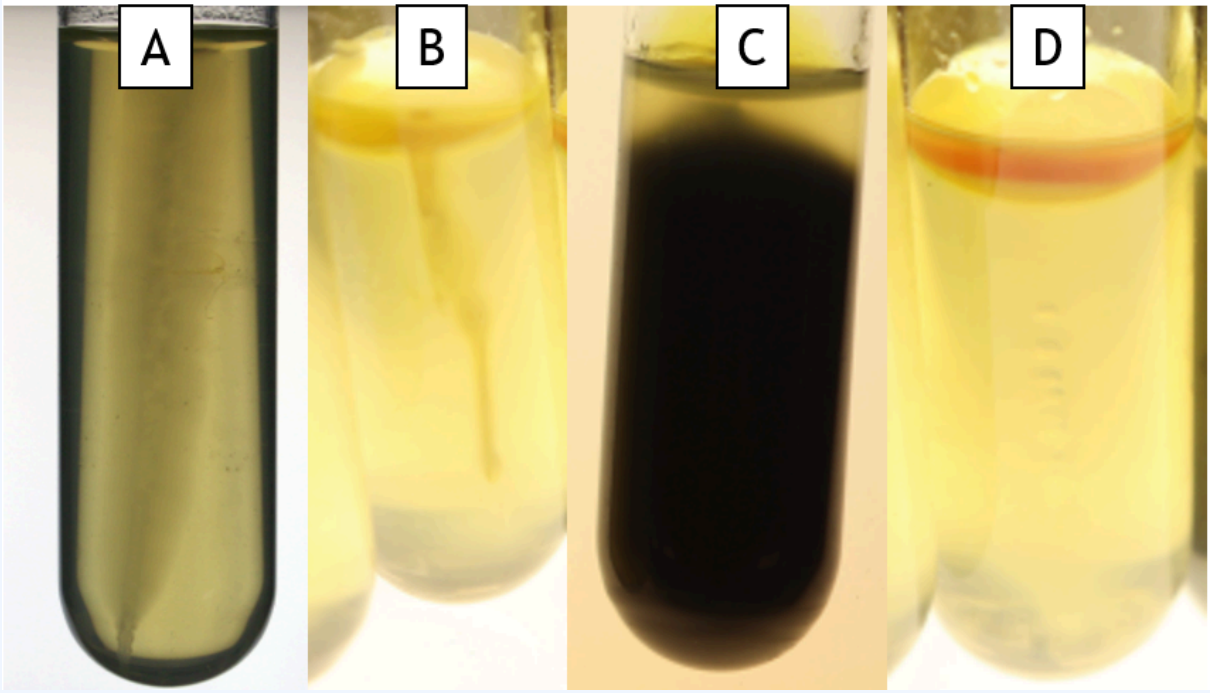

Figure 11.1 SIM medium

Oxidase Test

This tests the presence of cytochrome c, a component of the electron transport chain. When cytochrome c is present, the dye is oxidized, turning it from colourless to purple. Since only some bacteria possess cytochrome c, this is a useful test to differentiate bacteria.

Methyl Red and Voges-Proskauer tests

The methyl red test detects the ability of bacteria to ferment sugars by the mixed acid pathway. This results in a greater amount of acidic end products (as carbon is diverted into acids through fermentation). By adding the pH indicator methyl red after growth in MR-VP broth, a red colour indicates a pH below 4.4 (Fig. 11.2 Tube D, positive for methyl red) or yellow if the pH is higher (Fig 11.2 Tube C, negative for methyl red).

The Voges-Proskauer test detects the production of acetoin, an intermediate in pyruvate fermentation, into 2,3-butanediol. A red colour after the addition of Barrit's A and B reagents indicates the presence of acetoin, a positive reaction (Fig 11.2 Tube B). A yellow colour indicates no acetoin was produced (Fig 11.2 Tube A).

