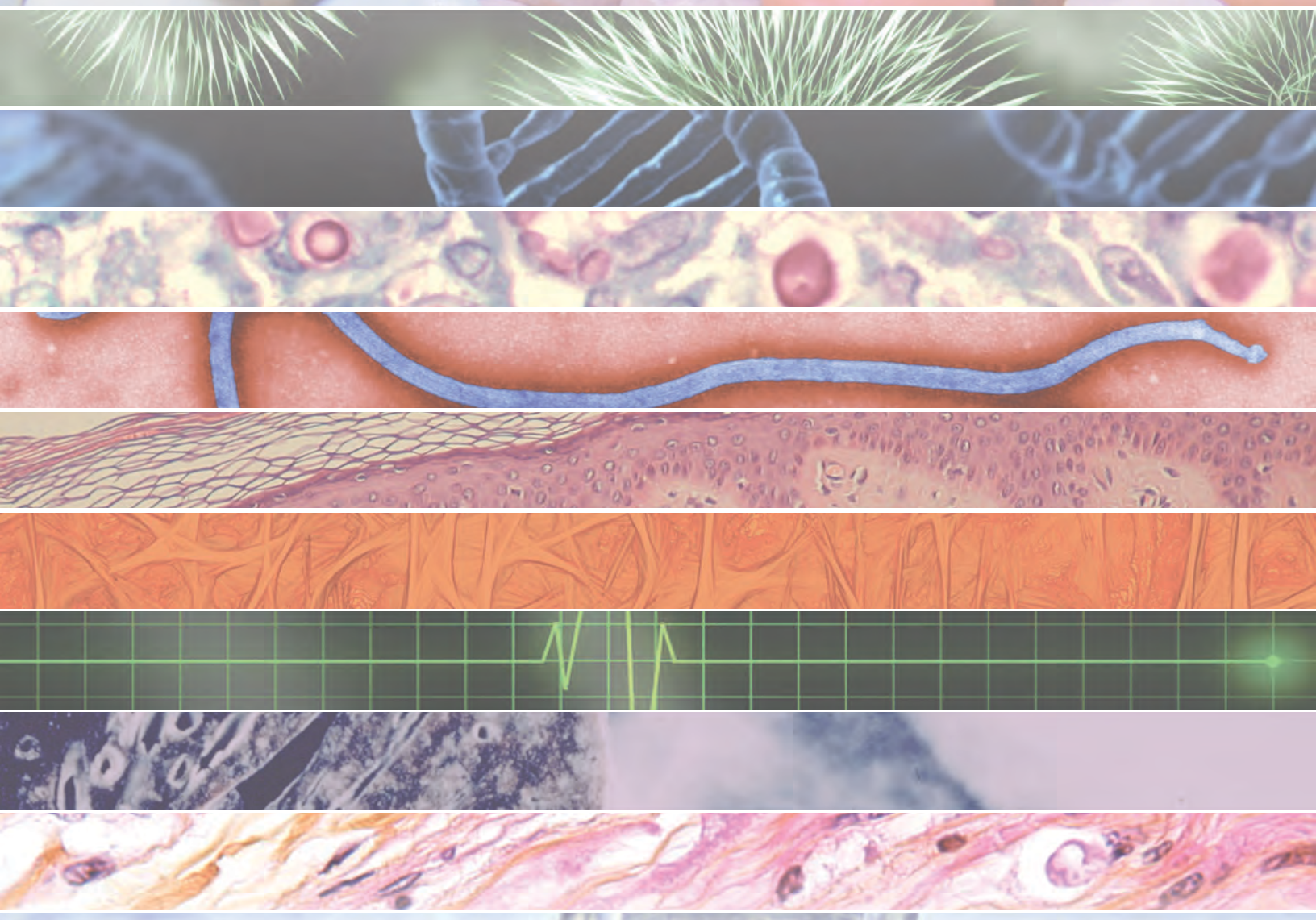
Lab Manual

| Cellular, Molecular and Microbial Biology



Written and adapted by Peggy O'Sullivan

Cellular, Molecular and Microbial biology
LAB MANUAL

(*2017 Edition*)

Adapted and written for CMMB 250: Microbiology

Except where otherwise noted, the content of this manual has been
adapted and written by Peggy O'Sullivan.

This project/resource was funded by the Alberta Open Educational Resources (ABOER) Initiative, which is made possible through an investment from the Alberta government.

Acknowledgements:
Special thanks to Denise Holt (Research Assistant), Tania Park (Biological Technician), Andrea Woods (Graphic Designer), and Barbara Mitchell (Educational Technology Specialist – Video Production).

Index

Microbiology Laboratory Safety

Introduction

The main concern in a microbiology lab is the safe handling and disposal of microorganisms and contaminated lab supplies. In addition to following general laboratory safety rules, additional rules must be implemented in the microbiology lab since students are working with living organisms and the risk of student exposure to these organisms must be minimized.

The four basic **routes of exposure** to microorganisms are:

1. contact with skin and mucous membranes
2. ingestion
3. inhalation
4. inoculation

Specific lab safety guidelines are designed to address each of these potential routes of exposure. **Contact with skin and mucous membranes** can be minimized by wearing proper personal protective equipment such as lab coats or aprons, gloves, goggles, respirators, and face shields. In addition, students should be prohibited from inserting contact lenses in the lab. **Ingestion** of microorganisms can be minimized by prohibiting eating, drinking, or applying cosmetics in the lab. **Inhalation** of microorganisms can be minimized by adopting measures which decrease the likelihood of generating aerosols. **Inoculation** can be minimized by instituting rigid protocols for the use and disposal of sharps (needles, slides, broken glass, etc.)

Biosafety Levels

Microorganisms are divided into 4 **Biosafety Levels (BSL)** by the Centers for Disease Control and Prevention (CDC). The microbes used in our micro lab fall into the BSL-1. The Public Health Agency of Canada (PHAC) divides organisms and toxins into four levels like CDC but these are called Risk Group Categories. There are four Risk Group Levels from 1 to 4 similar in definition to the CDC BSL's.

Visit the website:

Scan the QR code or use the link below and refer to section 2.3.1 “Pathogens and Risk Groups” of the *Canadian Biosafety Standards and Guidelines* web site for additional information on PHAC’s levels.

Canadian Biosafety Standards and Guidelines (Pathogens and Risk Groups)
http://canadianbiosafetystandards.collaboration.gc.ca/cbs-ncb/index-eng.php - a2.3

Biosafety Level 1 organisms are defined as well-characterized strains of microorganisms not known to cause disease in healthy human adults. Precautions in BSL-1 labs include general lab safety rules such as no eating or drinking, prohibition of mouth pipetting, practicing aseptic technique, and proper disposal of sharps and microbiological waste. Examples of BSL-1 organisms include non-pathogenic laboratory strains of *Escherichia coli*, *Staphylococcus epidermidis*, and *Bacillus megaterium*.

Biosafety Level 2 organisms are defined as moderate-risk microorganisms that are associated with less serious human diseases whose potential for transmission is limited and a proven treatment for the disease exists. Many BSL-2 pathogens are **opportunistic**, meaning they don’t ordinarily cause disease in healthy human adults, but may cause disease in children and immunocompromised adults. Additional precautions in BSL-2 labs include using personal protective equipment (PPE) such as disposable gloves and lab coats and limiting lab access to trained individuals. Examples of BSL-2 organisms include *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella sp*.

Biosafety Level 3 organisms are defined as high-risk microorganisms with a true potential for infection by aerosols and in which the resulting disease may have serious or lethal consequences. Researchers in BSL-3 labs generally wear double gloves, respirators, and disposable surgical scrubs and gowns, and work in biological safety cabinets in isolated, negative-pressure containment rooms. Examples of BSL-3 organisms include *Mycobacterium tuberculosis* and *Bacillus anthracis*.

Biosafety Level 4 organisms are defined as easily transmitted, very-high risk microorganisms which cause life-threatening diseases for which there is no vaccine or therapy. Workers in BSL-4 labs work in impermeable positive pressure “space suits” with an external oxygen supply, and precautions such as chemical showers must be taken before exiting the lab. Examples of BSL-4 organisms include Ebola virus, Marburg virus, and Lassa fever virus.

Complete the lesson:

Scan the QR code or use the link below to complete the *Centers for Disease Control and Prevention* "Recognizing the Biosafety Levels" training lesson.

Centers for Disease Control and Prevention (Recognizing the Biosafety Levels)
http://www.cdc.gov/training/QuickLearns/biosafety/

CMMB 250:
Student Conduct & Safety Rules

Laboratory safety rules must be followed to ensure the safety of all in the lab and of others that use our lab. Students that do not follow the laboratory safety rules will be asked to leave the lab.

Plagiarism and cheating will not be tolerated.
Unsafe conduct in the laboratory will not be tolerated.

1. Full length lab coats, fully buttoned, with full sleeves must be worn in the lab at all times. Lab coats must be washed after each microbiology lab. Open toes shoes/flip-flops and sandals not permitted.

2. Do not eat, smoke, drink or bring food or drinks into the lab to reduce a risk of infection. This includes chewing gum or candy.

3. Long hair (chin length or longer) must be tied back or worn under a cap.

4. Scarves and other hazardous long pieces of clothing & jewelry cannot be worn to prevent flammability and other safety risks when using open flames and when working with equipment. Note the positions of fire escape routes posted on the lab doors and fire extinguishers and fire blankets.

5. Cosmetics and contact lenses must not be applied in the lab. The instructor will show you where the eye wash fountain is located.

6. Fingers, gum, pencils, masking tape, and other objects must not be put in mouth during the lab. Do not wipe your eyes, face, etc… during the lab to prevent a risk of infection.

7. Do not remove any chemicals, microorganisms, and pieces of lab equipment from the lab.

8. Do not work with cultures, etc… on top of your lab notes or binder or books. Keep cultures on the bench tops and other surfaces where they can be decontaminated. Keep your coats, binders, and book bags away from your work area. If you have a locker, please leave extra materials you don't require in the lab in your locker.

9. Scrub up before and after the lab as instructed by your instructor. In most cases, 70% ethanol will be used on bench surfaces. In addition, 10% bleach will be used when spore forming bacteria are used and in some other cases. Hands will be scrubbed with antiseptic. If you must leave the lab during the lab period you must scrub your hands, remove your lab coat, leave it in the lab. Upon returning to the lab you will put on your lab coat and scrub your hands with antiseptic.

10. Come prepared to the lab – read the experiments before coming to the lab and prepare your own prep sheet before you come. Lab time is very busy with many experiments being started and completed weekly. Your safety and the safety of others in the lab can be compromised if you come unprepared to the lab.

11. Report all spills immediately to the instructor. Do not hurry through any lab procedure. Ask questions if you do not understand any procedure.

12. Turn off Bunsen burners and turn off microscopes when they are not in use. If the fire alarm rings, the lab gas supply will be turned off. Follow your instructor's instructions for fire drills and laboratory escape routes.

13. Your instructor will demonstrate the correct method to safely remove gloves if you are wearing them in the lab. Gloves are disposed of in the autoclave bags. If you are wearing gloves you must exercise caution when sterilizing instruments and glass wear using open flames.

14. Contaminated materials are autoclaved. Your instructor will instruct you on where to place contaminated materials and how they will be disposed of. In NO circumstances will you wash any contaminated materials down the drain or place them in the regular lab garbage. If you are unclear of where to place contaminated materials, ask your instructor before disposing of them.

15. Never place test tubes or pipettes directly on the bench. Place them in test tube racks or in special racks. Place loops and needles in their holders – never lay them on the bench surface.

16. Broken glass wear may be placed in the broken glassware boxes unless it is contaminated with microorganisms. In that case, ask your instructor where to dispose of it.

17. Food microbiology will be conducted in this lab. Do not consume products of your experiments or feed them to other humans or animals. This is an experimental lab – contaminated foodstuffs may not leave the lab.

18. Laboratory materials may not leave the lab. Do not take any laboratory items out of the lab for personal use (e.g. cultures, glass wear, petrie dishes, etc.)

WHEN IN DOUBT – ASK YOUR INSTRUCTOR

Sources

Biosafety Levels section
“Microbiology Lab Manual: Biol2421L” by Donna Cain, Hershell Hanks, Donna Cain, Mary Weis, Carroll Bottoms, & Jonathan Lawson, retrieved from https://microcosmrflores.wikispaces.com/file/history/Microbiology+Lab+Manual+--+Revised+Spring+2013.pdf
(Licensed under CC BY-SA)

CMMB 250

Student Conduct Contract

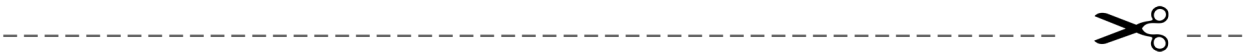
STUDENT COPY – Keep this copy in your lab manual.

1. I have read the CMMB 250 laboratory safety rules and agree to abide by them.
2. I am aware of the Academic Regulations outlined on pages 52 and 53 of the MHC 2016/17 calendar. I agree that plagiarism and cheating will not be tolerated in this course.
3. As part of research investigation and teaching practices, your instructor may be taking photographs/videos. The photographs/videos will be used for teaching purposes, project illustration, academic conference presentations, and may be placed on web sites for academic purposes. I give my consent for the use of photographs as outlined.
4. Microscope # Assigned: _____

   I agree to use the microscope assigned to me and to follow the instructions for the use and care of this microscope.

Name: _____

Date: _____ Lab Section: _____



CMMB 250

Student Conduct Contract

INSTRUCTOR COPY – Cut and give this portion to your instructor.

1. I have read the CMMB 250 laboratory safety rules and agree to abide by them.
2. I am aware of the Academic Regulations outlined on pages 52 and 53 of the MHC 2016/17 calendar. I agree that plagiarism and cheating will not be tolerated in this course.
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4. Microscope # Assigned: _____

   I agree to use the microscope assigned to me and to follow the instructions for the use and care of this microscope.

Name: (PRINT) _____

Signature: _____

Date: _____ Lab Section: _____

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Exercise 1
Hand Hygiene

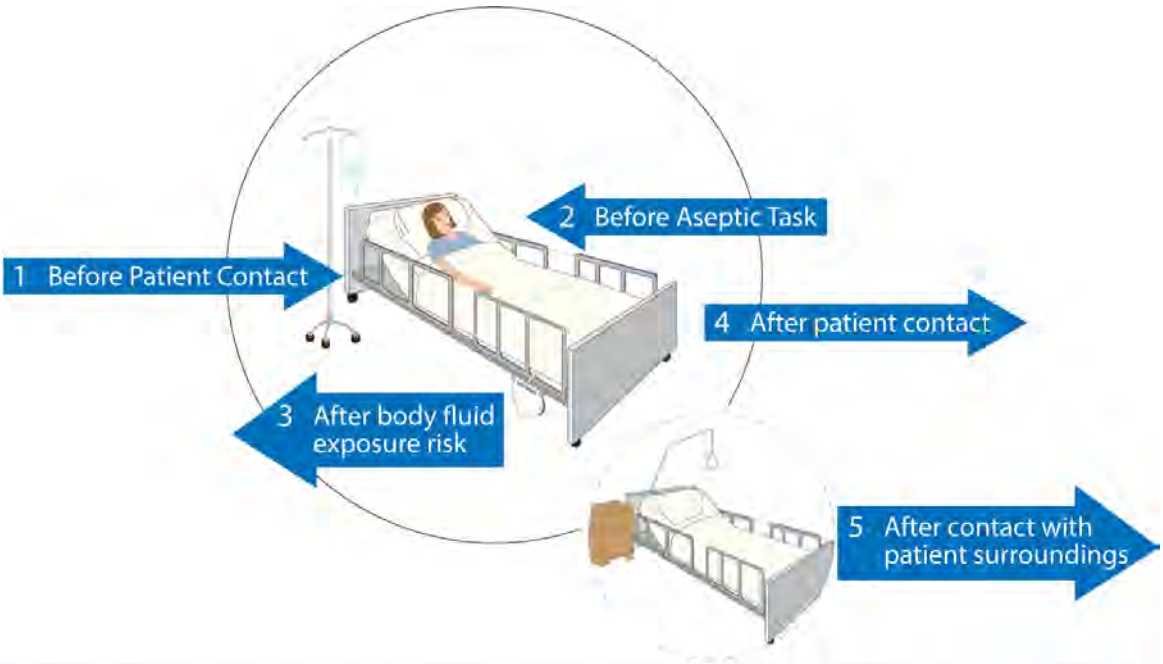
Do NOT sanitize your hands or lab benches until you complete the following experiment.

Effectiveness of Hand Hygiene

In 1847, Semmelweiss demonstrated the importance of hand washing in preventing disease. He attributed high rates of puerperal fever and death to lack of aseptic techniques by medical students assisting in childbirths who were washing their hands. Under his direction medical students began to wash their hands with a lime chloride solution and the death rate from puerperal fever dropped from 12% to 1.2 % in under a year.

According to the Public Health Agency (2012) “Hands are identified as the surfaces most at risk for contamination with microorganisms during the delivery of care. As such, hands are primary vectors for cross-transmission” (p. 5).

Hand hygiene is important before and after commencing working with microorganisms in the lab in order to reduce the numbers of microbes that can contaminate an individual during their work and that can contaminate microbial cultures that an individual is working with.



1 Before Patient Contact	When?	Clean your hands before touching a patient when approaching him or her
	Why?	To protect the patient against harmful germs carried on your hands
2 Before An Aseptic Task	When?	Clean your hands immediately before any aseptic task
	Why?	To protect the patient against harmful germs, including the patient's own germs, entering his or her body
3 After Body Fluid Exposure Risk	When?	Clean your hands immediately after an exposure risk to body fluids (and after glove removal)
	Why?	To protect yourself and the health-care environment from harmful patient germs
4 After Patient Contact	When?	Clean your hands after touching a patient and his or her immediate surroundings when leaving
	Why?	To protect yourself and the health-care environment from harmful patient germs
5 After Contact With Patient Surroundings	When?	Clean your hands after touching any object or furniture in the patients immediate surroundings, when leaving - even without touching the patient
	Why?	To protect yourself and the health-care environment from harmful patient germs

Figure 1.1 (above) Five moments in hand hygiene.

Figure 1.2 (left) Rub in between fingers and around fingers. Ensure all surfaces of the fingers are covered with soap, using friction to remove debris and oil.

Objectives:

- To evaluate the effectiveness of hand washing and surgical scrubbing procedures
- To differentiate between normal flora microorganisms and transient microbiota

Materials (per student):

- Nutrient agar plate (2)
- Sterile Surgical scrub brush
- Ivory Bar Soap
- Waterless hand cleaner

Procedure (Work on your Own):

1. Obtain two nutrient agar plates. Label one of the plates "WATER" plus your name, date and name of media. Label the plate on the bottom of the plate (agar side). Keep your writing small and around the periphery of the plate. Divide this plate into four quadrants. Label each quadrant from 1 to 4.
2. Label the other plate "SOAP" plus your name, date and type of media. Divide this plate into 4 quadrants. Label each quadrant 1 through 4.
3. Use the 'WATER' plate FIRST.
    a. Touch quadrant 1 with two fingers of your hand.
    b. Wash your hands with water as you normally would. Do not touch the faucet with your hands to turn on the water. Shake off the excess water.
    c. While your hands are still wet, touch the same two fingers to quadrant 2.
    d. Wash again using water, shake off excess water and touch quadrant 3.
    e. Repeat a final time and touch quadrant 4. Make sure you test the same two fingers each time.
4. Next obtain the 'SOAP' plate.
    a. Open the sterile package containing the surgical scrub. Place it by the sink where you will be washing your hands. Wash your same hand (as in Step 2 above) with soap and water. Wash as you normally would.
    b. Rinse, shake off excess water and touch two fingers (same as above) to section 1 of the NAP.
    c. Take the sterile scrub brush and scrub your hands using soap and water for 2 minutes. Rinse, shake off excess water and touch your fingers to quadrant 2 on the plate.
    d. Then once again scrub with soap and water for 5 minutes. Rinse, shake off excess water and touch your fingers to quadrant 3. Let your hands air dry and then use a waterless hand sanitizer on your hands. Let them air dry. Touch your fingers to quadrant 4.
5. Incubate plates at 37 °C for 24-48 hours.
6. Next lab: record growth as: (-) to (++++)
    a. no growth (-)
    b. minimum growth (+)
    c. moderate growth (++)
    d. the most growth (++++)

Questions:

1. Define ‘normal microbiota’, give examples of normal flora microorganisms and where they are found. Define ‘transient microbiota’, give examples of some disease causing transient microorganisms and explain their involvement in disease.