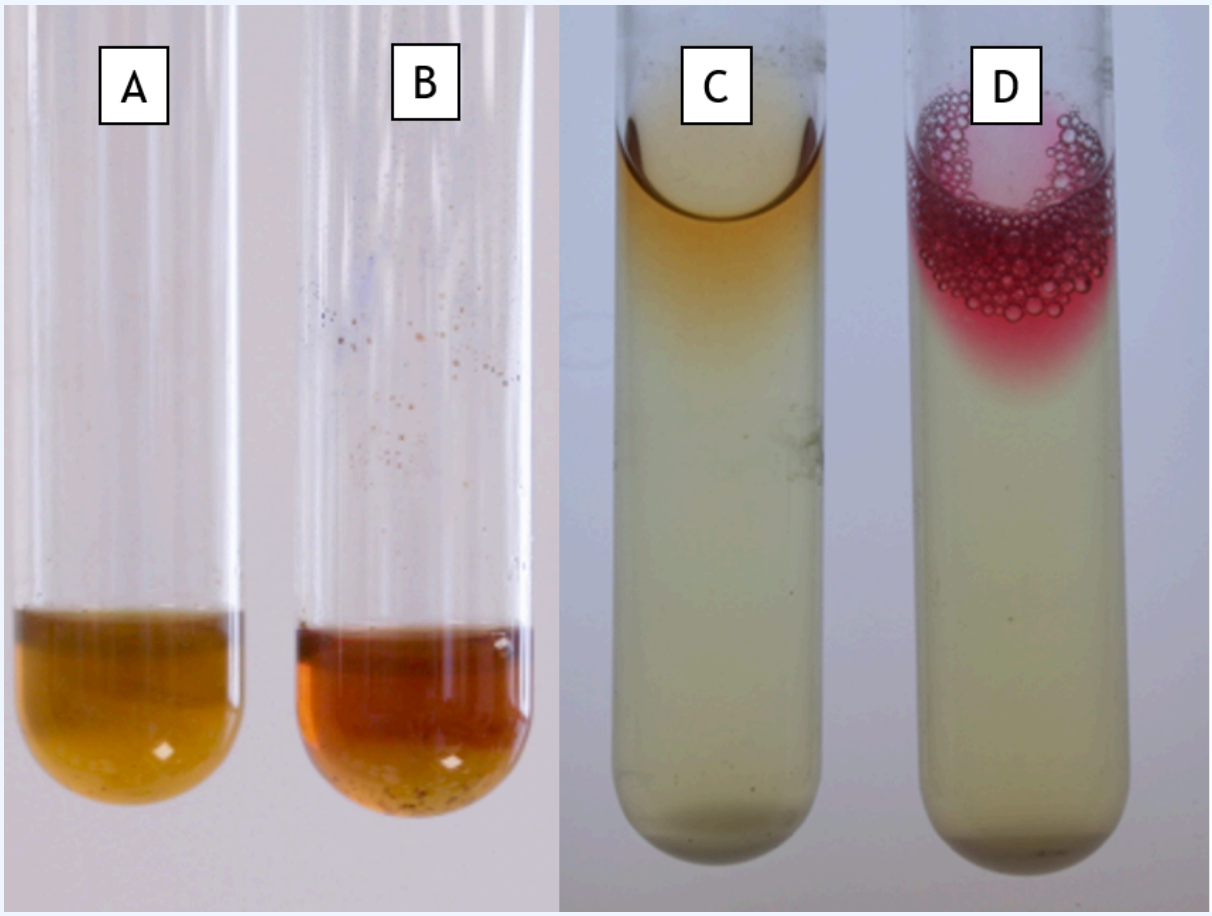

Figure 11.2 MR-VP tests. Tubes A and B: Voges-Proskeauer test. Tubes C and D: Methyl red test.

Catalase Test

Catalase is an enzyme produced by some bacteria to prevent damage due to hydrogen peroxide (a by-product of aerobic metabolism). If catalase is produced, hydrogen peroxide will be degraded into water and oxygen. Visible bubbling around the colony indicates catalase is working.

Carbohydrate Fermentation (Phenol Red Glucose, Sucrose or Lactose)

This media has one carbohydrate as the sole carbon source and phenol red, a pH indicator. If the bacteria consume the carbohydrate to produce acids (fermentation), phenol red turns from orange-red to yellow. If the bacteria consume the carbohydrate without fermentation, the medium does not turn yellow but will be turbid.

This medium is a broth and is performed in a test tube. Inside the test tube is an inverted miniature test tube called a Durham tube. If the bacteria produce gas, it will be captured by the Durham tube.

The results for this test could be the following:

- NG (no growth) (Figure 11.3, Tube A
- negative for consumption of the carbohydrate, but the strain grew (Fig. 11.3 Tube C)
- positive for consumption of the carbohydrate and no gas production
- positive for consumption of the carbohydrate with gas production (Fig. 11.3 Tube B)

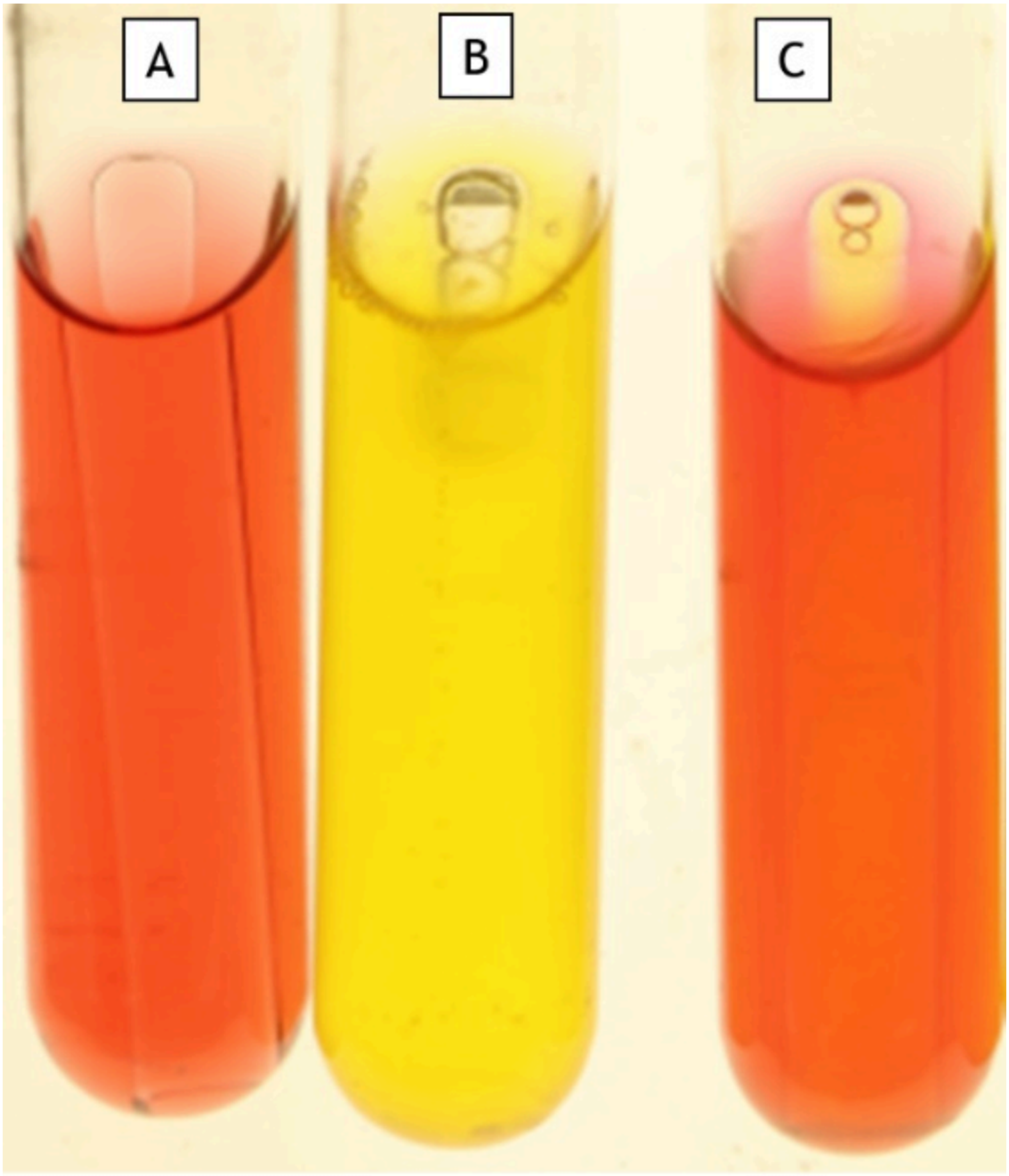

Figure 11.3 Carbohydrate fermentation test media

Citrate Utilization (Simmon's Citrate)

In this medium, citrate is the sole carbon source. If the bacteria possess citrate permease (a transporter to import citrate), they can grow on this medium. Simmon's citrate also contains ammonium phosphate as the sole nitrogen source. As the bacteria grow, this is converted into ammonia and ammonium hydroxide, both of which alkalinize the agar. Bromothymol blue is the indicator; as the pH increases, it turns from green at pH 6.9 to blue at pH 7.6.

For this test, growth is linked to the reaction. An organism that grows and consumes citrate will also raise the pH of the agar (Fig. 11.4 Tube B). Strains that are unable to use citrate will be unable to grow (Fig. 11.4 Tube A).