2. Do your results meet your expected results? Why or why not?

3. What do health care professionals attempt to accomplish with a 5 minute scrub followed by an antiseptic before surgery?

4. Why were there still organisms on your hands after scrubbing?

5. If normal flora microorganisms aren’t harmful, why is hand scrubbing used before surgical procedures?

Sources

Effectiveness of Hand Hygiene section
Public Health Agency of Canada. (2012). *Hand hygiene practices in healthcare settings.* Retrieved from http://publications.gc.ca/collections/collection_2012/aspc-phac/HP40-74-2012-eng.pdf

Figure 1.1
"Five moments in hand hygiene" by Glynda Rees Doyle & Jodie Anita McCutcheon, retrieved from https://opentextbc.ca/clinicalskills/chapter/1-6-hand-hygiene/
(Licensed under CC BY)

Figure 1.2
"Clinical Procedures for Safer Patient Care" by Glynda Rees Doyle and Jodie Anita McCutcheon, retrieved from https://opentextbc.ca/clinicalskills/chapter/1-6-hand-hygiene/ (Licensed under CC BY)

Exercise 2
The Microscope

Use and Care of the Compound Bright Field Microscope

The **compound microscope** is a system of highly corrected lenses arranged to give sharp clear magnified images of very minute objects. A microscope that allows light rays to pass directly through the microscope to the eye is called a **bright field microscope**. In this type of microscope, the light rays that strike an object in the specimen are bent and then refocused by the objective lens.

The compound microscope is one of the most expensive and indispensable instruments used by biologists. It is important that you become familiar with the various parts of the microscope which you will be using this session. Even if you have used a microscope before, it will be to your advantage to carefully work through the following procedures and observations. In this lab we use the Olympus CX31 microscope.

2.1 Parts of the Microscope

During this exercise in the lab you will become familiar with the components and operation of a compound microscope. The microscope which you have been assigned is a **binocular compound microscope**, so called because it has two ocular lenses and a further series of lenses which magnify the object being examined. It consists of a stand supporting a number of removable attachments.

Look at Figure 2.1 and locate the **arm** and the **base**. The instrument must always be carried by grasping the arm with one hand and supporting the base with the other. Always hold it in an upright position.

Materials:

- Compound microscope
- Prepared slides of silk fibers

Procedure:

You will be assigned a microscope number. Carefully remove the plastic cover from the microscope and bring it to your lab bench. Place the microscope on your desk identify each of the component parts of the microscope. Refer to Figure 2.1.

1. The **stand** consists of the **base** and **arm.** Material to be examined is placed on a glass slide over the circular opening in the **stage.**

2. The **lamp** shines light into the lens system. Find the on/off switch for the light source. The light intensity can be adjusted with a wheel that regulates the amount of current to the bulb. Higher magnification requires more light, which is obtained by adjusting the iris diaphragm.

3. Immediately above the light is the **condenser,** which focuses light from the light source onto the material being examined. At the bottom of the condenser there is a holder for a **ground glass filter** which diffuses light and reduces glare. The holder can be swung away from the body of the condenser if you need to remove the filter. The arm projecting from the condenser controls an **iris diaphragm**. This regulates the amount of light being projected onto the specimen. The position of the condenser relative to the stage (and hence also to the specimen) is controlled by a **knurled knob** on the left hand side of the instrument. The condenser housing is mounted on the stand with a rack and pinion focusing slide and so is moved up or down by turning the knob.

4. The top portion or head, of the microscope is an inclined tube fitted with two 10 X **ocular lenses**.

5. Underneath the head is a revolving **nosepiece** equipped with four **objective lenses**: the shorter 4 X or low-power objective, the large 10 X or medium-power objective, and a long 40 X or high-power objective. You should not attempt to remove the objectives from their housing. An objective is brought into position by rotating the nosepiece until it is lined up with the ocular. When moving an objective into position take great care that it does not strike the stage (see Figure 1.1). The fourth position on your microscope accommodates an objective of higher magnification - a 100 X oil immersion lens. This objective is used in combination with immersion oil when viewing very small objects such as bacteria.

6. The **stage** is equipped with a **mechanical stage** for holding and moving microscope slides. The controls for the mechanical stage are located below the stage on the right hand side of the microscope.

7. Focusing of specimens is accomplished by adjusting the position of the movable stage and condenser relative to the objectives. Movement is controlled by a large, outer **coarse focusing ring** and a small, inner **fine focusing knob**.

8. Before moving the coarse focusing ring, check to be sure the **white line** on the left hand fine focusing knob is just at the edge of the coarse focusing ring. This means that the fine focusing rotation is in the midrange of the total drive and helps you to use the fine focusing adjustment most effectively.

Label the parts of the microscope indicated with numbers.

Figure 2.1 Olympus CX 31 biological microscope

Watch the videos

Parts of the Olympus CX31 Microscope (video 0:54 minutes)

Scan the QR code or use the link below to view the video *Parts of the Olympus CX31 Microscope* by TXBIOLAB

https://www.youtube.com/watch?v=JxtSbtcqvqU

Using the Olympus CX31 Light Microscope (video 11:04 minutes)

Scan the QR code or use the link below to view the video *Using the Olympus CX31 Light Microscope* by Zach Pratt

https://www.youtube.com/watch?v=9RkKQsBW6zI

2.2 Properties of the Objective Lenses

Magnification

Magnification is a measure of how big an object looks to your eye. **The number of times that an object is magnified by the microscope is the product of the magnification of both the objective and ocular lenses**. The magnification of the individual lenses is engraved on them. Your microscope is equipped with an ocular which magnifies the specimen ten times (10 X), and four objectives which magnify the specimen 4 X, 10 X, 40 X and 100 X. These objectives are generally called low, medium, high power and oil immersion respectively. Each lens system magnifies the object being viewed the same number of times in each dimension as the number engraved on the lens. When using a 10 X objective, for instance, the specimen is magnified ten times in each dimension to give a primary or "aerial" image inside the body tube. This image is then magnified an additional ten times by the

ocular to give a virtual image that is 100 times larger than the object being viewed.

Figure 2.2 Objective lens

Resolution

Resolution is a measure of how clearly details can be seen and is distinct from magnification. **The resolving power of a lens system is its capacity for separating to the eye two points that are very close together**. It is dependent upon the quality of the lens system and the wavelength of light employed in illumination. The white light (a combination of different wavelengths of visible light) used as the light source in the lab limits the resolving power of the 100 X objective lens to about 0.25 µm. Objects closer than 0.25 µm cannot be resolved even if magnification is increased. Use of immersion oil with a 100 X special oil immersion objective lens can increase resolution to about 0.18 µm. Resolving power can be increased further to 0.17 µm if only the shorter (violet) wave-lengths of visible light are used as the light source. This is the limit of resolution of the light microscope.

The resolving power of each objective lens is described by a number engraved on the objective called **numerical aperture**. Numerical aperture (NA) is calculated from physical properties of the lens and the angles at which light enters and leaves it. Examine the three objective lenses. NA of the 10 X objective lens is 0.25.

Question:

Which objective lens is capable of the greatest resolving power?

1. **Working Distance**
   The working distance is measured as the distance between the lowest part of the objective lens and the top of the coverslip when the microscope is focused on a thin preparation. This distance is related to the individual properties of each objective.

2. **Parfocal Objectives**
   Most microscope objectives when firmly screwed in place, are positioned so the microscope requires only fine adjustments for focusing when the magnification is changed. Objectives installed in this manner are said to be **parfocal.**

3. **Depth of Focus**
   The vertical distance of a specimen being viewed that remains in focus at any one time is called the **depth of focus** or depth of field. It is a constant value for each of the objectives. As the microscope is focused up and down on a specimen, only a thin layer of the specimen is in focus at one time. To see details in a specimen that is thicker than the depth of focus of a particular objective you must continuously focus up and down, to build up a three dimensional picture from a series of optical sections.

Objective	Objective Magnification	Working Distance	N.A.	Field Diameter	
Low Power	4	15 mm	0.10	4.5 mm	(4.5 mm)
Medium Power	10	6 mm	0.25	1.8 mm	(1.5 mm)
High Power	40	0.5 mm	0.65	0.5 mm	(0.5 mm)
Oil Immersion	100	0.18 mm	1.25	0.18 mm	(0.2 mm)

Table 2.3 Properties of the objective lenses

Question:

As the magnification increases what happens to the working distance, resolution, and depth of focus?

2.3 Basic Rules for Microscopy

1. Carry the microscope with **both hands**. Always carry the microscope in an upright position. Adjust the microscope at your bench and your chair height so you do not have to tilt the microscope and you can use it comfortably.

2. Always use **both eyes open** when examining specimens. This is a binocular microscope. It is designed to be used with both eyes.

3. At the beginning of the laboratory period (and perhaps during it), gently clean the lens with the special **lens paper** (Kim wipes) supplied to you. **Never use anything else** such as Kleenex, paper toweling, or filter paper which might scratch the lenses. In addition to the ocular, objectives and top of the condenser, be certain that the slides you are to examine have also been cleaned with lens paper. Do not use any solvents (e.g. acetone) to clean prepared microscope slides. You may destroy the permanent mounting material on the slide and ruin it. Periodically, you should wipe off any dust or debris that collects on the body of the microscope, particularly the stage.

4. Focus by moving the lens away from the slide. **Do not raise the stage while looking through the microscope**. If you do this under high-power and miss the focal plane, you might drive the slide into the objective lens and cause damage to both. Always begin by locating the material with the low-power objective as outlined above before attempting more detailed work under high-power magnification. If you follow this procedure, only the fine focus adjustment need be used with the medium, high-power, and oil immersion objective. This is advisable since it is difficult to control movement of the stage with the coarse focusing control.

5. Never **force** any of the movable parts.

6. If the microscope does not seem to be in proper working order, **ask your instructor for assistance**.

Practical Procedure:

1. Obtain a slide of silk fibers and mount it on the microscope stage with the cover slip uppermost so that the specimen is centered over the opening in the stage.

2. With the low power objective in position, use the coarse adjustment to raise the stage until the objective is about 1 cm from the slide. It is best to look from the side while doing this.

3. Looking through the eyepiece, slowly lower the stage with the coarse adjustment ring until the specimen is visible. Then bring it to sharp focus. (Adjust the light).

4. Looking from the side move the nosepiece until the medium-power objective snaps into place. The specimen should be more or less in focus, a slight adjustment with the fine focus should bring the specimen into sharp focus. (Adjust the light).

5. Repeat with the high power objective. (Use only the fine adjustment while working on high power). Adjust the light.

2.4 Oil Immersion Microscopy

The 10X and 40X objectives give sufficient magnification for observation of eukaryotic cells. Since bacteria are much smaller, it is necessary to use the 100X objective to obtain sufficient magnification to view the microorganisms.

The 100X objective (sometimes called the oil immersion objective) is used with immersion oil. In order to decrease the refraction of light, oil is added to the slide and the lens is immersed in the oil.

NOTE: oil must never be applied to the 10X or 40X objectives. If this occurs, clean immediately with lens paper and lens cleaner.

1. Move the high dry lens (40X) out of position.

2. Place a small drop of immersion oil in the center of the slide directly over the specimen.

3. Move the immersion oil lens (100X) into position in the oil.

4. Focus using the **FINE FOCUS** adjustment know only.

5. When you are done making observations, lower the stage and remove the slide. Do **NOT** rotate the high dry objective (40X) into the oil. Wipe the oil off the slide using Kim wipes. Wipe the oil off the objective lens using Kim wipes. Do not let any of the other objective lenses (4X, 10X, 40X) come into contact with oil or you will ruin them.

Practical Procedure:

1. Obtain the slide labeled “Three Types of Bacteria”

2. Focus on the slide using the 4 X objective lens and the coarse focusing knob. Adjust the condenser and iris diaphragm.

3. Rotate the 10X objective lens into position. Focus using the coarse focusing knob. Can you make out any detail in the organisms you are examining? Rotate the 40X objective lens into position. Use the fine focus knob to focus on the bacteria. Can you see any detail in the organisms yet?

4. Now use the procedure above and examine the slide using the 100X objective lens and immersion oil. Record the shape and arrangement of the three types of bacteria on the slide. Sketch the organisms showing the relative sizes of each to the other.

Organism 1	Organism 2	Organism 3

Total magnification: _____X

2.5 Types of slides

In this course, you will use the following types of slides:

- **Prepared slides** of whole organisms or sections (thin slices) of organs or tissues. In the case of sectioned material, an organ or piece of tissue is first killed and 'fixed' in a preserving material and then infiltrated and embedded in paraffin. The resulting block is cut into thin sections usually 4-10 μm (micrometers) thick on a microtome and the sections are mounted on glass slides. The paraffin is removed and the material is stained usually following a specialized technique for the demonstration of particular structures or substances. Finally, the preparation is covered with a thin layer of balsam and a cover slip is placed on top.

- **Fresh mounts of living material**. This may involve whole organisms, free-hand sections cut with a razor blade of part or all of an organism, or material which is mechanically or chemically teased apart. The material is placed in a drop of physiological saline or water in the center of a clean slide. Some care must be taken in lowering the cover slip in order to avoid trapping air bubbles. This difficulty is overcome by first allowing only one edge of the cover slip to rest on the slide as shown in **figure 2.4**, and, while supporting the cover slip with a needle, slowly lowering it over the preparation. Since this type of preparation will dry up, a drop of saline or water must occasionally be added along one edge of the coverslip. Alternatively, you can reduce evaporation by ringing the edges of the cover slip with a thin film of Vaseline. Avoid getting any liquid on top of the cover slip since this interferes with focusing and might damage the objective.

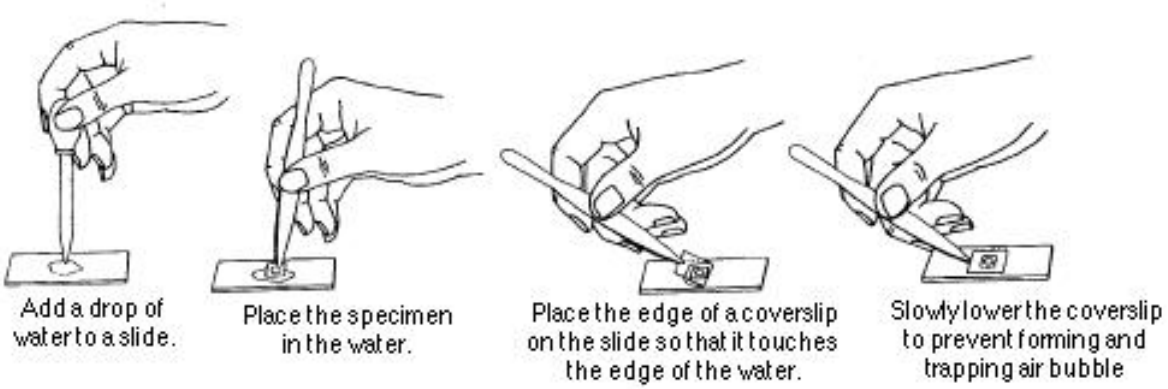


Figure 2.4 The proper technique for applying a coverslip

Practical Procedure:

1. Make a wet mount slide of pond water or hay infusion.
2. Examine the slide using 4X, 10X and 40X objective lenses.
3. Make observations of the organisms you see.
4. Examine the poster boards of organisms in the lab – can you identify any of the organisms you see?
5. Make sketches of the protozoa, algae, fungi and bacteria observed showing their relative sizes and shapes.

Sketch of Microorganisms from _____

Total magnification: _____X

Sources

Figure 2.1
Olympus. (2008). Olympus CX 31 biological microscope. Retrieved from http://www.manualslib.com/manual/795471/Olympus-Cx31.html

Figure 2.2
Olympus. (n.d.). 60x plan apochromatic objective. Retrieved from http://www.olympusmicro.com/primer/anatomy/specifications.html

Figure 2.4
"Wet Mount Slide Preparation", retrieved from https://cac-science8.wikispaces.com/Wet+Mount+Slide+Preparation (Licensed under CC BY-SA)

Exercise 3
Basic Culturing Techniques of Microorganisms

3.1 Isolation of Microorganisms from the Environment

Microorganisms are always present in the air, on laboratory surfaces and on your skin, clothing, hair, etc. They will serve as a source of external contamination and therefore interfere with experimental results unless proper techniques are used during sub culturing.

Bacteria in nature, whether they are in the soil, water, in our bodies, food or any other area, exist in mixed populations. To examine the cultural, morphological and physiological characteristics of an individual microorganism, it is essential that the microorganism be separated from the other microorganisms that are normally found in its natural habitat. A **pure culture** of the microorganism must be obtained.

In the laboratory, the isolation and identification of a particular microorganism is done by growing the microorganism on culture media.

Objectives:

- To define the term 'aseptic technique'
- To become proficient in performing various inoculation techniques for the purpose of transferring microorganisms from one culture medium to another using aseptic technique.
- To differentiate between different types of cultures: broth cultures, slant cultures, streak plates and deep cultures.

Materials:

Lab Bench

- Bunsen burner and striker
- inoculating loop
- inoculating stab
- holder
- marking pen
- masking tape

Supplies (Per Student)

- nutrient agar slant (1)
- nutrient broth (1)
- nutrient deep culture (1)
- nutrient agar plate (2)
- Czapek-Dox plate (1)
- sterile water for moistening dry swabs
- sterile swabs
- 24-48 hour nutrient broth culture of *Staphylococcus epidermidis*
- 24-48 hour nutrient broth culture of *Escherichia coli*

Procedure: (Each Student)

1. Obtain one plate of nutrient agar (NAP) and one plate of Czapek-Dox (C-Dox) agar.
2. Label your plates on the bottom (agar side of the plate). Write around the circumference of the plate using felt pen marking and small letters.

   Labels must always include the following four items:

   a. **Your Name**

   b. **Source of Inoculum or Name of Inoculated Organism**

   c. **Name of Medium**

   d. **Date of Inoculation**
3. Select ONE area of the college that you would like to sample. You must be able to sample that site and return to the lab in 10 minutes.
4. Run two damp sterile swabs over the surface you have chosen. Then gently run one swab over the surface of the nutrient agar plate as demonstrated and gently rub the second swab over the surface of the Czapek-Dox agar plate. Bring the contaminated swabs back to the lab to dispose of in the autoclave waste bags.
5. Place your plates (INVERTED) in the appropriate racks on the front bench for incubation. The instructor will give you the time and temperatures of incubation.

Next Laboratory Session:

Make observations of the numbers and types of colonies growing on your plates.

Questions:

1. Do you see any differences between the population of organisms on the NAP as compared to the C-Dox plates? Why or why not?
2. Why are plates incubated and handled in an INVERTED position?
3. Why were the NAP plates incubated at different temperatures than C-Dox plates?