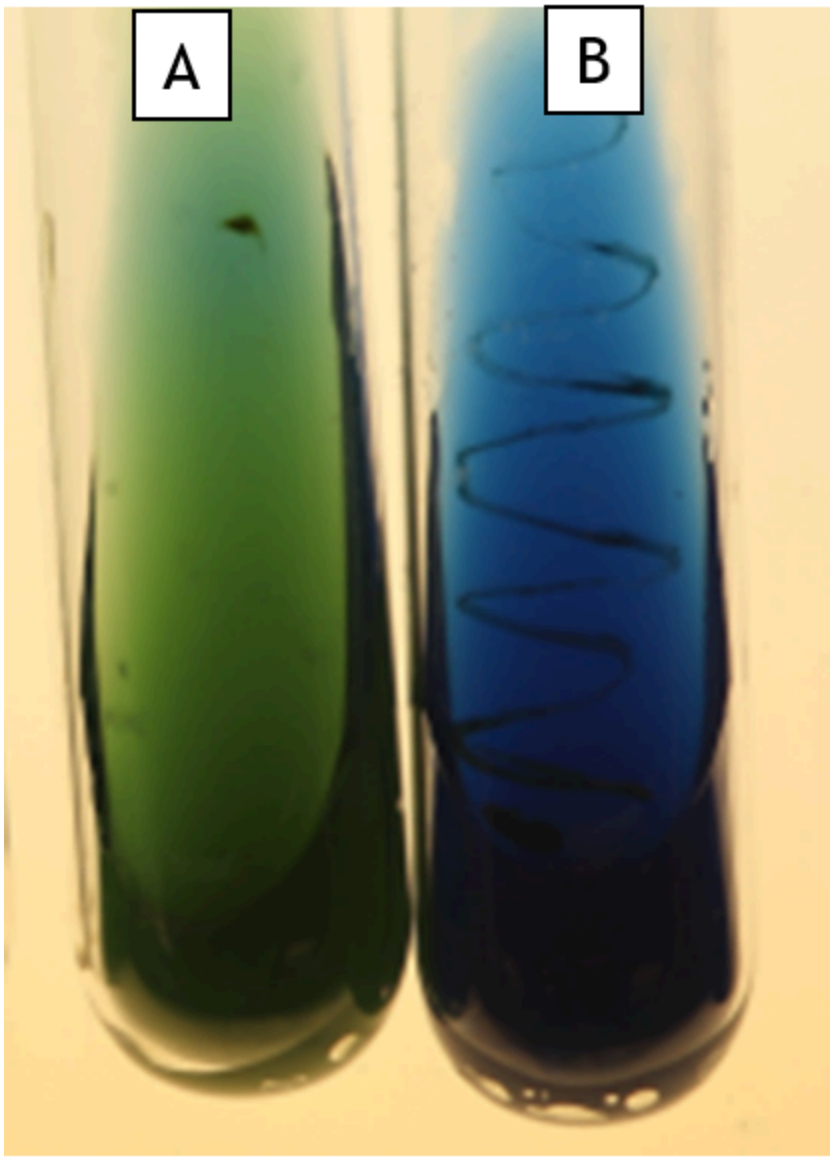

Figure 11.4 Simmon's citrate tubes.

Decarboxylase of Amino Acids (Lysine Decarboxylase)

This medium tests the ability of bacteria to decarboxylate an amino acid (remove the COOH-). It is used to differentiate enterics from other bacteria. The pH indicator, bromocresol purple, is purple at pH 6.8 and above and

yellow at pH 5.2 and below (Fig. 11.5 Tube A shows an uninoculated tube). Degradation of amino acids increases the pH of the medium, causing it to turn purple (Fig 11.5. Tube B and Tube C show a weak positive). If the bacteria do not degrade the amino acid but they make acid from glucose, then the tube will turn yellow (Fig 11.5, Tube D)

This test must be conducted anaerobically to cause expression of the decarboxylase enzyme; thus mineral oil is overlaid on the tubes.