3.2 Nomenclature

Most microorganisms (but not all) are named using rules of scientific nomenclature. In this course you must name bacteria and fungi using their correct Genus and species names (including correct spelling). Organisms are named using the following rules:

1. genus name (1st letter is capitalized; all others are lowercase)
2. species name (lowercase)
3. italicized (when printing both genus and species name must be underlined separately to indicate this)

Example:

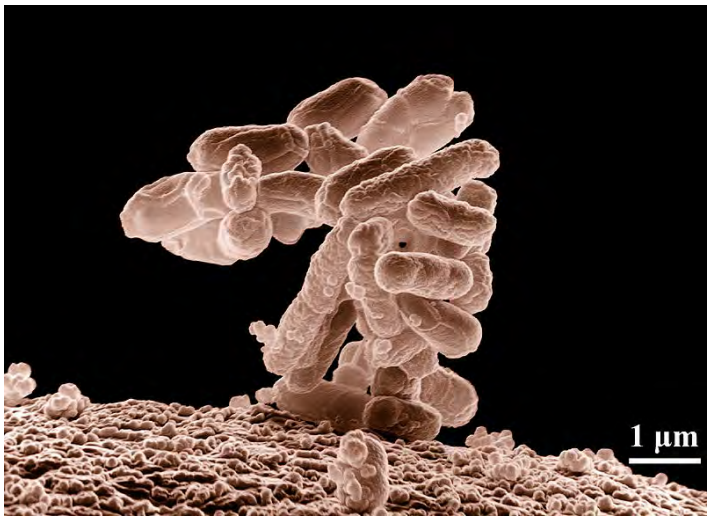


Figure 3.1 *Escherichia coli*

Escherichia coli
genus name = ***Escherichia***
species name = ***coli***

Note: If you are hand writing this organism's name you would write it as **Escherichia coli.**

(Both genus and species names are underlined separately when handwriting scientific names

e.g. in your lab notes or on a test/examination if you are handwriting your answers).

Questions:

1. Where would you find this organism in your body?
2. Why is it beneficial in your body?
3. How can some forms of this organism be detrimental?
4. Name the organism (using scientific nomenclature) that causes:
    a. Tetanus
    b. Chlamydia
    c. Syphilis
    d. Lyme's disease
    e. Tuberculosis
    f. Salmonellosis
    g. staphylococcal toxic shock syndrome
    h. rabbit fever
    i. Chagas disease
    j. malaria

3.3 Culture Techniques

There are several different formats of culture media that can be used to obtain pure cultures.

Liquid media
Liquid media are distributed in flasks or tubes plugged with non-absorbent cotton wool, specially designed rubber plugs, aluminum caps, or plastic caps. Alternatively, they may be kept in screw cap bottles. Liquid media are referred to as broths if organic components are a part of the media.

Solid media
If required, nutrients can be incorporated in a medium together with a solidifying agent. Generally, 1.5% - 2.0% agar is added. Agar, an extract of red algae, is a complex carbohydrate that is attacked by relatively few bacteria. Agar gels liquefy between 96 °C and 100 °C but do not set until the temperature has dropped to 42 °C. This must be borne in mind when using such media.
Solid media can be used in a variety of ways:

- **Slant (slope) cultures**
  Tubes of solid medium are heated to melt the agar. These are allowed to set in a sloping position, thus increasing the surface area of the medium. The inoculum is spread over the surface of the medium with the aid of a sterile wire loop.

- **Deep stab cultures**
  Tubes of solid medium are allowed to set in a vertical position and are inoculated by means of a straight wire stab plunged into the medium.

- **Shake cultures**
  Tubes of solid medium are heated and the molten medium is allowed to cool to 45°C. While still liquid, the medium is inoculated, mixed well by rotating the tube between the hands and then allowed to set.

Procedure:

Aseptic technique must be strictly adhered to so that the bacterial culture you are working with doesn't become contaminated or that you, or your immediate environment doesn't become contaminated. Have the inoculum, the inoculating loop or stab and the sterile, fresh medium all within easy reach before starting. Remove all papers from your work area. Work directly on the lab bench – it can be decontaminated with disinfectants if you have a spill. Do NOT work on paper towels. Never work over your books, notes or personal items. They cannot be sterilized or autoclaved easily if you have an accidental spill.

The following procedure is followed for all inoculations in this experiment.

Watch the video on transfer techniques before starting this procedure.

Scan the QR code or use the link below to view the video *Transfer Technique I: Transfer Organisms from Broth Culture to Broth Tube* (video 2:51 minutes)

https://www.youtube.com/watch?v=bYYYgUCLWeo&feature=youtu.be

1. With the marking pen provided, make a label with masking tape with your name and stick it to the tube. This label must be removed after you have finished with the tube.

2. Hold the loop or stab as if it was a pencil and sterilize it by holding it in the bunsen burner at about a 60° angle until the entire wire is red hot. Let it cool slightly by waiting for about 10-15 seconds.

3. The plate of inoculum is lid-side down. Lift the plate, invert and insert your loop or stab. When the loop or stab has cooled, gently pick up a single colony or a very small amount of a bacterial colony. Replace the plate in lid immediately. Do not place the plate on the bench.

4. Pick up the tube to be inoculated with the other hand. Remove the cap by grasping the cap with the little finger of the hand that is holding the inoculating loop or stab. Do not place the cap on the bench. This prevents the cap from becoming contaminated (figure 2.1).

5. Flame the mouth of the tube by passing it through the Bunsen burner. This sterilizes the air in and immediately around the mouth of the tube. Insert the loop or stab and dispense the microorganism in the appropriate manner. Remove the loop or stab and reflame the mouth of the tube. This will kill any bacterial cells that may have been deposited on the mouth of the tube when the loop or stab was inserted or withdrawn. Replace the lid immediately and place the tube in a test tube rack. Flame the inoculating loop or stab until red-hot and place it back into the holder.

6. Incubate at 37°C for 24 hours. The tubes and plate cultures will be removed by the instructors after incubation and refrigerated until your next lab session.

7. Using either the agar plate of *Staphylococcus epidermidis* or *Escherichia coli* provided prepare the following sub-cultures when you are sure you understand the above procedure for aseptic transfer of inoculum. Your partner should choose the other organism. During the next laboratory period, these cultures will be observed for growth, and the results will be recorded in data Table 2.1.

- **Nutrient agar slant**
  Follow the above inoculation procedure using the inoculating **loop** or **needle** using either *S. epidermidis* or *E. coli* (your partner should use the other organism). At step 5 the microorganisms are transferred to the **surface** of the slant with a zig-zag motion that starts at the bottom of the slant and ends at the top.

Watch the video on transfer techniques before starting this technique.

Scan the QR code or use the link below to view the video *Transfer Technique II: Transfer Organisms from Broth Culture to Nutrient Agar Slant* (video 2:57 minutes)

https://www.youtube.com/watch?v=4tHFUu_5DWk&feature=youtu.be

- **Deep Stab Culture**
  Follow the above inoculation procedure using the inoculating needle. Make sure it is fairly straight. At step 5, the microorganisms are transferred to the medium by carefully inserting the stab down the center of the medium to the bottom and then carefully removing the stab in the same manner. One partner should inoculate the nutrient agar deep with *E. coli* and the other partner should use *S. epidermidis*.

Watch the video on transfer techniques before you attempt this technique.

Scan the QR code or use the link below to view the video *Transfer Technique III: Transfer Organisms from Broth Culture to Nutrient Agar Deep* (video 2:47 minutes)

https://www.youtube.com/watch?v=pH4I6VEPoVY&feature=youtu.be

- **Nutrient broth**

Follow the above inoculating procedure using the inoculating **loop**. At step 5, the microorganisms are transferred to the liquid medium by gently swirling the loop in the broth. This will remove most of the cells from the loop. Use either *S. epidermidis* or *E. coli*.

Watch the video on transfer techniques before you attempt this technique.

Scan the QR code or use the link below to view the video

https://www.youtube.com/watch?v=bYYYgUCLWeo&feature=youtu.be

- **Nutrient agar streak plate – Multistreak Technique Method**
  Label the agar plate as demonstrated by your instructor. Follow the above inoculating procedure using the inoculating **loop**. Although there are many different methods of preparing streak plates for single-colony isolates, follow the method demonstrated by your instructor. Place your plate in the rack at the front to be incubated at 37°C for 24 hours. Incubate the plates agar side up so any condensation produced during incubation will fall on the lid and not disrupt the colonial growth on the plate.

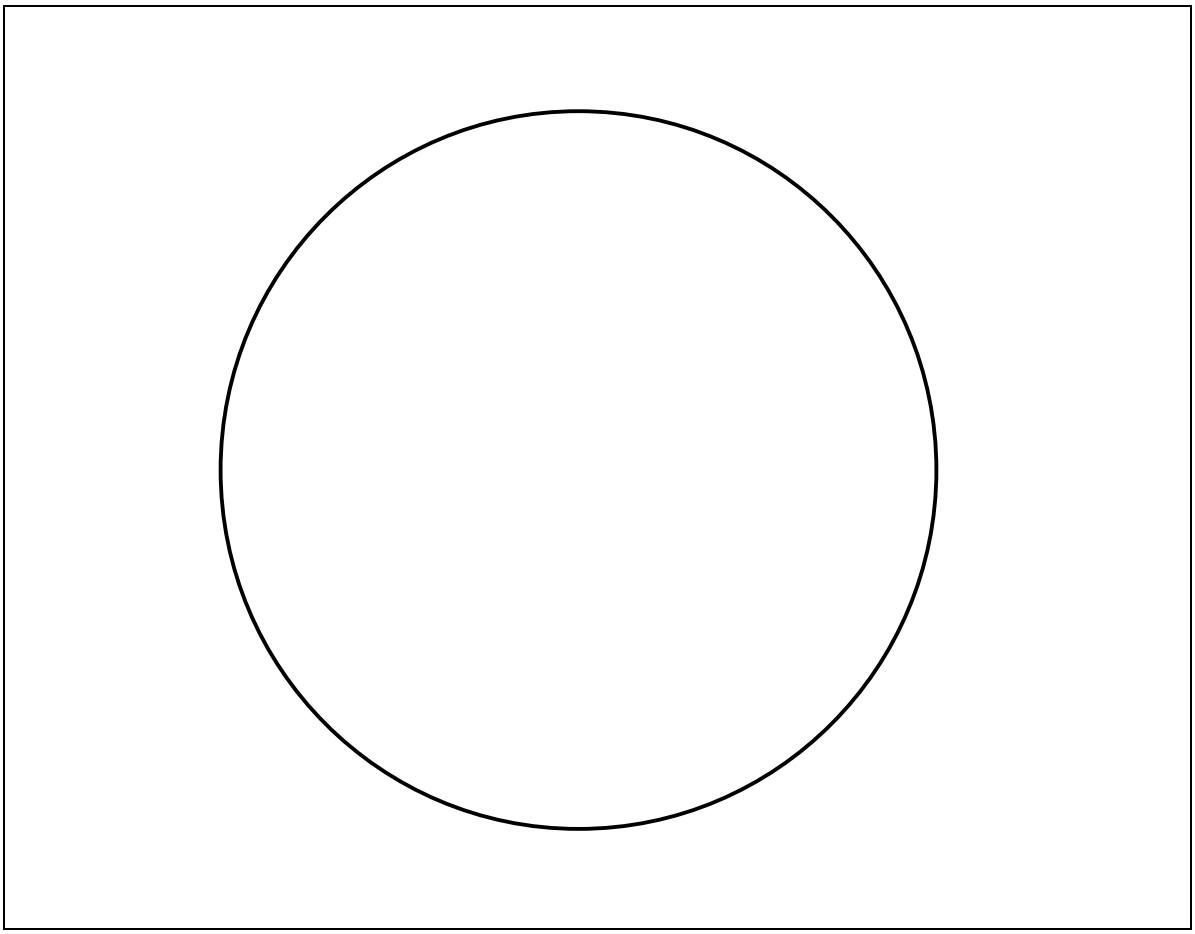
  The nutrient agar streak plate is one of the most important techniques used. The single colonies that are obtained are used for further tests or subcultures of the organism. The single colonies are also used to determine the colony morphology.

Watch the video on multistreak plate method before you attempt this technique.

Scan the QR code or use the link below to view the video *Multistreak Plate Method - Part 1* (video 1:09 minutes)

https://www.youtube.com/watch?v=_bAbQc3fKdc&feature=youtu.be

Draw a diagram of the Multistreak plate method in the space provided.



Questions:

1. What is meant by the term ‘aseptic technique’?
2. What is meant by the term ‘pure culture’?
3. What is meant by the term ‘mixed culture’?
4. Do organisms exist in pure culture in nature?
5. Define the term ‘sterile’.

Your cultures will be removed from the incubator after growth and will be stored in the fridge until your next laboratory period. Your instructor will tell you the temperature and times of incubation of your cultures.

Next Laboratory Session:

Record observations:

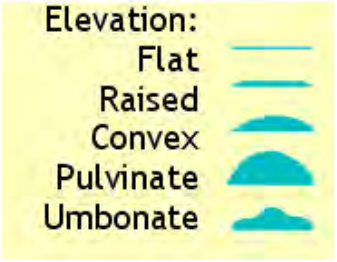
CAREFUL: Do NOT hold tubes by the plastic caps – they are sleeves and will slide off easily. Always handle/hold test tubes by the glass underneath the cap.

NOTE: DO NOT SHAKE or SWIRL liquid broth cultures or you will destroy the growth pattern.

Examine your nutrient agar slants, broths and deeps and record results in the table below (Table 2.1) using the following descriptions. Record results from your partner's media as well.

1. Evaluate the growth on the nutrient agar slant is as no growth, slight, moderate or abundant.
2. Evaluate the growth of organisms in the nutrient broth as:
    a. Uniform turbidity: - finely dispersed growth throughout.
    b. Flocculent: - flaky aggregates dispersed throughout.
    c. Pellicle: - thick, pad-like growth on surface.
    d. Sediment: - concentration of growth at the bottom of culture tube.
3. Record the appearance of the organism in the deep stab cultures. Did they grow throughout the tube? Was the growth pigmented? Was the growth light or heavy?
4. The **colony morphology** of an organism is that of a single isolated colony on an agar plate. Colonial morphologies are used to help identify bacteria and fungi (although please note that many organisms can take on a variety of colonial forms and morphologies – e.g. can have different phenotypes even though they have the same genus and species name).

    The following characteristics are those most commonly used to describe colony morphology:
    a. **Shape or form:**
    Figure 3.2 (top right) Shape of bacterial colony
    b. **Surface:**
    In order to access the surface, flame a loop and touch the surface of the colony.
        i. Smooth, glistening
        ii. Rough
        iii. Wrinkled
        iv. Mucoid, moist
        v. Dry, powdery
    c. **Elevation:**
    Figure 3.3 (bottom right) Elevation of bacterial colony
    d. **Size:**
    measure the diameter of a single colony with a ruler in millimeters.
    e. **Pigment:**
    Describe the color of the colony. For example, cream, white, beige, purple, red, pink, yellow, brown, blue, grey, etc. Some water soluble pigments diffuse into the media, if they do, make a note of it.



f. **Opacity:**
Transparent (can see through) Semi-transparent (partially able to see through colony) Opaque (not transparent or clear).

Table 2.1 Cultural characteristics of microorganisms.

Cultural Characteristics of Microorganisms	***Staphylococcus epidermidis***	***Escherichia coli***
1. Amount of growth on nutrient agar slant.		
2. Type of growth in the nutrient broth.		
3. Appearance in deep stab culture.		
4. Colonial Morphology on NAP incubated at _____°C for _____ hrs. :		
Shape		
Surface		
Elevation		
Size (mm)		
Pigment		
Opacity		

Sources

Figure 3.1
"E coli at 10000x, original" by Eric Erbe, digital colorization by Christopher Pooley, retrieved from https://commons.wikimedia.org/wiki/File:E_coli_at_10000x,_original.jpg (Available under Public Domain)

Figure 3.2
"Bacterial colony morphology" by Ewen, retrieved from https://commons.wikimedia.org/wiki/File:Bacterial_colony_morphology.png (Available under Public Domain)

Figure 3.3
"Bacterial colony morphology" by Ewen, retrieved from https://commons.wikimedia.org/wiki/File:Bacterial_colony_morphology.png (Available under Public Domain)

Exercise 4
Factors Affecting Microbial Growth & Control of Microbial Growth

4.1 Effect of Temperature on Microbial Growth

Every organism has an optimum temperature at which it grows best. The growth temperature range is the range of degrees between the minimum temperature, the temperature below which growth does not occur, and the maximum temperature above which the organism will not grow. These temperature distinctions are primarily made for convenience and the precise numbers should not be taken as absolute. Organisms can be classified into five groups depending on their temperature requirements.

- **Psychrophile**
  - Psychrophiles grow only at low temperatures with an optimal range of growth is between 0°C and 20°C.

- **Psychrotroph**
  - Psychrotrophs prefer to grow between 20 and 30°C.

- **Mesophile**
  - Mesophilic organisms have optimal temperatures between 25°C and 40°C.

- **Thermophile**
  - Thermophilic organisms prefer growth at temperatures above 45 and 65°C.

- **Hyperthermophile**
  - Hyperthermophilic organisms only grow at 80°C and higher.

Bacteria and Archaea are more heat resistant than most other forms of life. High temperature can be used to control microbial growth however moisture content, cell density, pH, and medium composition can all affect the heat sensitivity of an organism and whether it can enhance a microorganism's growth or prove lethal to it. Usually heat is used to sterilize a variety of items used in the lab or a hospital health care setting. Heat can be dry or moist.

Dry heat can kill microorganisms by denaturing enzymes, oxidizing, or dehydrating the cells. Dry heat can be applied from ovens, flaming over an open flame, or in incinerators. Usually heat is applied in hot air ovens at 170°C for 2 hours.

Moist heat is normally more effective in killing microorganisms. It includes pasteurization, boiling and autoclaving. Moisture in hot air transfers more heat energy to microbial cells than hot dry air. Autoclaving is used in labs and health care facilities as the most effective means of moist heat sterilization. It involves heating substances at 121°C at 15-20 psi pressure for 15-20 minutes (depending on the load being autoclaved). This kills endospores produced by some bacteria and makes sterilizes heat and moisture resistant materials.

Figure 4.1 **Ariel view of Grand Prismatic Spring, Yellowstone National Park.** Thermophiles are found in various geothermally heated regions of the Earth, such as hot springs like those in Yellowstone National Park and deep sea hydrothermal vents, as well as decaying plant matter, such as peat bogs and compost.

Figure 4.2 **Watermelon snow streaks (left) and watermelon snow pits (right).** Watermelon snow, also called snow algae, pink snow, red snow, or blood snow, is *Chlamydomonas nivalis*, a species of green algae containing a secondary red carotenoid pigment (astaxanthin) in addition to chlorophyll. Unlike most species of fresh-water algae, it is cryophilic (cold-loving) and thrives in freezing water. This type of snow is common during the summer in alpine and coastal polar regions worldwide, such as the Sierra Nevada of California. Here, at altitudes of 10,000 to 12,000 feet (3,000–3,600 m), the temperature is cold throughout the year, and so the snow has lingered from winter storms. Compressing the snow by stepping on it or making snowballs leaves it looking red. Walking on watermelon snow often results in getting bright red soles and pinkish trouser cuffs.

Objectives:

- Classify bacteria on the basis of their temperature requirements.
- Compare effectiveness of dry heat and moist heat in controlling bacterial growth.
- Define pasteurization, incineration, autoclaving and for each, show its use in health care and laboratory settings.

Materials (Per Bench):

- Brain Heart Infusion (BHI) agar plates (4)
- Sterile swabs
- 24 - 48 hour broth cultures of:
    - *Bacillus stearothermophilus*
    - *Micrococcus luteus*
    - *Psychrobacter immobilis*
    - *Staphylococcus epidermidis*

Procedure: (Per Bench)

1. Divide each of 4 BHI agar plates into quadrants. Label one plate 15°C, the second 25°C, the third 37°C and the fourth 56°C. the plates should also be labeled with organism's names, your bench number and type of media.

2. Using a sterile swab and the broth culture provided, streak *B. stearothermophilus* on one quadrant of each plate. Use a fresh swab on each plate. Repeat the procedure using the other three organisms: *M. luteus, P. immobilis and S. epidermidis.* The plates will be incubated for 24 -48 hours at the appropriate temperature.

Next laboratory period:

Score plate growth as (-) to (++++) as follows:

- no growth (-)
- slight growth (+)
- moderate growth (++)
- heavy growth (+++)
- maximum growth (++++)

Classify each organism as a psychrophile, psychrotroph, mesophile, thermophile or hyperthermophile.

Questions:

1. Differentiate between incineration, pasteurization, autoclaving. Define each.
2. How can heat be used to control microbial growth?
3. Compare the effectiveness of autoclaving and dry heat.
4. What are bacterial endospores? How are they heat resistant?

5. *Geobacillus stearothermophilus* is a biological indicator that is used in autoclaves when materials are sterilized? Why? And how does it accomplish this purpose?
6. How would you sterilize the following items? inoculating loop, inoculating needle, vitamin solution, cotton swab, patient's bed frame, patient's used Kleenex, soiled bandages?

4.2 The Effect of Oxygen on Microbial Growth

Oxygen is very important to microbial growth. Different organisms have different requirements for molecular oxygen. They use oxygen for respiration and their differences in oxygen requirements reflect differences in their enzymes for bio oxidation. Microorganisms can be classified into five major groups based on their oxygen needs:

1. **Obligate aerobes** These organisms require atmospheric oxygen for growth. They use oxygen as the final hydrogen (electron acceptor) in the oxidative degradation of high energy molecules like glucose.

2. **Microaerophiles** These organisms require limited amounts of oxygen for growth. They grow best in an atmosphere that contains increased carbon dioxide (5-10%) and reduced oxygen. These microorganisms are often grown in a candle jar (also called a CO_2 jar). Inoculated plates/tubes are placed inside the jar, a candle is lit (or a CO_2 generating packet is placed in the jar) and the lid is screwed onto the jar. This produces a carbon dioxide rich environment for the organisms to grow in.

Figure 4.3 **Candle Jar.** Anaerobiosis is produced by a sealed jar and a candle inside, so microorganisms in the culture plates can survive and grow. The candle is lit, chamber sealed and when the candle is extinguished, the oxygen in the jar is reduced.

3. **Obligate anaerobes** These organisms cannot tolerate oxygen and it is lethal to them. In these organisms (as in aerobes) oxygen results in the formation of toxic metabolic end products like superoxide and hydrogen peroxide. These organisms lack the enzymes, superoxide dismutase and catalase. Superoxide dismutase breaks down superoxide to hydrogen peroxide and catalase breaks down hydrogen peroxide to water. They have to be grown in the absence of oxygen in anaerobic jars where the oxygen is replaced with carbon

dioxide or other gases. They can also be grown in reducing media (thioglycolate broth) and in anaerobic incubators and glove boxes.

Figure 4.4 **Gas-Pak jar.** Gas-pak is a method used in production of an anaerobic environment. It is used to culture bacteria which die or fail to grow in presence of oxygen.

4. **Aerotolerant anaerobes** These are fermentative organisms and so they don't use oxygen as a final electron acceptor. Unlike obligate anaerobes they produce catalase and/or superoxide dismutase and are not killed by the presence of oxygen. They don't use oxygen but can tolerate it. Their growth is enhanced by microaerophilic growth conditions generally.

5. **Facultative anaerobes** Facultative organisms grow in the presence or absence of oxygen. They prefer to use oxygen for aerobic respiration. In an oxygen poor environment, they can carry out fermentation processes or use compounds like nitrates, sulfates as final hydrogen acceptors in cellular respiration.

Oxygen requirements of bacteria can be determined in a variety of ways. In a deep tube stab, organisms will grow at a depth where they have their optimum oxygen concentration.

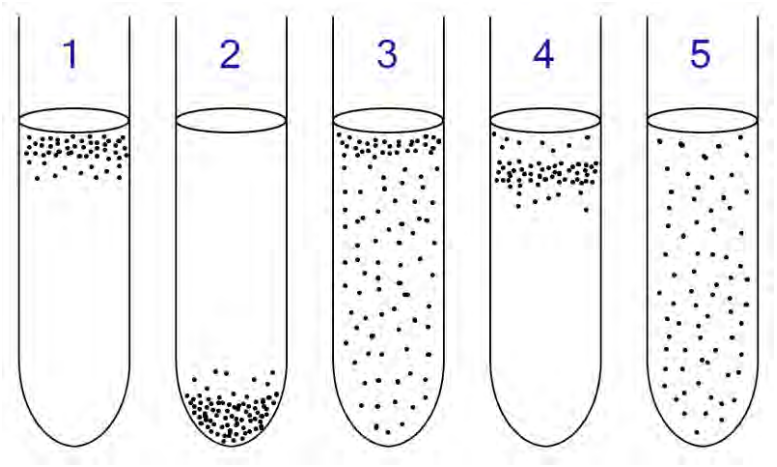


Figure 4.5 **Aerobically different bacteria behave differently when grown in liquid culture:**

- **Obligate aerobic** bacteria gather at the top of the test tube in order to absorb maximal amount of oxygen.

- **Obligate anaerobic** bacteria gather at the bottom to avoid oxygen.
- **Facultative** bacteria gather mostly at the top, since aerobic respiration is advantageous (energetically favorable); but as lack of oxygen does not hurt them, they can be found all along the test tube.
- **Microaerophiles** gather at the upper part of the test tube but not at the top. They require oxygen, but at a lower concentration.
- **Aerotolerant** bacteria are not affected at all by oxygen, and they are evenly spread along the test tube.

Reducing media like thioglycolate broth can be used to reduce concentration of oxygen in media so anaerobic organisms that can be cultured in open air. Reducing media contains chemicals that combine with oxygen and reduce the concentration of oxygen.

Oxygen can also be used to control microbial growth. Exposing anaerobic organisms to oxygen will kill them.

Objectives:

- Classify microbial growth as to oxygen requirements
- Examine three methods of culturing anaerobes
- Examine candle jars and anaerobic jars and identify their use
- Perform a catalase and oxidase test on bacteria and interpret the results

Materials: (Per bench)

- thioglycollate broth (4)
- NAP (2)
- Hydrogen peroxide (3%) for catalase test
- Oxidase test strips
- scissors
- inoculating loop
- anaerobic jar and gas pak system
- 24-48 hour cultures of:
    - *Alcaligenes faecalis*
    - *Clostridium pasteurianum*
    - *Escherichia coli*
    - *Lactococcus lactis*

Procedure:

1. Obtain four tubes of thioglycollate broth. Label them with your bench number, organism name, name of media and date. Make observations. **Handle this medium very carefully so the oxygenated layer is not disturbed. (Do NOT shake these tubes) Why not?**

2. Inoculate four thioglycollate broth tubes below the upper blue pigmented layer (which contains dissolved oxygen) with a loopful of each organism. The lower colorless section of Thioglycollate broth is anaerobic. Methylene blue is used to indicate the presence or absence

of oxygen, being blue when oxidized and colorless when reduced. Incubate at 37°C.

3. Obtain 2 nutrient agar plates. Divide each plate into four quadrants.

4. Label one plate AEROBIC ($+O_2$) and the other plate ANAEROBIC ($-O_2$)

5. Label plates with your bench number, media, date and name of organisms in each quadrant.

6. Inoculate each quadrant by streaking a single line of the bacterium in each appropriate quadrant. Place the plate labeled AEROBIC in the incubator at 37°C. Place the ANAEROBIC plate in the anaerobic jar. The instructor will show you how to use the anaerobic-incubation system. When the anaerobic jar is sealed it will be incubated at 37°C.

Next Laboratory Session:

- Examine the types of thioglycollate medium. Record the appearance of growth in each tube. Classify the organisms on the basis of their oxygen requirements.

- Examine the NAP's. Compare the growth of organisms on the plates grown in oxygen (air) with the ones grown in the anaerobic jar (no oxygen). Can you classify the organisms' requirements for oxygen based on your results? What are they?

- Perform the catalase test on the organism from the NAP's. Drop 3% hydrogen peroxide on one of the colonies on the nutrient agar.

  A positive catalase test is indicated by oxygen bubbles in a white froth. In this case hydrogen peroxide is broken down by catalase to water and oxygen. Alternatively, a loopful of culture can be placed on a glass slide and hydrogen peroxide can be dropped onto the culture material. Bubbling indicates catalase production by the organism.

- Perform an oxidase test on each of your organisms from the NAP's. Take a sterile loopful of culture and rub it on the oxidase test strip. If the streak line turns blue immediately, oxidase is present. Oxidase is used by organisms to break down superoxide radicles.

Questions:

1. Define and describe the terms aerobic, microaerophilic, facultative anaerobe, aerotolerant and anaerobic.
2. Why can't obligate aerobes grow in the absence of oxygen?
3. Do aerobes produce catalase and oxidase?
4. What defense mechanism does *Clostridium* sp. have to survive exposure to oxygen?
5. What does the appearance of a blue color in the thioglycolate tube mean?
6. Will an obligate anaerobe grow in thioglycolate?
7. Describe three methods of cultivating anaerobic bacteria.

8. The catalase test is used clinically to distinguish between two genera of gram positive cocci: _____ and _____ and two genera of gram positive bacilli: _____ and _____

4.3 The Effect of Ultraviolet Radiation on Microbial Growth

Radiation comes to the Earth from the sun and other sources. Radiation differs in energy and wavelength. Shorter wavelengths of radiation have more energy. There are two forms of radiation – ionizing and non-ionizing radiation.

Ionizing radiation include X rays and gamma rays – they ionize water into reactive free radicals that can break strands of DNA. This can be used to control microbial growth and is dependent on the age of microbial cells, composition of the medium and the temperature.

Some non-ionizing wavelengths is necessary for life. Processes like photosynthesis by plants and algae has wavelengths in the visible range of the electromagnetic spectrum ranging from 380 - 750 nm. Animal cells synthesize vitamin D in the presence of light at about 300 nm. Non ionizing light in the 15-400 nm spectrum is called Ultraviolet light (UV). It is just below the visible spectrum of light. It is divided into three types: UVA, UVB, and UVC. UVA wavelengths are the longest (320-400 nm). They are known as the 'tanning spectrum'. This energy causes increased melanin production by melanocytes found just below the epidermis of the skin. Although still used in tanning salons, UVA radiation is now known to penetrate the skin causing the skin to age 40 times faster than normal. As well, UVA light is associated with cataract formation and with malignant melanomas, the deadliest form of skin cancer. UVB radiation (wavelengths 290-320 nm), known as the 'burning spectrum', is most energetic and is associated with sunburn and the most common form of cancer, basal cell carcinoma. UVC radiation (200-290 nm) are biocide and the most lethal wavelengths. These wavelengths correspond to the optimal absorption wavelengths of DNA. Wavelengths below 200 nm are absorbed by air and don't reach living organisms.

UV light is a powerful **mutagen.** It can damage DNA by the induction of pyrimidine dimers (example thymine dimers) resulting in mutation. This is a situation where two adjacent pyrimidine residues along the DNA backbone become "fused" to each other rendering them unable to hydrogen bond with the complementary base on the opposite DNA strand. Other changes to DNA structure, which can occur as a result of exposure to UV light, are strand breakages, duplications, deletions, inversions and translocations. Any one of these alterations in DNA if not repaired will ultimately disrupt DNA replication and protein synthesis. This can result in cell death unless the damage is repaired. Cells may repair themselves using light repair (photo reactivation) or dark repair. Light repair occurs when thymine dimers are exposed to visible light photolyases are activated that split the dimers repairing DNA and restoring it to its undamaged state.

Dark repair occurs without light. Dimers are removed by endonuclease. DNA polymerase replaces the nucleotides and DNA ligase seals the sugar-phosphate backbone.

UV radiation is used as a sterilizing agent but it is limited in its use because it has poor penetrating

power. It can be used to control microbial growth (sterilize) surfaces in hospital operating rooms, laboratories and food processing plants, to disinfect water and sewage in water and sewage treatment plants, and to sterilize some heat labile solutions.

Objectives:

- Examine effects of radiation on bacteria
- Observe and describe 'dark repair' and 'light repair'

Materials: (Per bench)

- NAP (3)
- UV light source (260 nm)
- Covers
- Protective goggles
- Micropipette + tips
- Alcohol for flame sterilizing
- spreader
- 24 hour nutrient broth cultures of:
    - *Bacillus subtilis*
    - *Serratia marcescens*
    - *Staphylococcus epidermidis*
    - *Micrococcus luteus*

NOTE: Each bench will be assigned a **different** bacterium to test.

Procedure:

1. Label plates with your name, organism, date, media. Label one plate A, one B, and the last C. Divide each plate in half.

2. Label one side of each plate + UV (TEST) and the other – UV (CONTROL).

3. Pipette 100 µL of bacterial culture onto each plate. Spread the culture evenly over the surface of the plate using an alcohol flamed spreader.

4. Hold the plates under the UV light source (wear protective goggles). Expose the plate to the UV light on the side of the plate labeled +UV light as follows:

    a. Plate A – expose to UV for 30 sec. Incubate @ room temperature in DARK (cover plate in tin foil to eliminate light)
    b. Plate B – expose to UV for 30 sec. Incubate @ in light (sunlight or visible light source) at room temperature
    c. Plate C – expose to UV for 60 sec. Incubate @ room temperature in DARK. (cover plate with tin foil to eliminate all light)

5. Incubate plates for 3-4 days.

Next Laboratory Period:

- Record growth. Score growth on plates from (-) to (++++)
- Record class results. Interpret your results.

Questions:

1. What was the effect of UV light on each of the organisms?
2. Which organism(s) underwent dark repair?
3. Which organism(s) underwent light repair?

4.4 The Effect of Heavy Metals on Microbial Growth

Heavy metals such as copper, silver or mercury are capable of exerting a lethal effect upon bacteria.

To show this effect, a sterile paper disc that has been dipped into a 1% silver nitrate solution is placed on a nutrient agar pour plate that has been inoculated with *E. coli.* After incubation, a zone of inhibition will surround the disc indicating that the bacteria have been killed. This is called the **oligodynamic zone**. Normal growth will occur in the remainder of the agar.

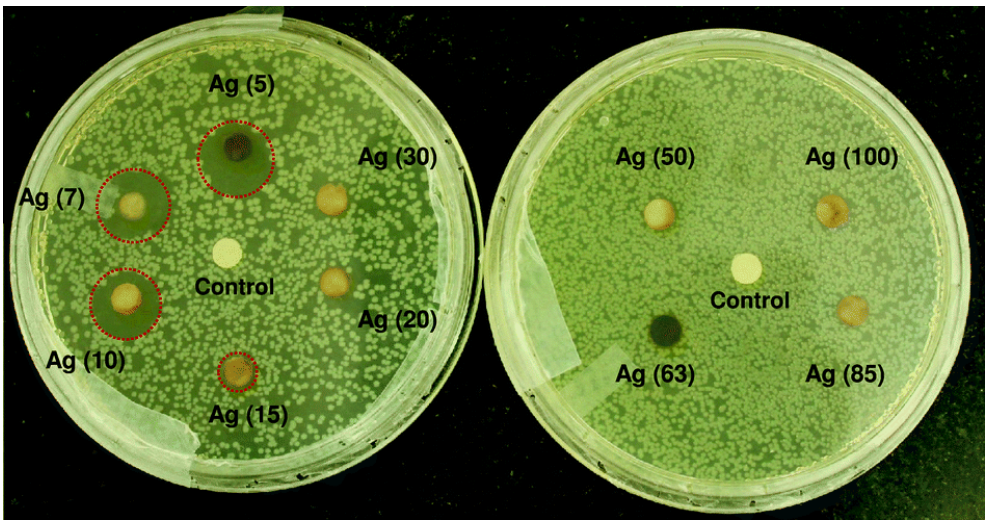


Figure 4.6 **Disk diffusion tests for different sized silver nanoparticles against the E. coli MTCC 443 strain.** The zone of inhibition is highlighted with a dashed circle indicating a noticeable antibacterial effect.

Objectives:

- Show that silver and other heavy metals have an effect on bacterial growth
- Define the term 'oligodynamic'

- List the mode of action of silver and other heavy metals on bacteria

Materials: (Per pair of students)

- BHI plate (2)
- Silver nitrate
- Sterile discs
- Forceps
- Spreader
- Beaker
- Micropipette and tips
- Alcohol for flame sterilizing instruments
- 24-48 hour cultures of
    - *S. epidermidis*
    - *E. coli*

Procedure:

1. Label the BHI plate with your bench number, organism, date, type of media. Divide it in half. Label one side CONTROL, the other side TEST.

2. Using a micropipette, Inoculate 100 µl of a 24-48 hour nutrient broth culture of *E.coli* onto the surface of a BHI plate . Alcohol flame sterilize a spreader and spread the *E. coli* over the surface of the plate evenly.

3. Sterilize forceps by dipping in ethanol and flaming and pick up a sterile paper disc. Place the disc gently on the surface of the media on the side of the plate labeled CONTROL.

4. Sterilize forceps again by dipping them in ethanol and flaming and then pick up another sterile filter paper disc. Dip this disc into the solution of 1 % silver nitrate. Blot off any excess solution by touching the side of the container and place the disc on the surface of the medium on the side of the plate labeled 'TEST'.

5. Repeat the procedure (Steps 1-5) using *S. epidermidis.*

6. Place at 37 °C to be incubated for 24-48 hours.

Next Laboratory Session:

Examine the plates for bacterial growth and zones of inhibition. Measure the diameter of the zone of inhibition. Record results.

Questions:

1. What is meant by the term 'oligodynamic'?
2. What was the mode of action of silver nitrate on *E. coli* cells?

3. List examples of how silver is used as an antimicrobial agent.
4. What other heavy metals are used to control microbial growth in a health care setting? List the heavy metal, organism inhibited and application.

4.5 The Effects of Antiseptics and Disinfectants on Bacterial Growth

Antimicrobial substances have two kinds of activity, one is bactericidal or viricidal concerned with the killing of microorganisms; the other is bacteriostatic, or growth-inhibiting.

The terms antiseptic and disinfectant are used to describe antimicrobial activity. An **antiseptic** is generally considered to be bacteriostatic and has sufficient antimicrobial activity to interfere with the development of infection. However, it is nontoxic when applied superficially to the skin. A **disinfectant** that is bactericidal is a more potent substance that destroys nearly all pathogenic microorganisms, but can be applied only to inanimate material because of its toxicity. Both terms must be defined with respect to the microorganisms against which the substance is to be used.

Objectives:

- Differentiate between disinfectants and antiseptics
- Differentiate between antiseptics and disinfectants.
- Evaluate the effectiveness of antiseptics and disinfectants on two normal flora bacteria.
- Define the term 'DRT'. Correlate DRT's to efficacy of disinfectants.

Materials: (Per pair)

- BHI plate (2)
- Sterile filter paper discs
- Selection of antiseptics and disinfectants
- Forceps
- Spreader
- Micropipette
- Alcohol for flame sterilizing
- Beaker
- 24-48 hour culture of
    - *S. epidermidis*
    - *E. coli*

Procedure: (Please work in pairs)

1. Choose ONE antiseptic and ONE disinfectant that you and partner will test. You will test the SAME substance on both *S. epidermidis* and *E. coli* and then compare their effectiveness on the gram positive and the gram negative organism. Record the names of the antiseptic and disinfectant you have chosen.