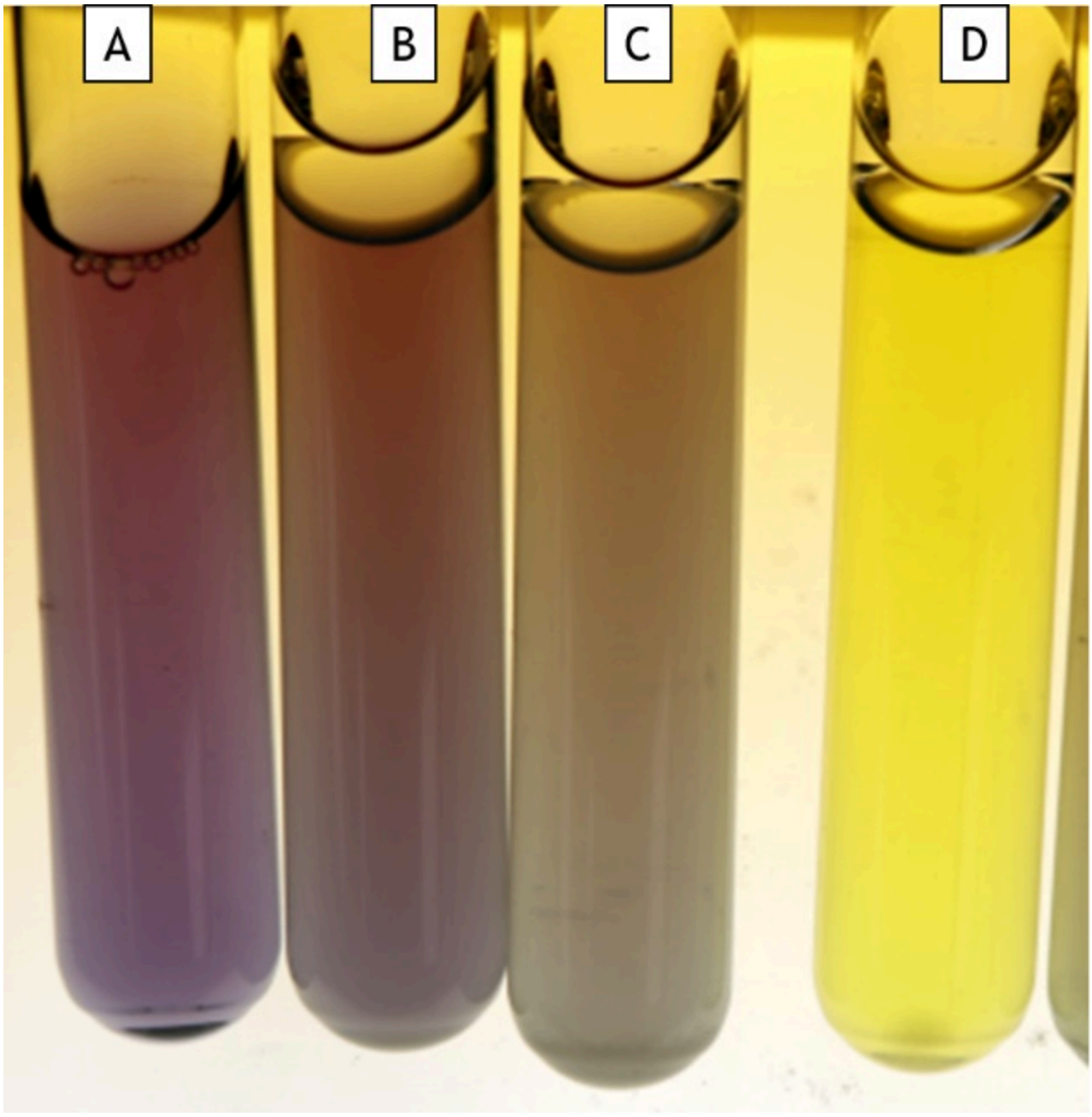

Figure 11.5 Decarboxylate broth

Indole production (tryptone broth)

The ability of bacteria to produce indole from tryptophan is a way of differentiating closely related strains. By

including tryptophan in the growth medium, indole is produced if the organism is able to. SIM media also contains tryptophan, to allow the indole test to be completed.

Indole is detected after growth by adding Kovac's reagent (containing dimethylaminobenzaldehyde (DMABA) and HCl in alcohol). The DMABA reacts with any indole present to produce a red colour.

Nitrate Reduction

This tests the ability of a strain to reduce nitrate to nitrite or nitrate to nitrogen gas. Nitrate respiration is a form of anaerobic respiration, with nitrate used in place of oxygen as the terminal electron acceptor. When nitrate accepts electrons, it becomes nitrite. If the bacteria possess the correct enzymes, nitrite can then be reduced into nitrogen gas.

This is a broth medium, and the test tube contains an inverted Durham tube. After growth, nitrate A and B (sulfanilic acid and a-naphthylamine) are added to the tube. If nitrite is present, the medium will turn red due to nitrite reacting with the reagents (Fig. 11.6 Tube A). If the nitrite has been reduced to nitrogen gas OR there is no nitrite present, the medium remains colourless (Fig. 11.6 Tube B). If this is the case, powdered zinc is added. The zinc will reduce any remaining nitrate into nitrite, which then reacts with nitrate A and B, causing the red colour (Fig. 11.6 Tube D). Thus, a red colour after the addition of zinc is negative for nitrate reduction, while a colourless result indicates nitrate was reduced to nitrogen gas or other nitrogenous compounds (Fig. 11.6 Tube C). Gas in the Durham tube indicates the presence of nitrogen gas.