2. Label the BHI plate with your names, organism, date, type of media. Divide it in half. Label one half ANTISEPTIC and the other half 'DISINFECTANT'.

3. Using a micropipette, Inoculate 100 µl of a 24-48 hour nutrient broth culture of *E.coli* onto the surface of a BHI plate. Alcohol flame sterilize a spreader and spread the *E. coli* over the surface of the plate evenly.

4. Sterilize forceps by dipping in ethanol and flaming and pick up a sterile filter paper disc. Place the disc gently into an antiseptic of your choice and blot off the excess. Then place the disc on the surface of the media on the side of the plate labeled 'ANTISEPTIC'.

5. Sterilize forceps again by dipping them in ethanol and flaming and then pick up another sterile filter paper disc. Dip this disc into the solution of the disinfectant. Blot off any excess solution by touching the side of the container and place the disc on the surface of the medium on the side of the plate labeled 'DISINFECTANT'.

6. Repeat the procedure (Steps 1-5) using *S. epidermidis.*

7. Place the plates at 37°C to be incubated for 24-48 hours.

Next Laboratory Session:

Observe and measure the zone of inhibition around the discs. Record your results. Determine which substances were more effective against the Gram positive organism or the Gram negative organism.

Questions:

1. Define the terms 'bacteriostatic' and 'bactericidal'.
2. DRT (Decimal reduction times) are used to select disinfectants for use at health care facility. **DRT is the time it takes to kill 90% of a test microbial population**.
3. Four disinfectants were tested against a gram positive organism. The following values were obtained. Answer the following questions:

Disinfectant	DRT Value (minutes)
Disinfectant 1	2.8
Disinfectant 2	3.1
Disinfectant 3	150
Disinfectant 4	400

   a. Which disinfectant is most effective?
   b. What is the minimum time that a piece of catheter tubing that is contaminated with 100 bacteria should be soaked in Disinfectant 1?

c. What is the minimum time the same piece of catheter tubing should be soaked in Disinfectant 4?

4.6 Effects of Chemical Agents on Microbial Growth: Antibiotics

NOTE: The antibiotic penicillin is known to produce a hypersensitive reaction in a small percentage of the population. If any student has ever had an allergic reaction to penicillin, it is advised that he/she notify the instructor for further instructions.

Chemotherapeutic drugs are chemicals used to treat disease. They include the antibiotics, a group of compounds originally produced by metabolic reactions of bacteria and fungi, which kill or inhibit the multiplication of bacteria. The two bacterial genera *Streptomyces* and *Bacillus* and the fungal genus *Penicillium* produce most antibiotics. Among them, these genera produce many different antibiotics.

Not every antimicrobial kills all bacteria. Some chemotherapeutics are broad spectrum - effective against a wide range of bacteria, both Gram-positive and Gram-negative. Others have a narrow spectrum of activity, only a few species are killed or inhibited by these agents.

In 1929, Alexander Fleming reported that a mold, which appeared as an air contaminant in laboratory bacterial cultures, produced a powerful antibacterial substance. Filtrates of a broth culture of this organism were called "penicillin". The active substance affected bacteria in different degrees, its action being very marked on the Gram-positive bacteria while the Gram-negative bacteria groups were quite insensitive to it. The air contaminant was identified as a fungus, *Penicillium chrysogenum.*

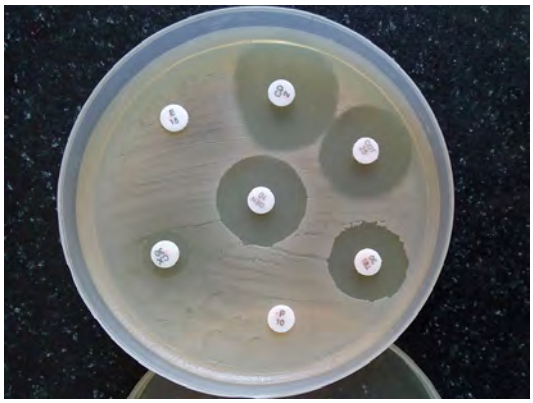


Figure 4.7 Antibiotic sensitivity testing: zones of inhibition

Penicillin is primarily only effective against Gram-positive bacteria, therefore considered to be a narrow spectrum antibiotic. This is because penicillin inhibits the formation of the cross-links in the peptidoglycan of the cell wall. Since Gram-positive bacteria have a much greater amount of peptidoglycan in their cell walls, they are affected to a greater degree.

A bacterial lawn is made on Kirby Bauer media onto which antibiotic impregnated paper disks are placed. Organisms susceptible to the antibiotic show a zone of inhibition (clearing zone) around the disc where the organism has been inhibited by the antibiotic. Discs showing growth up to the disc are not affected by the antibiotic.

Abbreviation on Disk	Antibiotic	Disk Content	Diameter of Zones of Inhibition (mm)		
			Resistance	Intermediate	Susceptible
AM	Ampicillin when testing gram negative bacteria	10 µg	≤13	14-16	≥17
	Ampicillin when testing gram positive bacteria	10 µg	≤18	19-21	≥22
C	Chloramphenicol	30 µg	≤12	13 - 17	≥18
CAZ	Ceftazidime	30 µg	≤14	15 - 17	≥18
CB	Carbenicillan	100 µg	≤19	20 - 22	≥23
CF	Cephalothin	30 µg	≤14	15 - 17	≥18
CIP	Ciprofloxacin	5 µg	≤15	16 - 20	≥21
E	Erythromycin	15 µg	≤13	14 - 22	≥23
G	Sulfisoxazole	25 µg	≤12	13 - 16	≥17
GM	Gentamicin	10 µg	≤12	13 - 14	≥15
K	Kanamycin	30 µg	≤13	14 - 17	≥18
N	Neomycin	30 µg	≤12	13 - 16	≥17
NB	Novobiocin	30 µg	≤17	18-21	≥22
P	Penicillin when testing Staphylococci	10 units	≤28	-	≥29
	Penicillin when testing other bacteria	10 units	≤14	-	≥15
R	Rifampin	5 µg	≤16	17 - 19	≥20S
S	Streptomycin	10 µg	≤11	12 - 14	≥15
TE	Tetracycline	30 µg	≤14	15 - 18	≥19
VA	Vancomycin	30 µg	≤9	10 - 11	≥12

Table 4.8 Interpreting inhibition zones of test cultures – disc diffusion tests

Adapted from *Performance Standards for Antimicrobial Susceptibility Tests,* by Clinical and Laboratory Standards Institute, 2014.

The Kirby-Bauer antibiotic disc diffusion test is a rapid, inexpensive simple test used in diagnostic laboratories to determine the effectiveness of an antibiotic on a particular strain of bacteria. The procedure is designed to evaluate one variable, the sensitivity (susceptibility) of a pathogen to assorted antibiotics, all other variables are held constant.

Variables that can alter results of an antibiotic disc diffusion test include the concentration and rate of diffusion of the antibiotic in each disc, the density of bacterial growth, thickness of medium, and temperature and length of incubation. The discs are prepared commercially by adding known amounts of the antibiotic to the disc. During incubation, the antibiotic diffuses from the filter paper into the agar, the further it gets from the filter paper, the weaker the concentration of the antibiotic.

Objectives:

- Perform antibiotic sensitivity testing on bacteria.
- Compare the effectiveness of several antibiotics on gram positive and gram negative organisms using the Kirby-Bauer sensitivity testing method.
- Define the terms 'antibiotic', antimicrobial drug, pathogen.
- Classify organisms as Resistant, Intermediate or Susceptible to antibiotics in antibiotic sensitivity tests.
- Use standard tables to interpret inhibition zones in antimicrobial susceptibility testing.

Materials: (Per Pair)

- Mueller Hinton Agar plates (2)
- Micropipette
- Spreader
- forceps
- Alcohol for flame sterilizing
- Beaker
- Antibiotic impregnated discs
- ruler
- 24-48 hour cultures of
    - *S. epidermidis*
    - *E. coli*

Procedure:

1. Each partner should prepare a lawn of 100 μL bacteria on a labeled Mueller Hinton agar plate. One partner will use *S. epidermidis*. The other partner will use *E. coli.* Plates should be labeled with your name, the name of the media, organism and date. Antibiotic discs are already labeled so you do not need to write the name of the antibiotic tested on your plate.

2. Commercially prepared antibiotic testing discs are placed evenly on the surface of plates. Six antibiotics can be tested per plate. Ensure that the discs have made firm contact with the agar by gently pressing the disc with forceps that have been sterilized in alcohol and flamed. Discs are coded with an alpha numeric code – for example: Penicillin (P10), Streptomycin (S10), and Erythromycin (E15). Choose 6 antibiotics you wish to test. Record the names, symbols and concentration of the antibiotics you choose (this is found on the disc and on the container packaging)

3. Each partner should use the same antibiotics so that a comparison between the effectiveness of the antibiotics on Gram positive and Gram negative organisms can be made.

4. Incubate plates at 37°C for 24 – 48 hours.

Next Laboratory Session:

- Observe and measure the zone of inhibition around the discs.
- Compare the zones of inhibition to the 'Performance stands for Antimicrobial Susceptibility Tests' tables available in the lab.
- Determine the effectiveness of the various antibiotics against the Gram positive organism and the Gram negative organism.

Questions:

1. For each antibiotic used, look up the mode of action of the antibiotic and its application (e.g. when/where is this antibiotic used?)
2. Which antibiotics tested were effective against the gram positive bacterium?
3. Classify each organisms as S, R, I to each antibiotic. What do the initials S, R, and I mean?
4. Which antibiotics tested were effective against the gram negative bacterium?

Sources

Figure 4.1
"Grand prismatic spring" by Jim Peaco, retrieved from https://en.wikipedia.org/wiki/File:Grand_prismatic_spring.jpg (Available under Public Domain)

"Thermophile", retrieved from https://en.wikipedia.org/wiki/Thermophile (Licensed under CC BY-SA)

Figure 4.2
"Watermelon snow streaks" by Will Beback, retrieved from https://commons.wikimedia.org/wiki/File:Watermelon_snow_streaks_3.jpg (Licensed under CC BY-SA)

"Watermelon snow pits" by Will Beback, retrieved from https://commons.wikimedia.org/wiki/File:Watermelon_snow_pits.jpg (Licensed under CC BY-SA)

Figure 4.3
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Figure 4.4
"Gas-Pak jar" by Netha Hussain, retrieved from https://commons.wikimedia.org/ wiki/File:Gas-Pak_jar.jpg (Licensed under CC BY-SA)

Figure 4.5
"Anaerobic" by Pixie, retrieved from https://commons.wikimedia.org/wiki/File: Anaerobic.png (Available under Public Domain)

Figure 4.6
"Size-controlled Silver Nanoparticles Synthesized Over the Range 5–100 nm Using the Same Protocol and Their Antibacterial Efficacy," by S. Agnihotria, S. Mukherjiabc, & S. Mukherji, November 2013, *RSC Advances*, 4, p. 3980. doi: 10.1039/C3RA44507K (Licensed under CC BY)

Figure 4.7
"D test" by Gsbhalla, retrieved from https://commons.wikimedia.org/wiki/File:D_test.jpg (Licensed under CC BY-SA 4.0)

Exercise 5
Staining Methods for the Examination of Cellular Characteristics of Microorganisms

Bacterial specimens are often stained prior to microscopy studies to allow for better visualization of specimens. A stain is a substance that adheres to a cell, giving the cell color. Different stains have different affinities for different organisms, or different parts of organisms. They may be used to differentiate different types of organisms or to view specific parts of organisms.

There are several types of stains which are commonly used in microbiology. The first is a **simple stain**, which uses only one reagent which provides contrast between the background and the heat-fixed bacterium itself. The bacterium takes up stain and becomes colored, while the background remains unstained. Simple stains are typically used on bacterial smears which have been heat-fixed and thus contain non-living microbes. Methylene is often used to prepare simple stains.

A second type of stain is a **negative stain**, which uses a single reagent to provide contrast between the background and the living bacterium. Thus, the background is "stained", while bacterium does not take up any stain. Negative stains are typically used when observing live bacteria is desired. The capsule stain is an example of a negative stain.

A **differential stain** is a type of staining that allows you to distinguish between types of bacteria or between specific structures in a bacterium. A differential stain typically uses two or more reagents – a primary stain and a counter stain. Gram stains and acid fast stains are examples of differential stain.

Chemically, there are two main types of stains: **basic stains**, which have a positive charge (cationic) and **acidic stains**, which have a negative charge (anionic). Basic stains have an affinity for negative components of cells, and include dyes such as methylene blue, crystal violet, and carbol fuchsin. Acidic stains have an affinity for positive components of cells, and include dyes such as nigrosin, India ink, and picric acid. Since cell walls are negatively charged, a positive dye will be attracted to and stain the cell wall, whereas a negative dye will be repulsed by the cell wall and not directly stain the cell.

Objectives:

- To understand the purpose of staining bacterial cells.
- To be able to observe a microorganism microscopically and determine the cellular morphology including drawing a diagram of the microorganism to scale.
- To explain the mechanism and application of the Gram stain technique
- To perform a Gram stain on bacteria.
- To perform an endospore stain on a bacterial organism.
- To explain the mechanism and application of endospore stains.
- To examine negative stains and identify bacterial capsules.
- To identify acid fast organisms in an acid fast stain.
- To classify bacteria according to their flagella by examining flagella stains of bacteria.

Materials:

- gram stain reagents - crystal violet, Gram's iodine, decolorizer (alcohol), safranin
- endospore stain reagents - malachite green, safranin
- sporulation agar streak plates of *Clostridium pasteurianum* grown for 48 hours.
- 24-48 hour cultures of *S. epidermidis* and *E. coli*

Demonstration Bench

- endospore-stained slide of *Clostridium*
- flagella-stained slide of *Pseudomonas* and *Proteus*
- capsule stains (negative stains) of various bacteria

Generally, bacterial cells are fixed to a microscope slide and stained for microscopic observation. There are many different staining procedures available to stain bacteria including simple stains that will show the shape and size of the bacterial cell; differential stains, the most common being the Gram stain which will differentiate Gram positive and Gram negative bacteria; and special stains which are useful for staining such specialized structures as the endospore and capsule.

Procedure:

Using the plate provided on your bench, prepare three Gram stains. Make a gram stain of *Staphylococcus epidermidis*, *Escherichia coli*, then a mixture of both species.

5.1 The Preparation of Smears for Staining

To make a good film for staining, obtain a clean grease-free slide. Place a drop of sterile water from the container onto the center of a glass slide. Do not allow the tip of the medicine dropper to touch the glass slide or you will contaminate it and the water in the container. With a sterile loop remove a **small** quantity of surface growth from the agar plate, mix this with the water to make a smooth suspension of the cells, and allow to **air-dry**. The loop must be flamed again before replacing it in the holder. When dry, **the film should be only faintly visible; a thick opaque film where the bacteria are piled on top of one another is useless**. Because of their small size, bacteria dry without great distortion and so the only fixation required is to pass the slide approximately 3 times through the bunsen flame after the smear is AIR DRIED. If the slide is too hot to touch, then the bacteria will probably have been cooked and will consequently be misshapen when observed under the

microscope. If the preparation is not heat fixed adequately, the bacterial cells will wash off during the staining process.

Question:

1. What are two reasons for heat fixing bacterial/fungal smears prior to staining?

5.2 Gram Stain

In 1884, Christian Gram developed a staining technique that separated bacteria into two groups: gram-positives and gram-negatives. This makes the Gram stain very useful for classification and identification of microorganisms. The procedure is based on the ability of gram-positive bacteria to retain the purple color of crystal violet during decolorization with alcohol and therefore remain purple. Gram-negative bacteria are decolorized by the alcohol and become colorless. When the safranin (a red dye) is added at the last step in the Gram staining procedure, the gram-negative bacteria become red (or pink).

It is now thought that the difference between gram-positive and gram-negative cells occurs because of the structure of the cell walls. Thus gram-positive cells have cell walls which prevent the 'leaching out' of the stain with alcohol while gram-negative cells do not. Variation in the structure of cell walls would explain the intermediate forms that occur and also the change in reaction that may occur with the aging of the cell. It is important, especially for gram-positive cells, that the Gram stain be done on cultures that are a maximum 48 hours old. As the culture ages, many gram-positive cells lose their ability to retain the primary dye. Therefore, they may appear to be gram-negative even though they really are not. Gram-positivity is a property of bacteria that correlates with a number of other properties besides the structure of the cell wall, for example susceptibility to certain antibiotics.

Watch the videos on Gram Staining before attempting this technique.

Scan this QR code or use the link below to view the video *Gram Stain - Part 1* (video 2:18 minutes)

https://youtu.be/H9ex4T69-Qo

Scan this QR code or use the link below to view the video *Gram Stain - Part 2* (video 2:26 minutes)

https://youtu.be/StJRIEE6yXY

Practical Procedure:

1. Make a smear of *S. epidermidis*, one of E. coli and a mixture of both organisms. Allow to air dry. Heat fix. Place the prepared and heat-fixed smears on the staining rack. Wear gloves if necessary.

2. Flood the smear with crystal violet solution for 1 min. Wash with tap water for 2-3 seconds and remove the excess water by tapping the slide gently on the staining rack.

3. Add Gram's iodine solution to the slide for 1 minute. Wash with tap water and remove as before.

4. Decolorize with 95% ethanol. Since the time for decolorization varies with the thickness of the bacterial smear and the type of bacteria being stained, the alcohol is dripped down the slide for approximately 10 sec or less. If too much alcohol is added the gram-positive organisms may become gram-negative.

5. Rinse with water.

6. Counterstain the slides with safranin for 1 minute. Rinse with water.

7. Gently blot the slide dry with paper towels, and examine under the oil-immersion lens.

Step	Reagent	Purpose	Time (sec)	Color of gram + cells	Color of gram – cells
1	Crystal violet	Primary stain	60	Purple	Purple
2	Iodine	Mordant	60	Purple/black	Purple/black
3	Ethanol	Decolorizer	<10	Purple	Colorless
4	Safranin	Counterstain	60	Purple	Red

Gram-positive organisms stain PURPLE | Gram-negative organisms stain RED

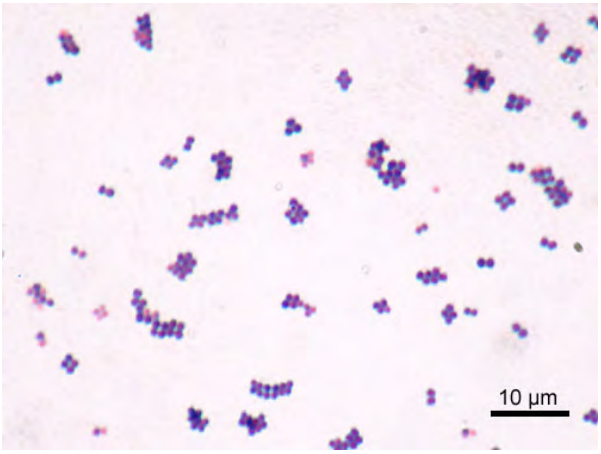

Figure 5.1 **Gram stain of *S. saprophyticus* cells which typically occur in clusters. The cell wall readily absorbs the crystal violet stain.** *Staphylococcus aureus* is a gram-positive coccal bacterium that is a member of the Firmicutes, and is frequently found in the nose, respiratory tract, and on the skin. Although *S. aureus* is not always pathogenic, it is a common cause of skin infections such as abscesses, respiratory infections such as sinusitis, and food poisoning. Pathogenic strains often promote infections by producing potent protein toxins, and expressing cell-surface proteins that bind and inactivate antibodies. The emergence of antibiotic-resistant strains of *S. aureus* such as methicillin-resistant *S. aureus* (MRSA) is a worldwide problem in clinical medicine.