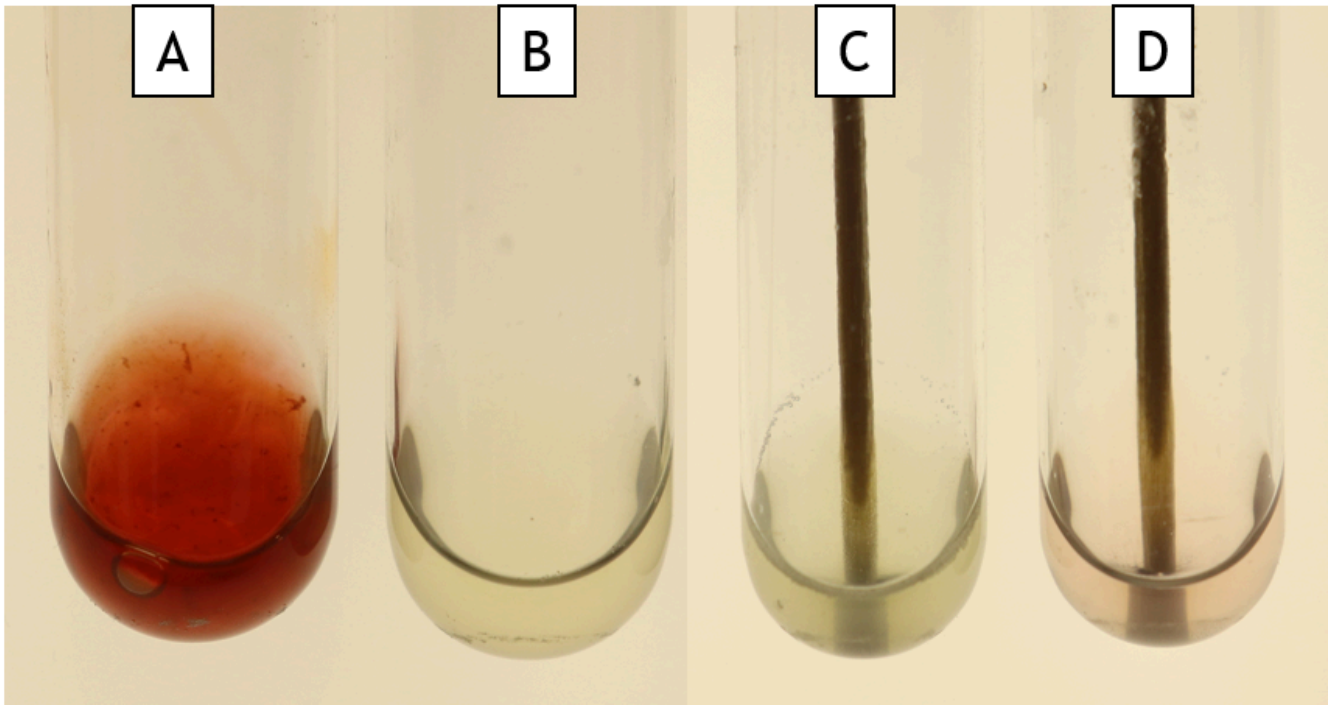

Figure 11.6 Nitrate reduction test after reagents A and B are added to the tubes.

Urease Production

This differentiates organisms based on their ability to degrade urea. If bacteria possess urease, urea is converted into ammonia and carbon dioxide. Phenol red is the pH indicator. Ammonia raises the pH, causing phenol red to

turn from orange-red below pH 8.4 (Fig. 11.7 Tube A) to magenta pink at a pH greater than 8.4 (Fig. 11.7 Tube C). Bacteria that acidify the media below pH 6.2 cause phenol red to turn yellow (Fig. 11.7 Tube B).