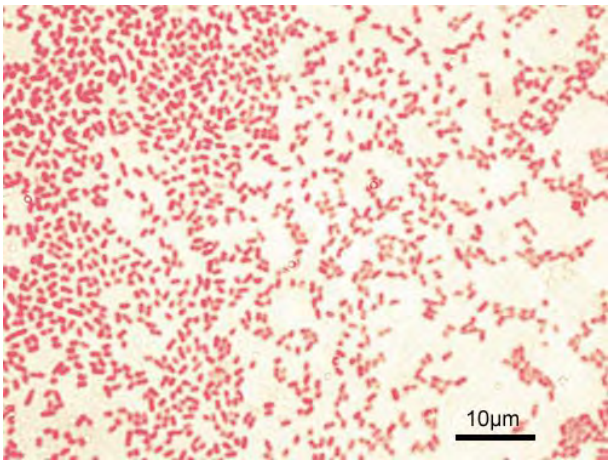

Figure 5.2 **Gram-stained *P. aeruginosa* bacteria (pink-red rods).** *Pseudomonas aeruginosa* is a common gram-negative, rod-shaped bacterium that can cause disease in plants and animals, including humans. A species of considerable medical importance, *P. aeruginosa* is a prototypical "multidrug resistant (MDR) pathogen" recognized for its ubiquity, its intrinsically advanced antibiotic resistance mechanisms, and its association with serious illnesses – especially nosocomial infections such as ventilator-associated pneumonia and various sepsis syndromes.

Cellular morphology:

By observing the cells microscopically, the **cellular morphology** of a microorganism can be obtained. Descriptions of cellular morphology include:

- **Shape** of one individual cell: bacillus, coccus, spirillum
- **Arrangement** of the cells: chains, clusters, pairs, random, singles, or a combination thereof
- **Gram reaction**: gram-positive or gram-negative

Usually a diagram drawn to scale accompanies the cellular morphology.

Record your observations on the data table that follows:

Table 3.1 Cellular Morphology Bacteria		
Cellular Morphology	***Staphylococcus epidermidis***	***Escherichia coli***
Shape		
Arrangement of cells		
Color of cells & Gram reaction		
Diagram to scale of *Staphylococcus epidermidis*. (Total Magnification – 100X)		Diagram to scale of *Escherichia coli*. (Total Magnification – 100X)

5.3 Endospore Production in Bacteria

Endospores are bacterial spores formed within the vegetative cell. The endospore is a part of the life cycle of the spore-forming bacteria. They are formed because of changes in the nutritional environment of the bacteria. Endospores are mainly found in Gram-positive organisms, including the Gram-positive bacilli - *Clostridium* and *Bacillus.*

Endospores are highly resistant to heat, chemical disinfectants or to desiccation and, therefore, allow the bacterial endospore to survive much more rigorous conditions than the vegetative cells. Endospore resistance is due to several factors which include:

1. decrease in the amount of water compared to vegetative cells.
2. increase in the amount of dipicolinic acid and calcium ions.
3. enzymes that are more resistant to heat.
4. spore coat that is impermeable to many substances

Endospores may be formed in a central, terminal, or sub-terminal position in the cell and their shape varies from ellipsoidal to spherical. The portion of the vegetative cell which forms the endospore, the sporangium, may or may not become swollen as the endospore matures. The location of the endospore in the cell is usually characteristic of the species. For example, the location and shape of the *Bacillus cereus* endospore is different from the location and shape of the *Clostridium* endospore. Therefore, the presence or absence of endospores and the description of the endospore is useful to a microbiologist as an aid in identification.

Endospore Stain

Only true endospores will stain with this procedure. Endospores resist more dyes, therefore, heating is necessary to stain the endospore but once stained it does not decolorize.

Make a film from the culture of *Clostridium pasteurianum* provided and stain following Conklin's endospore staining procedure. The endospore is able to take up the malachite green when heated and because of its special outer layers, the dye is not released when the film is washed. The vegetative cells decolorize when washed with water and restain pink/red after the addition of safranin.

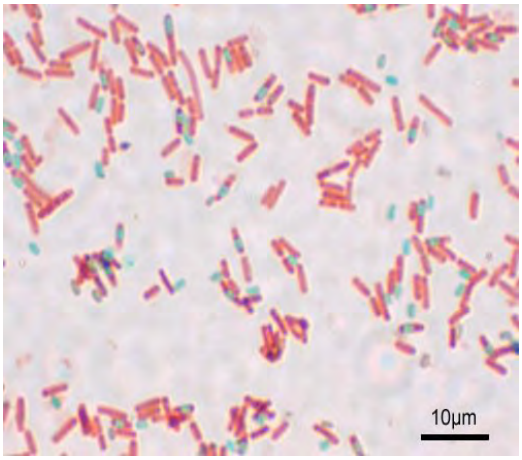

Figure 5.3 A stained preparation of *Bacillus subtilis* showing endospores as green and the vegetative cell as red.

Practical Procedure:

1. Prepare a smear of *Clostridium pasteurianum* and a mixed smear of *Cl. pasteurianum* and *E. coli* and place the slides on a staining rack to dry. Heat fix the slides.

2. Add enough malachite green to saturate the film and leave for 15-20 minutes (do not let the film dry out). Add more malachite green as required. Rinse thoroughly with tap water to remove stain. Remove excess water and replace slide on the staining rack in the sink.

3. Counterstain with safranin for 60 seconds.

4. Wash and blot dry and examine with the oil immersion lens.

   Usually, the endospores that are still the in the vegetative cells will not absorb the malachite green stain. In this case, the endospore will appear as a clear area in the pink cell. Endospores will stain green, vegetative cells will stain pink/red.

5. Make observations by drawing a diagram to scale of both slides. Label appropriately. Note colors of structures on your drawing.

   Note whether the organism has more than one spore/cell, note the location of the endospore(s). These characteristics can be used to help identify organisms.

Clostridium pasteurianum	*C. pasteurianum & E. coli* (mixed smear)
Total magnification: 100X	Total magnification: 100X

5.4 Capsule Stain

Capsules are a polysaccharide layer formed outside the cell wall by some bacteria. The amount or type of capsule formed depends upon the cultural conditions with respect to nutrients available (a high sucrose concentration often produces a larger capsule), temperature, pH and the growth stage of the organism.

Capsules are protective inasmuch as they decrease the rate of desiccation and may prevent phagocytosis by other microorganisms and by phagocytic leukocytes. They have a connection with the virulence of the organism. A bacterium that is non-encapsulated is non-pathogenic since the non-encapsulated organism can be readily digested by phagocytes whereas an encapsulated bacterium is pathogenic. The capsule may also be the antigen in an antigen-antibody reaction. Capsules may be involved in the storage of reserve materials or may accumulate wastes excreted from the cell. The capsule also enables the bacterial cells to stick to each other or to inert surfaces if the organism produces fimbriae.

Observe the prepared slide of bacterial capsules. Since capsules are destroyed by heat, they cannot be stained by regular methods where heat-fixing to the slide is required. Therefore, a negative stain is used to detect the presence of capsules. The negative stain increases contrast by increasing the opacity of the background. The carbol fuchsin particles are too large to enter the cell which results in the capsules appearing as a clear zone surrounding the cell wall, and the background staining grey/black.

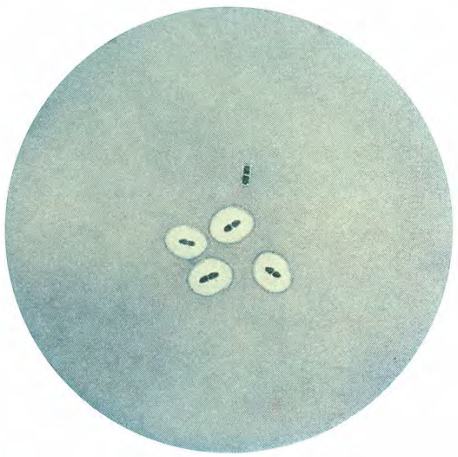

Figure 5.4 Photomicrograph of *Streptococcus pneumoniae* bacteria revealing capsular swelling using the Neufeld-Quellung test. This organism causes respiratory infections such as pneumonia and sinusitis, as well as bacteremia, otitis media, meningitis, peritonitis and arthritis. The Neufeld-Quellung test is used in pneumococcus typing.

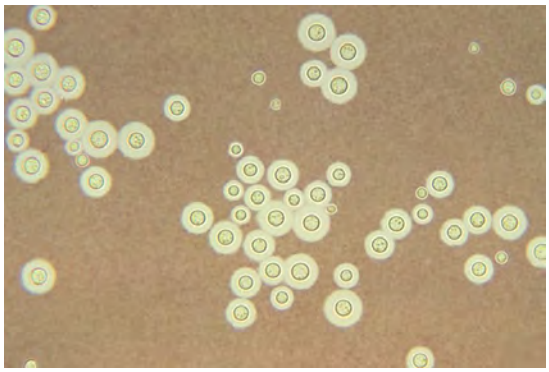

Figure 5.5 This photomicrograph depicts *Cryptococcus neoformans* using a light India ink staining preparation. Life-threatening infections caused by the encapsulated fungal pathogen *Cryptococcus neoformans* have been increasing steadily over the past 10 years because of the onset of AIDS, and the expanded use of immunosuppressive drugs.

Procedure:

1. Remove a small loop of culture from the *Klebsiella pneumoniae* streak plate.

2. Place the loop with the bacteria in a drop of carbol fuchsin in a watch glass. Stain for 30 seconds.

3. Remove the loop and carefully remove excess stain by rinsing the loop in a vial that is half filled with water.

4. Add the bacteria to a small drop of carbol fuchsin on a slide and mix until all the bacteria have been transferred to the slide. Flame the loop.

5. Using the end of a second slide draw the carbol fuchsin to the opposite end of the slide. The carbol fuchsin should form a thin translucent layer on the slide.

6. Allow to air dry. Observe with the oil immersion objective.

Cells are pink/grey, capsules are clear and the background is grey/black. Differentiate between bacterial cells and artifacts on the slide. When the edge of the capsule is in focus, the cell should be visible.

Record on the data sheet by drawing a diagram to scale of the *Klebsiella* capsule and cell. Label appropriately.

Klebsiella pneumonia
Total magnification: _____X

5.5 Flagella Stain

Flagella are below the visual limit in size for light microscope observation, and so the best method of study is to use the electron microscope. For practical purposes, it is possible to increase the diameter of the flagella and therefore make them visible in the light microscope by adding a component to the stain that will coat and bind to the cell. This component is called a **mordant**. Further difficulties are encountered when staining flagella since flagella are very delicate and easily lost or obscured. To minimize these difficulties great care must be taken at all stages of the staining process.

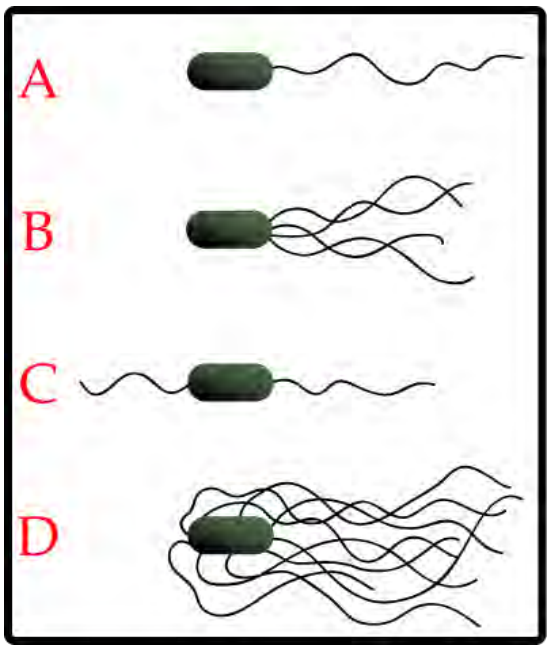

Figure 5.6 Examples of bacterial flagella arrangement schemes: (A) Monotrichous, (B) Lophotrichous, (C) Amphitrichous, (D) Peritrichous

Procedure:

The stain used is Leifson's flagella stain, containing basic fuchsin which gives the stain the red color and a mordant, tannic acid.

Observe the two prepared slides on the demonstration bench. The cells have been stained with Leifson's flagella stain.

On the data sheet, draw diagrams to scale of *Pseudomonas* and *Proteus.*

Proteus sp.	*Pseudomonas* sp
Total magnification: 100 X	Total magnification: 100 X

Sources

Staining Methods section
"Microbiology Lab Manual: Biol2421L" by Donna Cain, Hershell Hanks, Donna Cain, Mary Weis, Carroll Bottoms, & Jonathan Lawson, retrieved from https://microcosmorflores.wikispaces.com/file/history/Microbiology+Lab+Manual+--+Revised+Spring+2013.pdf (Licensed under CC BY-SA)

Figure 5.1
"Staphylococcus aureus Gram" by Y. Tambe, retrieved from https://commons.wikimedia.org/wiki/File:Staphylococcus_aureus_Gram.jpg (Licensed under CC BY-SA)

"Staphylococcus aureus", retrieved from https://en.wikipedia.org/wiki/ Staphylococcus_aureus (Licensed under CC BY-SA)

Figure 5.2
"Pseudomonas aeruginosa Gram" by Y. Tambe, retrieved https://commons.wikimedia.org/wiki/File:Pseudomonas_aeruginosa_Gram.jpg (Licensed under CC BY-SA)

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Figure 5.3
"Bacillus subtilis Spore" by Y. Tambe, retrieved from https://commons.wikimedia.org/wiki/File:Bacillus_subtilis_Spore.jpg (Licensed under CC BY-SA)

Figure 5.4
"Pneumococcus CDC PHIL 2113" by Centers for Disease Control and Prevention, retrieved from https://commons.wikimedia.org/wiki/File:Pneumococcus_CDC_ PHIL_2113.jpg (Available under Public Domain)

Figure 5.5
"Cryptococcus Neoformans using a Light India Ink Staining Preparation PHIL 3771 Lores" by Centers for Disease Control and Prevention, retrieved from https://commons.wikimedia.org/wiki/File:Cryptococcus_neoformans_using_a_light_India_ink_staining_preparation_PHIL_3771_lores.jpg (Available under Public Domain)

Figure 5.6
"Flagella" by Mike Jones, retrieved from https://en.wikipedia.org/wiki/File:Flagella.png (Licensed under CC BY-SA)

Exercise 6
Differential and Selective Media

Microbiologists often use multiple types of media to cultivate microorganisms in order to either specifically grow a particular organism, or to obtain information about the biochemical properties of the organisms that grow. Three commonly used types of media are selective media, differential media, and enriched media.

Selective media is designed to suppress the growth of some microorganisms while allowing the growth of others (i.e., it *selects* for certain microbes). Solid media is usually employed with selective media so that individual colonies may be isolated. Examples of selective media include mannitol salt agar, MacConkey agar, and eosin-methylene blue agar.

Differential media allow the growth of more than one microorganism of interest but with morphologically distinguishable colonies based on specific biochemical properties of the organisms. Most differential media contain a substrate and a chemical indicator, often a pH indicator. As with selective media, solid media is usually employed so individual colonies may be observed. Examples of differential media include mannitol salt agar, DNAse agar, blood agar, MacConkey agar and eosin-methylene blue agar.

Enriched media contains specific growth factors needed by fastidious bacteria to support their growth. Examples of enriched media include blood agar and chocolate agar.
Described below are the specific properties of some of the more commonly used selective, differential, and enriched media. Note that a particular type of media can have multiple properties – e.g. it can be both selective and differential!

Examples of Differential and Selective Media:

Blood Agar:
Blood agar is a differential medium. It is also commonly used as an enriched medium for growing fastidious bacteria. Some bacteria produce exotoxins called hemolysins that cause lysis of red blood cells. The degree of the hemolysis is an especially useful tool for differentiation among Gram-positive cocci. Usually sheep's blood is used in the medium. The three types of hemolysis are:

- **Alpha (α) hemolysis** refers to the partial lysis of red blood cells and hemoglobin. This results in a greenish-grey discoloration of the blood around the colonies.

- **Beta (β) hemolysis** - the complete lysis of red blood cells and hemoglobin. This results in complete clearing of the blood around colonies.

- **Gamma (γ) hemolysis** is indicated by no hemolysis and results in no change in the medium.

Figure 6.1 Hemolyses of *Streptococcus*: (left) Alpha-hemolysis (middle) Beta-hemolysis (right) Gamma-hemolysis. Hemolysis (from Greek "αιμόλυση" which means blood breakdown) is the breakdown of red blood cells. The ability of bacterial colonies to induce hemolysis when grown on blood agar is used to classify certain microorganisms. This is particularly useful in classifying streptococcal species. A substance that causes hemolysis is a hemolysin.

Alpha

When alpha hemolysis (α-hemolysis) is present, the agar under the colony is dark and greenish. Streptococcus pneumoniae and a group of oral streptococci (Streptococcus viridans or viridans streptococci) display alpha hemolysis. This is sometimes called green hemolysis because of the color change in the agar.

Beta

Beta hemolysis (β-hemolysis), sometimes called complete hemolysis, is a complete lysis of red cells in the media around and under the colonies: the area appears lightened (yellow) and transparent. Streptolysin, an exotoxin, is the enzyme produced by the bacteria which causes the complete lysis of red blood cells.

Gamma

If an organism does not induce hemolysis, the agar under and around the colony is unchanged, and the organism is called non-hemolytic or said to display gamma hemolysis (γ-hemolysis

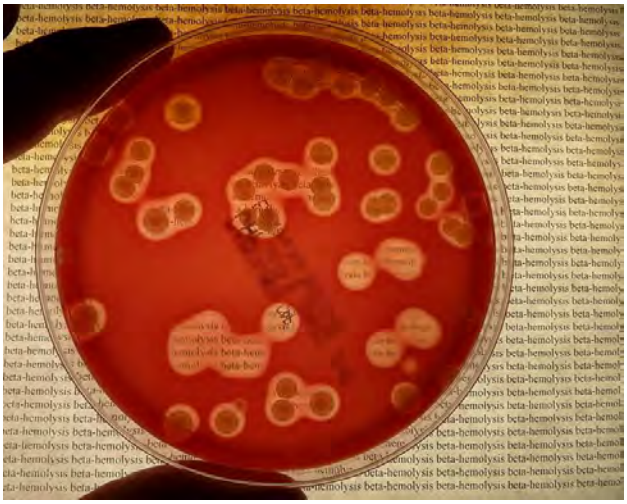

Figure 6.2 Beta hemolysis on blood agar, cultivation 48 hours, 37°C. Staphylococcus aureus and Streptococcus agalactiae (smaller colonies, incomplete beta hemolysis).

Mannitol Salt Agar:
Mannitol salt agar (MSA) is both selective and differential. MSA contains 7.5% NaCl, which selects for halo tolerant organisms such as members of the *Staphylococcus* genus. The media also contains the carbohydrate substrate mannitol, and a pH indicator, phenol red. Organisms which are able to ferment the mannitol will produce acid fermentation products which lower the pH, causing the phenol red indicator to turn yellow.

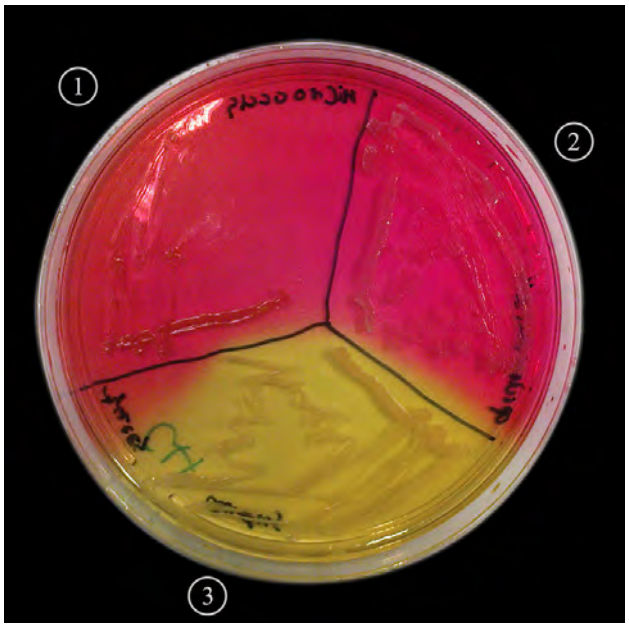

Figure 6.3 An MSA plate with (1) *Micrococcus* sp. (2) *Staphylococcus epidermidis* (3) and S. *aureus* colonies

MSA encourages the growth of a group of certain bacteria while inhibiting the growth of others. This medium is important in medical laboratories by distinguishing pathogenic microbes in a short period of time. It contains a high concentration (~7.5%-10%) of salt (NaCl), making it selective for Gram positive bacterium *Staphylococci* (and *Micrococcaceae*) since this level of NaCl is inhibitory to most other bacteria. It is also a differential medium for mannitol-fermenting staphylococci, containing carbohydrate mannitol and the indicator phenol red, a pH indicator for detecting acid produced by mannitol-fermenting Staphylococci. *Staphylococcus aureus* produce yellow colonies with yellow zones, whereas other *Staphylococci* produce small pink or red colonies with no colour change to the medium. If an organism can ferment mannitol, an acidic byproduct is formed that will cause the phenol red in the agar to turn yellow. It is used for the selective isolation of presumptive pathogen *Staphylococci*.

Gram + *staphylococcus*: fermenting mannitol: Media turns yellow (eg. S. aureus)
Gram + *staphylococci*: not fermenting mannitol. Media does not change color (eg. S. epidermidis)
Gram + *streptococc*i: inhibited growth
Gram - :inhibited growth

MacConkey Agar:
MacConkey (Mac) agar is both selective and differential. This media contains crystal violet and bile salts, which inhibit most gram-positive organisms and select for gram-negative organisms. It also contains the substrate lactose and the pH indicator neutral red, which allow differentiation among gram-negative bacteria based on their ability to ferment lactose. When lactose is fermented by coliforms such as *Escherichia coli*, acid end-products lower the pH of the media below 6.8, with the resulting colonial growth turning pinkish-red. If an organism is unable to ferment lactose, the colonies will be colorless, taking on the color of the medium.

Figure 6.4 MacConkey's agar showing both lactose and non-lactose fermenting colonies. Lactose fermenting colonies are pink whereas non-lactose fermenting ones are colorless or appear same as the medium.

Eosin Methylene Blue Agar:
Eosin Methylene Blue (EMB) agar is both selective and differential. This media contains the dyes eosin and methylene blue, which inhibit gram-positive growth and select for gram-negative organisms. It also contains lactose, allowing differentiation between organisms which ferment lactose and produce acid end-products, and organisms that do not ferment lactose. Small amounts of acid production result in a pink colored growth (e.g. *Enterobacter aerogenes*) while large amounts of acid cause the acid to precipitate on the colony, resulting in a characteristic greenish, metallic sheen (e.g. *E. coli*). Organisms which do not ferment lactose will be colorless, taking on the color of the medium.

Objectives:

- Differentiate between differential and selective media.
- Use differential and selective media to isolate and identify microorganisms from skin, genitourinary tract, gastrointestinal tract, mouth and respiratory tract of humans.
- Differentiate between pathogens and normal microbiota.

Materials:

- Blood agar plates (1/bench +1/student)
- Mannitol salt agar plates
- Eosin Methylene Blue agar plates
- MacConkey agar plates
- 24-hour broth cultures of *E. coli, S. salivarius, S. equisimilis, S. species (beta hemolytic)*

NOTE: DO NOT handle any person's biological specimens other than your own. Why not?

A. Blood Agar & Organisms of the Upper Respiratory System

Blood Agar Plates (BAP) are often used to aid in the identification of organisms in throat cultures. The throat (upper respiratory system) is a warm moist environment that contains many normal flora microorganisms such as *Staphylococcus, Streptococcus, Neisseria* and *Haemophilus* species of bacteria. The normal microbiota of the throat helps to minimize the rate of infection of the upper respiratory tract through a process of microbial antagonism. Potential pathogens compete for nutrients with normal flora microbes and many normal flora microbes produce inhibitory substances that control pathogens. Never the less, the upper respiratory tract can be contaminated by many pathogens. (Note – the lower respiratory tract is normally sterile. Why?). Blood agar is used to isolate and identify pathogens on the basis of their hemolytic reactions.

Materials:

- Per bench (1BAP + 1BAP/student)
- Inoculating loop

Procedure:

1. **Per bench:** Label plates. Divide one BAP into 4 sections. Inoculate each quadrant using your inoculating loop with a thin inoculum of each of the organisms – *E. coli, S. salivarius, S. equisimilis,* and *S. sps. (beta).*

2. **Per student**: Label 1 BAP. Swab your throat with a sterile cotton swab. Swab between the 'golden arches (glossopalatine arches). Do not hit your tongue with the swab. Swab the BAP. Discard of the swab in the autoclave bag.

3. Incubate plates at 37°C for 24-48 hours.

4. Observe plates for hemolysis. Compare your throat culture to the group plate. What type of hemolytic organisms did you observe?

B. MacConkey Agar & Organisms of the Genitourinary Tract

The genitourinary system comprises the urinary and genital systems. The upper urinary tact and urinary bladder are generally sterile. The urethra contains some normal flora organisms including species of *Streptococcus, Bacteroides, Neisseria, Mycobacterium* and enterics. Many infections of the urinary tract such as cystitis and pyelonephritis are caused by opportunistic pathogens are related to fecal contamination of the urethra (especially in women) and to medical procedures such as catheterization. The presence of bacteria in urine is generally not considered indicative of a urinary tract infection unless there are greater than 100 coliforms/mL or urine or if there are more than 10^5 colony forming units (CFU)/mL.

Materials:

- MacConkey agar plate (MAC) – 1/bench and 1/student
- Blood agar plate -1/bench and 1/student
- EMB agar plate (EMB) – 1/bench and 1/student
- Urine collection specimen cup (1/student)
- Disposable sterile loops
- Loops
- 24-hour broth cultures of *E. coli, S. epidermidis, A. faecalis, Ps. putida*

Procedure:

1. **Per bench** – obtain one Mac, 1 BAP, and 1 EMB. Label plates. Divide each plate into quadrants. Inoculate each quadrant with a thin inoculum of each of the above organisms.

2. **Per student** – obtain a 'clean-catch' (mid-stream) urine sample as instructed by your instructor using the sterile specimen collection container. Obtain 1 BAP, 1 Mac and 1 EMB plate. Label each plate. Using the disposable plastic loop inoculate each plate with your urine sample as instructed by your instructor. Dispose of the contaminated plastic loops in the autoclave bag. Use a new disposable loop for each new plate. Dispose of your left over urine sample as instructed.

3. Incubate all plates at 37°C for 24-48 hours.

4. Make observations. Observe plates for lactose fermenting organisms. Compare your urine sample to the bench plate of known gram positive and gram negative organisms your bench inoculated. Perform an oxidase test on colonies. What do the results indicate?

C. Mannitol Salt Agar and Organisms of the Skin

Human skin is a protective barrier for the human body. It inhibits many organisms because it is protected by dry layers of keratin containing epidermal cells that most organisms can't colonize. In addition, sebum secreted by oil glands inhibits some organisms. Salt and chemicals in perspiration create a hypertonic and inhibitory environment for other organisms. There are some normal microbiota that can use perspiration and sebum as nutrients and the skin has a normal microbiota that provides a protective biofilm against invasive pathogens.

Organisms like *Propionibacterium, Staphylococcus, Methylophilus* species and other organisms are found on the skin and in hair follicles. Most organisms on the skin are gram positive and salt tolerant. Mannitol Salt agar is an agar that selects for salt tolerant organisms. It differentiates between them on the basis of their ability to ferment mannitol.

Materials:

- Mannitol Salt agar plate (1/bench and 1/student)
- Sterile cotton swabs
- Sterile saline
- 24 hour broth cultures of *S. epidermidis, E. coli, M. luteus, A. faecalis*

Procedure:

1. **Per bench**: obtain one MSA plate. Label it. Divide it into quadrants. Using an inoculating loop, inoculate each quadrant with a thin Inoculum of each of the organisms above.
2. **Per student**: Obtain 1 MSA plate. Label it. Using the sterile swab dipped in saline, inoculate the plate as instructed. Dispose of the swab in the autoclave bag.
3. Incubate plates at 37°C for 24-48 hours.
4. Make observations of the bench plate. Compare your skin plate to it. Do you notice any mannitol fermenting organisms? What do you think they are?
5. Do a catalase test on any mannitol fermenting organisms? Make observations.

Questions:

1. What property or properties of gram-negative organisms would likely make them more resistant to crystal violet and bile salts than gram-positive organisms?
2. An unknown bacterium produces colorless colonies when inoculated onto an EMB plate. Predict what you would see if you inoculated that same unknown onto the following media, MacConkey agar and why.
3. What type of agar would you use to differentiate between Salmonella species? Why? What disease(s) do Salmonella cause?
4. If you found a mannitol fermenting organism from a skin sample, why might you perform a coagulase test on it in addition to a catalase test?

Sources

Differential and Selective Media section
"Microbiology Lab Manual: Biol2421L" by Donna Cain, Hershell Hanks, Donna Cain, Mary Weis, Carroll Bottoms, & Jonathan Lawson, retrieved from https://microcosmorflores.wikispaces.com/file/history/Microbiology+Lab+Manual+--+Revised+Spring+2013.pdf (Licensed under CC BY-SA)

Figure 6.1
"Streptococcal hemolysis" by Y. Tambe, retrieved from https://commons.wikimedia.org/wiki/File:Streptococcal_hemolysis.jpg (Licensed under CC BY-SA)

"Hemolysis (microbiology)", retrieved from https://en.wikipedia.org/wiki/Hemolysis_(microbiology) (Licensed under CC BY-SA)

Figure 6.2
"Beta Hemolysis on Blood Agar" by Hans N, retrieved from https://commons.wikimedia.org/wiki/File:Beta_hemolysis_on_blood_agar.jpg (licensed under CC BY-SA)

Figure 6.3
"Chapmanes" by Navaho, retrieved from https://commons.wikimedia.org/ wiki/File:Chapmanes.jpg (Licensed under CC BY-SA)

"Mannitol salt agar", retrieved from https://en.wikipedia.org/wiki/Mannitol_salt_agar (Licensed under CC BY-SA)

Figure 6.4
"MacConkey Agar with LF and LF Colonies" by Medimicro, retrieved from https://commons.wikimedia.org/wiki/File:MacConkey_agar_with_LF_and_LF_colonies.jpg#file (Available under Public Domain)

Exercise 7
Biofilms & Bacteria of the Mouth

Biofilms are communities of microorganisms that are attached to a solid surface in the presence of moisture. They are characterized by the production of an extracellular polysaccharide substance (EPS). Microbes in a biofilm play a key role in disease. *Pseudomonas aeurginosa* produces permanent biofilms in lungs of cystic fibrosis patients. Many microorganisms produce biofilms in catheters and implanted prostheses. The microorganisms in biofilms are of concern because they are more resistant to antibiotics and difficult for the immune system to combat. The organisms in biofilms can communicate via a process called quorum sensing. This allows them to interact and change their gene expression, making them more of a problem in the human body and the environment. Biofilms are also helpful, for example they are used in land reclamation and sewage treatment plants to remove some organisms from the environment and from sewage.

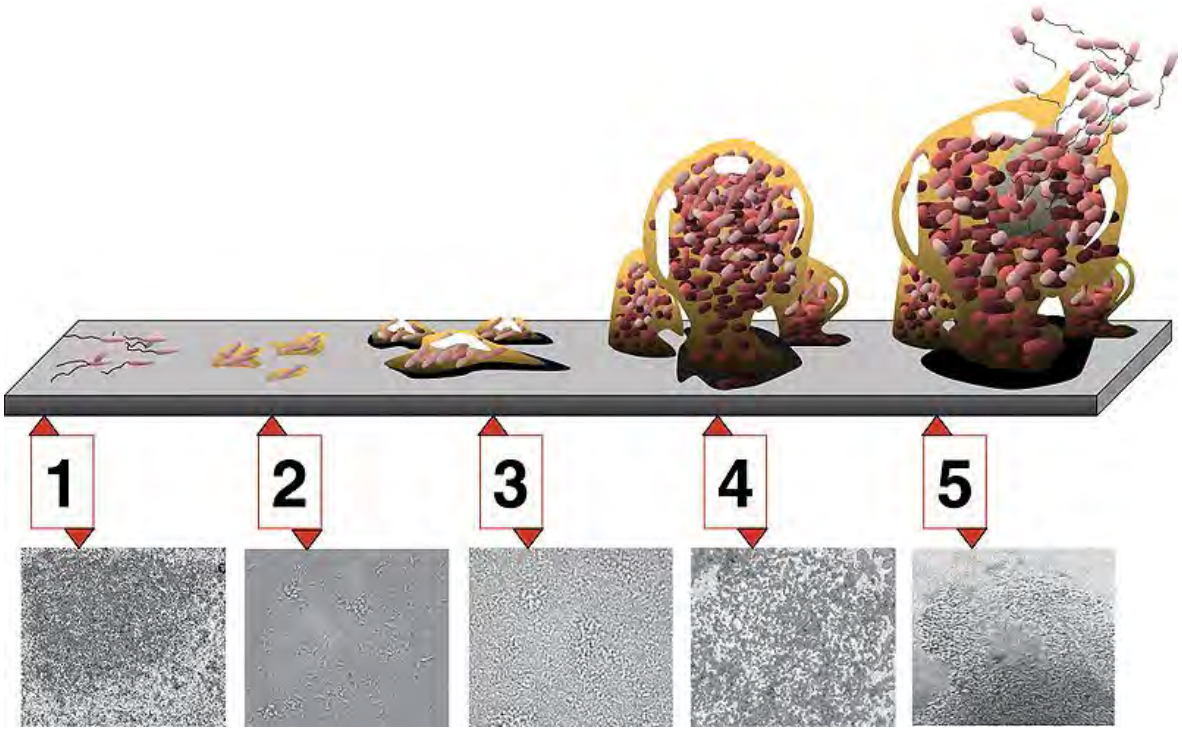

Figure 7.1 Five stages of biofilm development: (1) Initial attachment, (2) Irreversible attachment, (3) Maturation I, (4) Maturation II, and (5) Dispersion. Each stage of development in the diagram is paired with a photomicrograph of a developing *P. aeruginosa* biofilm. All photomicrographs are shown to the same scale.

The mouth contains millions of bacteria in each mL of saliva. Some of these microorganisms are transient and carried on food and beverages. Some are part of the normal flora of the mouth and teeth. Microorganisms in the mouth and on teeth form protective biofilms. They can also form biofilms that cause dental caries. Differential and selective media can be used to isolate and identify organisms in mouth and on teeth. Many organisms in the mouth are gram positive streptococci and lactobacilli.

That "fuzzy" feeling that can be felt on unbrushed teeth is an accumulation of a biofilm of bacteria. There are several bacteria within a person's mouth which make up this biofilm and are **cariogenic**, meaning they have a tendency to cause **dental caries**. These include *Lactobacillus acidophilus*, *Streptococcus mutans* (and *S. gordonii, S. salivarius*), and *Actinomyces odontolyticus*. These organisms are able to ferment carbohydrates to lactic acid, thus lowering the pH at the surface of the enamel which can **decalcify** the enamel and lead to cavities. Enamel decalcification will begin with a pH of 5.5 and accelerates as the pH reaches 4.4. For example, *S. mutans* produces the enzyme **dextransucrase**, which polymerizes sucrose to form a polysaccharide called **glucan**. *L. acidophilus* produces a similar polymer called **lexan**. Glucan and lexan are both **glycocalyces** which bind tightly to teeth, allowing the bacteria to form a biofilm. The bacteria then produce acids as a product of carbohydrate fermentation.

Objectives:

- To examine biofilms in the human mouth and to discuss how microorganisms interact in a biofilm.
- To use differential and selective media to examine and identify organisms in oral biofilms.

Materials: (Per student)

- Sterile saline
- Glass slides
- Sterile toothpicks
- EMB, MSA, BHI, EMB plate – 1/student
- Sterile swabs
- Mouthwash
- Dixie cups
- Gram stain reagents

Procedure (per student):

1. Place a drop of sterile saline on the center of a clean glass slide.

2. Collect biofilm from your mouth by using the sterile toothpick provided. Hold a toothpick by the pointed end and gently scrape a tooth in your mouth gently several times.

3. Rinse the plaque on the toothpick into the saline on the slide and mix it around. WORK WITH YOUR OWN PLAQUE ONLY. YOU ARE NOT ALLOWED TO WORK WITH ANOTHER STUDENT'S SAMPLE FOR SAFETY REASONS. Air dry, heat fix and gram stain your slide. Examine your

stained biofilm using oil immersion. Record observations on gram reactions and cellular morphology of organisms present.

4. Rinse your mouth with mouthwash using the Dixie cups and mouthwash provided. Swirl the mouthwash in your mouth for 1 minute. Expel mouthwash into the sink and throw the cup in the garbage when you are done.

5. Repeat the experiment above. Collect biofilm from your teeth using a sterile toothpick and make a second smear and gram stain of your mouth after rinsing with mouthwash. Make observations.

6. Wait 30 minutes and collect a third biofilm sample from your tooth. Make a third smear and fixed slide as above. What differences do you observe cell types and numbers? What conclusions can you draw from this experiment?

7. Bring an old used toothbrush from home. Suspend the toothbrush in a test tube of sterile nutrient broth. Swirl it around. Using a sterile cotton swab, make a smear from the nutrient broth on BHI, MAC, MSA, EMB plates. Incubate at 37°C for 24-48 hours. Examine plates next week. Report your results.

Question:

1. Do biofilm organisms live in communities? How do you know?

Sources

Figure 7.1
"Biofilm" by D. Davis, retrieved from https://commons.wikimedia.org/wiki/File:Biofilm.jpg (Licensed under CC BY-SA)

Bacteria of the Mouth section
"Microbiology Lab Manual: Biol2421L" by Donna Cain, Hershell Hanks, Donna Cain, Mary Weis, Carroll Bottoms, & Jonathan Lawson, retrieved from https://microcosmorflores.wikispaces.com/file/history/Microbiology+Lab+Manual+--+Revised+Spring+2013.pdf (Licensed under CC BY-SA)

Exercise 8
The Identification of Bacteria using Biochemical Testing

8.1 The Inoculation, Incubation and Interpretation of Various Biochemical Tests Using Known Microorganisms

Objectives:

- To determine the ability of microorganisms to utilize various carbohydrates or an organic acid as a source of energy for metabolism, and to determine the end products produced as a result of metabolism.
- To determine the presence of various enzymes in microorganisms by testing for end products of enzymatic degradation.

8.1.1 Carbohydrate Metabolism

Heterotrophic bacteria may utilize various carbohydrates as a source of energy for growth and the synthesis of new cellular material. Polysaccharides must be hydrolyzed by the complex enzyme systems of the organism to a monosaccharide before they can be used by the cell.