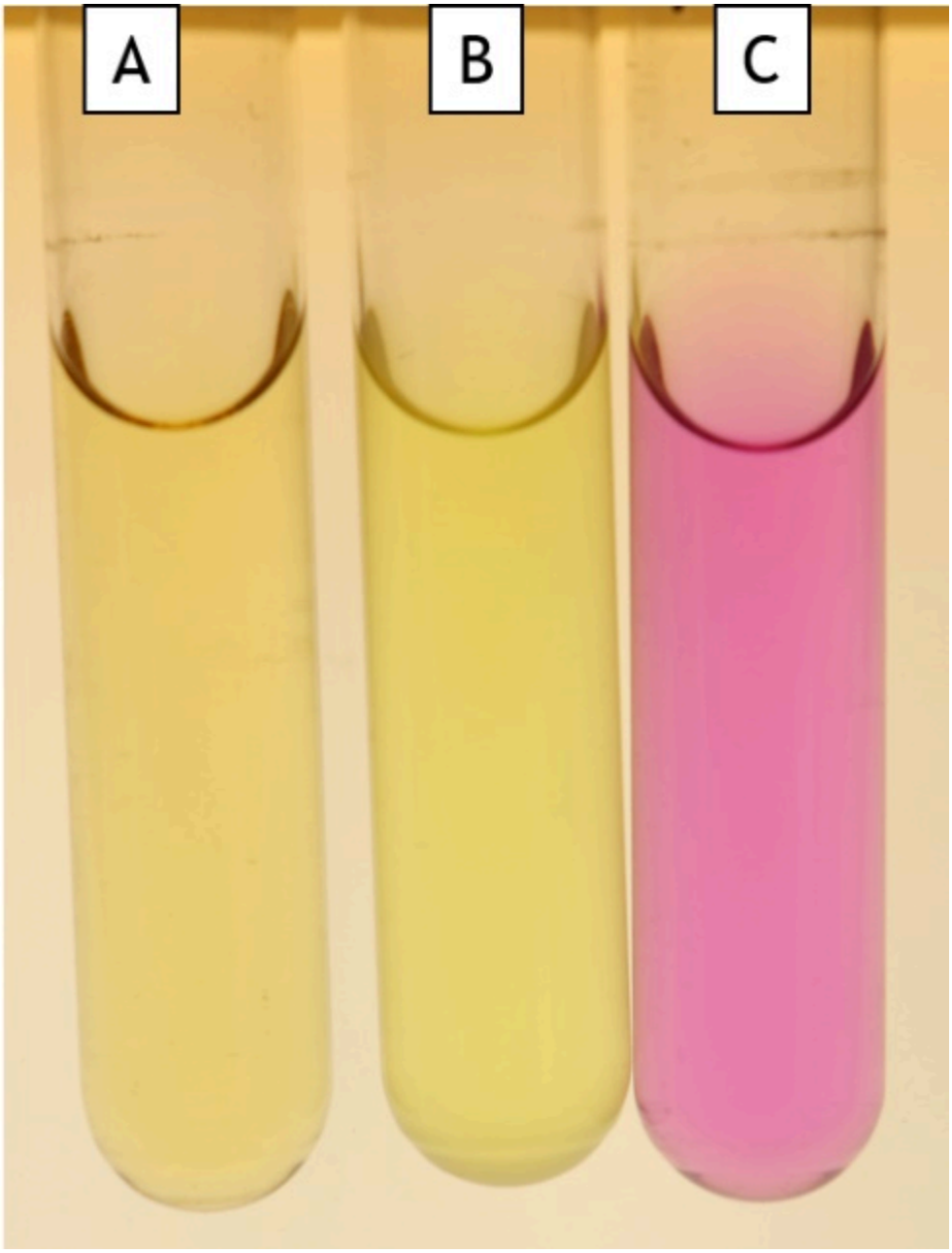

Figure 11.7 Urea broth

IMViC Tests

Together, the indole, methyl red (MR), Voges-Proskauer (VP), and citrate tests comprise the IMViC tests. This panel of tests is important in distinguishing *Enterobacteriaceae* members from each other. Table 1 shows the commonly encountered species in the *Enterobacteriaceae* and the typical test results.

Table 11.1: Typical Enterobacteriaceae culture results.

Genus	Indole	MR	VP	Citrate	H2S	Motility	Lactose fermentation	Gelatin hydrolysis
Salmonella	–	+	–	+	+	+	–	–
Proteus	V	+	–	–	+	+	–	+
Klebsiella	–	–	+	+	–	–	+	–
Enterobacter	–	–	+	+	–	+	+	–
Citrobacter	+	+	–	+	+	+	+	–
Escherichia	+	+	–	–	–	+	+	–
Serratia	–	–	+	+	–	+	+	+

+ indicates a positive reaction
– indicates a negative reaction
V indicates a variable reaction for species within a genus

Once you have all the test results for your organism, you then need to figure out which species you have. The table above can be useful, as can the more extensive table and dichotomous key on FOL. The dichotomous key is simply a series of yes/no questions about your test results. As you work through the questions, you will eventually arrive at a species name. Once you have a name, you can search to see if this species fits with your other observations. Remember, you need to justify the species that you ultimately arrive at in your conclusions.

Unknown Identification Exercise

Materials

- Gram staining kit
- Slides
- Cover slips
- Inoculating needle and loop
- Unknown plate
- Oxidase reagent
- Hydrogen peroxide
- Barritt's A and B, Nitrate A and B, Kovac's
- TSIA, SIM, nitrate broth, decarboxylase, MR-VP, phenol red glucose, phenol red lactose, Simmon's citrate, gelatin, urea
- Filter paper

Method

Colony Morphology

1. From the streak plate, make observations on colony morphology.
   Cellular morphology

2. Make a wet mount of the bacteria and observe cell arrangement and motility under the microscope: With a pipette, transfer 25 ul of sterile water onto a slide. Resuspend bacteria from an agar plate in the water, then add a coverslip. View immediately under the microscope.
    - You will be testing for motility and cell arrangement using other tests; however, it is always best to have multiple tests to justify your conclusions.
3. Gram stain: Make a Gram stain to confirm your culture is Gram negative. Make observations on cell morphology and arrangement.

Oxidase Test

4. Soak a small filter paper in oxidase reagent in the lid of a petri plate. Use a loop to pick up bacteria from an agar plate and rub onto the filter paper. Record the colour of the bacterial culture. If there was no colour change within 2 minutes of smearing on the bacteria, then the bacteria are negative for the oxidase test.

Catalase Test

5. Using a toothpick, pick up a cells (these can be from the first streaking on the plate) and smear onto a slide. Drop hydrogen peroxide onto the cells and observe for immediate bubble formation.
    - You may need to use a magnifying glass to observe tiny bubbles if the strain is a weak positive.

Media

6. Inoculate the following media: TSIA, SIM, gelatin (stab into the butt of the tube), phenol red glucose, phenol red lactose, phenol red sucrose Simmon's citrate slant, nitrate broth, decarboxylase broth (with oil on the top of the tube), urea broth, MR-VP tubes.
7. Incubate your cultures:

24 hours	48 hours
SIM, TSIA, PR glu/suc/lac, nitrate, urea	citrate, MR-VP, gelatin, decarboxylase

8. Interpret the test results:
    - VP (from the MR-VP tube): Aliquot 1 ml of culture into a new test tube. Add approximately 6 drops Barritt's A (a-naphthol) and approximately 2 drops Barritt's B (potassium hydroxide). Incubate 30 minutes. A red colour indicates the presence of acetoin.
    - MR (from the MR-VP tube): incubate for 48 hours at 37 C. Aliquot 1 ml into a new test tube and add 5 drops of methyl red. Interpret test results immediately- red indicates a positive result, yellow indicates a negative result.
    - Gelatin hydrolysis: incubate 48 hours at 37 C. Gelatin liquefies at 28 C, so before reading the test result, place the gelatin tube and a control tube (which was also incubated, uninoculated, at 37 C) in an ice bath for 15 minutes. The control tube should be solid (if it isn't, keep incubating in the ice bath until it is). Observe

whether the test tube is solid or liquid. If you incubate too long on ice, all tubes will appear negative.

- SIM: Incubate for 24 hours at 37 C. Observe the presence of hydrogen sulphide (black precipitate) and motility (turbidity migrating from the stab). Drop Kovac's reagent onto the tube and observe indole production as the reagent turns red.
- After 24 hours:
    - Interpret TSIA, phenol red tubes, nitrate broth and urea.
    - Nitrate Test: Observe if the inverted Durham tube has gas.
        - Transfer 1 ml culture to a new test tube.
        - Add 2 drops each of Nitrate A and Nitrate B to the tube. Read the results after two minutes. If the tube turns red, then the test is complete.
        - If the tube is still colourless after two minutes, dip a wooden applicator in zinc powder and stir into the culture tube. Note the colour change.
- After 48 hours, interpret the decarboxylase broth, MR-VP, gelatin, and citrate tubes.
- **Positive and negative controls:** These will be inoculated by your professor and will be available for you to compare your results to.

Interpretation Using Media

7. Use your results to determine the genus of your unknown culture.

    - Is your unknown a member of the Enterobacteriaceae?
    - If yes, you can use Table 1 and the table posted on FOL to see which is the best fit.
    - Use the dichotomous key to arrive at a species.
    - Look your species up on Microbe Wiki or in Bergey's Manual of Determinative Bacteriology to determine if this genus is a good fit

      Interpretation using DNA sequence
    - Using the DNA sequence provided from sequencing your PCR product or from your professor, search the DNA sequence into the BLAST search tool. Paste your sequence into the box under 'Enter Query Sequence', then hit the blue BLAST button at the bottom of the page. Examine the top hits with the most sequence identity with your sequence to determine the probable species.

LAB DOWNLOADS

Lab Worksheets

The following files can also be accessed directly from each lab section in this guide:

- Lab 2 Worksheet Techniques (.Word) and Lab 2 Worksheet Techniques(.pdf)
- Lab 3 Worksheet Growth (.Word) and Lab 3 Worksheet Growth (.pdf)
- Lab 4 Worksheet Isolation (.Word) and Lab 4 Worksheet Isolation (.pdf)
- Lab 5 Worksheet Smears and Stains (.Word) and Lab 5 Worksheet Smears and Stains (.pdf)
- Lab 6 Worksheet Streaks (.Word) and Lab 6 Worksheet Streaks (.pdf)
- Lab 7 Worksheet Molecular I (.Word) and Lab 7 Worksheet Molecular I (.pdf)
- Lab 8 Worksheet Molecular II (.Word) and Lab 8 Worksheet Molecular II (.pdf)
- Lab 9 Worksheet Water (.Word) and Lab 9 Worksheet Water (.pdf)
- Lab 10 Worksheet food (.Word) and Lab 10 Worksheet food (.pdf)
- Lab 11 Worksheet Unknown (.Word) and Lab 11 Worksheet Unknown (.pdf)

VERSION HISTORY

This page provides a record of changes made to the open textbook since its initial publication. If the change is minor, the version number increases by 0.1. If the change involves substantial updates, the version number increases to the next full number.

Version	Date	Change	Affected Web Rage
1.0	January 11, 2024	Publication	N/A
1.1	January 23, 2024	Updated Lab 6 Worksheet	Lab 6: Bacterial Media