Depending upon the organism and what metabolic pathway is used, various end products are formed: organic acids (lactic acid, acetic acid), neutral products (ethyl alcohol, acetate) and various gases (hydrogen, carbon dioxide). Detection of acid production from the utilization of carbohydrates is determined by adding a pH indicator to the media. Therefore, the color of the media will change if the organism produces acid or alkaline end products.

Carbohydrates can be fermented or respired depending upon the culture conditions (aerobic or anaerobic) and the enzyme systems of the organisms. **Respiration** is the process by which a compound is oxidized using oxygen. The carbohydrate (electron donor) is oxidized and the final electron acceptor is oxygen. Therefore, this type of oxidation can only occur under aerobic conditions and is mediated by the electron transport chain. Intermediate products are organic acids and the carbohydrate may be completely oxidized to CO_2. **Fermentation** is an energy-yielding process where the carbohydrate (electron donor) is oxidized but there is not external electron acceptor. Instead, the process relies on substrate level phosphorylation and an internally generated electron acceptor. Fermentation usually occurs under anaerobic conditions and does not involve the electron transport chain. Organisms that ferment carbohydrates are strict anaerobes, facultative organisms grown in anaerobic conditions, or a small group of organisms that do not possess an electron transport chain,

i.e. lactic acid bacteria.

Materials:

A. Brain Heart Infusion Agar (BHI) streak plates of stock microorganisms listed in each test

- Phenol Red glucose
- Phenol Red lactose
- Phenol red broth + mannitol
- MR - VP broth
- Citrate agar slant
- Peptone water broth
- Urea broth
- Hydrogen peroxide for catalase test
- methyl red indicator for methyl red test
- α naphthol for Voges Proskauer test
- 40% KOH for Voges Proskauer test
- Kovac's reagent for Indole test

Microbiologists use many types of media and different tests to cultivate microorganisms and identify and classify them. them. In the previous lab you used differential and selective media to grow particular bacteria and gain information about their metabolic properties. In this laboratory you will use a variety of different types of media to assist in identifying bacteria. There are many types of biochemical tests designed to demonstrate the biochemical and physiological characteristics of various microorganisms. The following is a brief selection of the many tests available that will assist in the identification of heterotrophic bacteria.

8.1.2 Phenol Red Carbohydrate Stabs

The components of Phenol Red medium are:

- Peptone
- beef extract
- sodium chloride
- phenol red pH indicator
- agar
- carbohydrate (e.g. glucose, lactose, mannitol)

The indicator is phenol red. It is red above pH 8 and yellow below pH 6.6

Utilization of the carbohydrate, either glucose or lactose, results in the production of acidic end products or intermediates. Excretion of these acidic compounds into the media results in the pH indicator changing from red to yellow. This is considered a positive result for carbohydrate utilization. The direction of the color change, from red to yellow is also important. If the organism is aerobic and requires oxygen, then the color change will occur from the top downward, i.e. it will become yellow at the top of the medium. If the organism is anaerobic and is inhibited by oxygen then the change occurs from the bottom upwards, i.e. it will become yellow at the bottom of the medium. If the organism is facultative, the medium will change to yellow entirely. The oxygen requirements of the organism can only be determined if the result is positive for carbohydrate utilization.

Procedure: (per bench)

1. Record the appearance of the media before inoculating. Stab inoculate 4 tubes of Phenol red glucose and phenol red lactose media with the following organisms: (1 per tube).
    - *Alcaligenes faecalis*
    - *Enterobacter aerogenes*
    - *Escherichia coli*
    - *Serratia marcescens*

2. Incubate tubes at 37°C for 24 – 48 hours.

3. Observe and record results.

8.1.3 Phenol Red Carbohydrate Broths

Another method for testing for carbohydrate utilization is using a nutrient liquid medium with phenol red as the indicator. Phenol red is red at pH 8.0 and yellow at pH 6.6. A carbohydrate such as mannitol, sucrose or sorbitol is added to the medium in a 1.0% concentration. If acid is produced by the organism upon fermentation of the carbohydrate, the medium will turn yellow. If the organism cannot use the carbohydrate, the medium will remain red.

Procedure:

1. Record the appearance of your uninoculated media. Inoculate the following two organisms into one tube each of phenol red broth + mannitol using your inoculating loop:
    - *Staphylococcus epidermidis*
    - *Bacillus subtilis*

2. Incubate tubes at 37°C for 24-48 hours.

3. Make observations and record your results.

8.1.4 Methyl red - Voges Proskauer Tests (MR-VP)

The medium used in the MR-VP tests is called MR-VP broth. It contains 0.5% glucose, 0.5% peptone and 0.5% K_2HPO_4 in distilled water. These tests are used to determine two possible metabolic end products produced from glucose metabolism. Glucose is metabolized to pyruvic acid which is the key intermediate in glycolysis. From pyruvic acid there are many pathways a bacterium may follow depending on the enzymes the organism possesses.

In the MR test, the organism ferments the glucose in the medium to pyruvate which is then reduced to the end products acetic, lactic and succinic acids, and ethanol, CO_2 and H_2. This is called mixed-acid fermentation. This results in a high concentration of organic acids so that the pH becomes very acidic. The addition of methyl red, a pH indicator that is red at pH 4.4 and yellow at pH 6.2, indicates the amount of acid production. A red color will indicate that the organism has produced a large amount of acid and is giving a positive result. A yellow color indicates that only a small amount of acid has been produced by the organism and the result is negative.

In the VP test, the organism ferments the glucose in the medium to pyruvate, but the main product of pyruvate metabolism is acetoin which is then further reduced to 2, 3-butylene glycol (2, 3-butanediol fermentation). After incubation α naphthol, which acts as a color intensifier, and KOH, which provides an alkaline environment, are added to the tube. The tube is shaken gently to expose the medium to oxygen in order to oxidize the acetoin, if present, to diacetyl. This then provides the red color to indicate a positive test. If there is no acetoin present, the reagents will turn a brown color.

Procedure:

1. Inoculate a tube of MR-VP broth with *E. coli*.

2. Inoculate a second tube with Enterobacter *aerogenes*.

3. Incubate at 37°C for 24-48 hours.

The Following Week:

- Perform the MR and VP test as follows:

  Remove 1 mL of the culture and place in a separate small test tube for the VP test.

  Voges-Proskauer test - Add 15 drops of a 5% α naphthol solution to the solution. Gently aerate. Add 5 drops of 40% potassium hydroxide. Gently aerate with the vortex mixture and then remove the cap off. A positive reaction is indicated by the development of a distinct red color which occurs in about 5 minutes.

  Methyl red test – to the remaining culture in the large test tube Add 5 drops of methyl red solution to the remaining culture. Shake gently. A red color is positive and yellow is negative.

Figure 8.1 Methyl red test: Escherichia coli (left), showing a positive result (the microorganism has produced a large amount of acid during fermentation of glucose, which is detected by the pH indicator), and Enterobacter cloacae (right) showing a negative result (the microorganism has produced a small amount of acid or no acid during fermentation of glucose).

8.1.5 Organic Acid Metabolism

Citrate Slants:

Some bacteria are able to utilize certain organic acids as a sole carbon source. Citrate medium contains nutrients plus 0.3% sodium citrate as the sole carbon source. The indicator bromthymol blue is added to determine if an alkaline reaction is produced. The ability of an organism to use citrate depends on the presence of a citrate permease that facilitates the transport of citrate into the cell. The sodium citrate is acted upon enzymatically to produce sodium carbonate which results in the medium becoming alkaline and therefore turning blue. If the organism cannot utilize the citrate, slight growth may occur on the slant but the medium will remain green.

Procedure:

1. Using the **loop**, streak a citrate slant with *E. coli* and a second slant with *Enterobacter aerogenes*.

2. Incubate at 37°C for 24-48 hours.

3. Observe and record your results.

8.1.6 Enzymatic Degradation

Bacteria possess many different enzymes that enable them to degrade various nutrient substances in the medium either as a source of energy or as a method of removing a toxic end product. The presence of these enzymes can be detected by testing for the end products of the enzymatic reaction.

Indole Test

Some bacteria have the ability to break down the amino acid tryptophan which results in the production of indole. The medium used in this test is a peptone water broth that contains all the amino acids including tryptophan. Tryptophan is translocated from outside to inside the cell, where an enzyme called tryptophanase splits tryptophan into indole, ammonia and pyruvate. The indole and ammonia are excreted from the cell as waste products, while pyruvate is oxidized to provide energy for the cell. The presence of indole is indicated by a rosy-pink color at the interface of the medium and the reagent after the addition of a few drops of Kovac's reagent and shaken well. If the organism has not produced indole, the reagent remains the original yellow-brown color.

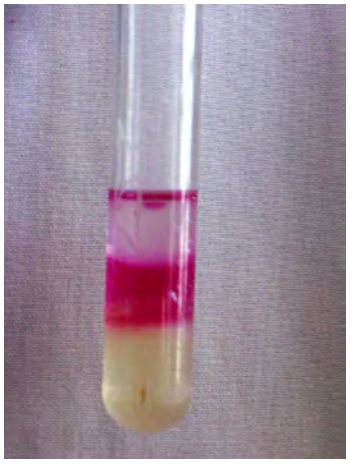

Figure 8.2 Indole test positive: appearance of pink layer at top (e.g. *Escherichia coli*)

Procedure:

1. With the loop, inoculate two tubes of peptone water, one tube with *E. coli* and the second tube with *Enterobacter aerogenes*.

2. Incubate tubes at 37°C for 24-48 hours

Next Laboratory Session:

- Add five drops of Kovac's reagent, shake well and observe the color produced.

NOTE: *E. coli* and *E. aerogenes* can be differentiated by the IMViC tests.

IMViC stands for:

I	-	indole
M	-	methyl red
V	-	Voges-Proskauer
C	-	citrate

The expected results are as follows:

Organism	I (indole)	M (methyl red)	V (Voges Proskauer)	C (citrate)
E. coli	+	+	-	-
E. aerogenes	-	-	+	+

Did your results correspond with the above expected results?

Urease Test

Urea is excreted by all mammals and some other animals as an end product of protein metabolism. Urease is a hydrolytic enzyme that attacks the nitrogen and carbon bond in amide compounds such as urea and forms the alkaline end product ammonia. The presence of urease is detected when the organism is grown in a urea broth medium containing the pH indicator phenol red. The indicator phenol red is yellow at pH 6.6 and red at pH 8.0. As the substrate urea is degraded into ammonia and carbon dioxide, the presence of ammonia creates an alkaline environment that causes the phenol red to turn a deep pink. If the organism cannot utilize the urea, the medium remains yellow.

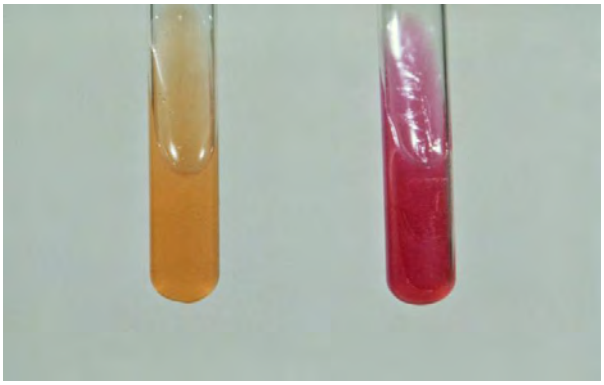

Figure 8.3 This urease test, based on the process involving the hydrolysis of urea, was performed to help identify the Gram-negative enteric bacterium *Yersinia enterocolitica*.
The hydrolysis of urea is confirmed by the formation of ammonia, and CO_2, which alkalinizes the medium, and results in a pH shift. A "hot-pink" coloration, as seen in the tube at right, indicates a positive reaction, i.e., urea hydrolysis, and a yellow or peach/gold color, as in the tube on the left, indicates a negative reaction. *Yersinia enterocolitica* bacteria contain the urease gene cluster that enables them to hydrolyze urea, however, though this test may yield positive results, *Y. enterocolitica* is not the only organism that can perform this process, and further testing would be required in order to rule out the presence of other urease producing bacteria.

Procedure:

1. Using the loop, inoculate two tubes of urea broth with:
    - *Escherichia coli*
    - *Serratia marcescens*

Catalase Test

The enzyme catalase reduces hydrogen peroxide to water and free oxygen. The enzyme is essential in most aerobic organisms that utilize the electron transport chain for energy production. The electrons from the organic substrates in the TCA (Krebs) cycle are passed to the flavoproteins which are reduced (FADH to $FADH_2$). In turn, the electrons are then passed down the transport chain via quinones and cytochromes to oxygen, the final electron acceptor. When the flavoprotein is re-oxidized after the electrons are passed to the quinone compounds, hydrogen peroxide is formed by a by-product. Since peroxide is highly toxic to cells, it must be reduced immediately, and this is usually carried out by the catalase enzyme resulting in water and free oxygen.

Question:
Formula: _____

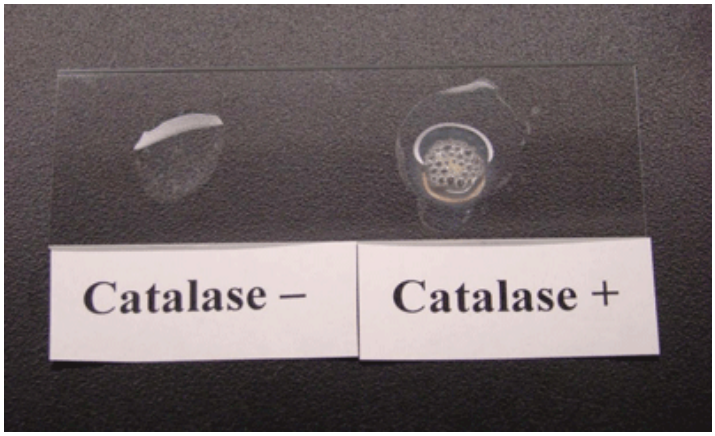

Figure 8.4 Negative and positive catalase test results.

Procedure:

Using a sterile loop, place an inoculum of *Staphylococcus epidermidis* and *Lactobacillus plantarum* on a glass slide. Add one drop of 3% hydrogen peroxide solution to the cells on the slide. Bubbling, which is the free oxygen being evolved due to the action of catalase, is a positive result.

8.2 The Identification of an Unknown Microorganism

There are essentially three different methods of classifying bacteria.

1. The **classical** method uses the morphological and biochemical characteristics of an organism. They are determined and the identity of the organism is established by comparing these characteristics to already described organisms. Bergey's *Manual of Determinative Bacteriology*, 8th ed., 1974 or Bergey's *Manual of Systematic Bacteriology*, 9th ed., 1984 (since updated into so many volumes that the library refused to buy them) are considered to be the microbiologists "bible" and are used extensively in the identification of bacteria using this method.

2. The **numerical** method of taxonomy uses the same characteristics as the classical approach but instead places equal weighting on them and uses a computer to compare all the characteristics of each organism with those of others. Therefore, large numbers of organisms, recognizing their similarities and differences, can be analyzed.

3. **Molecular** characteristics are also used in taxonomy. Comparisons of protein sequences, nucleic acid base composition, nucleic acid hybridization and nucleic acid sequences yield considerable information about true relatedness.

To aid in the identification of your unknown bacterium a dichotomous key is provided. This key begins with an obvious characteristic which in this case is the cellular morphology of the Gram reaction. After determining the cell shape and Gram reaction, you are presented with an either/or

situation of a combination of characters. You choose one character and continue with this process until you have identified your organism.

Objectives:

- To determine the cellular, cultural and metabolic characteristics of an unknown microorganism for identification with the key.

Materials:

Demonstration Bench

- One demonstration bench contains positive and negative biochemical tests showing how to interpret the results for identifying bacteria with the key below.
- The second demonstration bench has an unknown organism which can be identified using the biochemical test and the key provided.

Procedure:

Identify the bacteria on the demonstration bench each week according to the directions given by your instructor. This display will change for each lab. A copy of the key used on the demonstration bench follows.

Key to the Identification of an Unknown Bacterium		
Step No.	**Description**	**Go to Step No.**
1.	Bacillus shape	2
	Coccus shape	3
2.	Gram positive	6
	Gram negative	7
3.	Catalase negative	4
	Catalase positive	5
4.	Growth in 6.5% NaCl nutrient broth Phenol red broth + mannitol positive	*Streptococcus faecalis*
	No growth in 6.5% NaCl nutrient broth Phenol red broth + mannitol negative	*Streptococcus lactis*
5.	Cellular arrangement: grape-like clusters Acid from phenol red glucose Acid from phenol red broth with mannitol	*Staphylococcus aureus*
	Cellular arrangement: cubical packets No acid from phenol red glucose No acid from phenol red with mannitol	*Micrococcus luteus*

6	Colony morphology on BHI: large, flat, granular colonies Gelatinase positive	*Bacillus subtilis*
	Colony morphology on BHI: smooth, regular colonies Gelatinase negative	*Bacillus circulans*
7.	Acid from phenol red glucose Colony morphology on BHI: non-pigmented colonies	8
	Acid from phenol red glucose Colony morphology on BHI: pigmented colonies	9
	No acid from phenol red glucose	12
8.	Acid from phenol red lactose	10
	No acid from phenol red lactose	11
9.	Colony morphology on BHI: red or red and white colonies	*Serratia sp.*
	Colony morphology on BHI: violet to purple/black colonies	
	Colony morphology on BHI: green, yellow or brown colonies.	*Pseudomonas aeruginosa*
10.	Colony morphology on EMB: metallic sheen Methyl red positive Voges Proskauer negative	*Escherichia coli*
	Colony morphology on EMB: large, mucoid, no metallic sheen. Methyl red negative Voges Proskauer positive.	*Enterobacter aerogenes*
11.	Urease positive Motility media positive Tryptophanase positive	*Proteus sp.*
	Urease negative Motility media negative Tryptophanase negative	*Salmonella sp.*
	Urease positive Motility media positive Tryptophanase negative	*Providencia rettgeri*
12.	Litmus milk alkaline Motility media positive (aerobic)	*Alcaligenes faecalis*
	Litmus milk alkaline Motility media negative	*Acinetobacter sp.*

Results:

Table 1: Explanation of Biochemical Tests (see demonstration on side bench)				
	Appearance of Uninoculated Medium	**Positive Reaction**	**Negative Reaction**	**Explanation**
Phenol red Glucose				
Phenol red Lactose				
Phenol Red Broth + Mannitol				
Methyl Red (MR-VP broth)				
Voges-Proskauer (MR-VP broth)				
Growth in 6.5% NaCl				
Catalase				
Urease – Urea Broth				
Gelatin hydrolysis				
Citrate Slant				
Indole – Peptone Water Broth				
Eosin Methylene Blue Agar (EMB)				
Litmus Milk Test				

Table 2: Results of Biochemical Tests of Known Microorganisms		
	Phenol Red Glucose	**Phenol Red Lactose**
Serratia marcescens		
Escherichia coli		
Alcaligenes faecalis		
Enterobacter aerogenes		
	Phenol Red Broth + Mannitol	
Staphylococcus epidermidis		
Bacillus subtilis		
IMVIC tests	**Methyl red**	**Voges Proskauer**
Escherichia coli		
Enterobacter aerogenes		
	Citrate slant	
Escherichia coli		
Enterobacter aerogenes		
	Indole (Peptone Water Broth)	
Escherichia coli		
Enterobacter aerogenes		
	Urea	
Serratia marcescens		
Escherichia coli		
	Catalase	
Staphylococcus epidermidis		
Lactobacillus plantarum		

Sources

Figure 8.1
"Methylrot Probe Methyl Red Test" by A. Doubt, retrieved from https://commons.wikimedia.org/wiki/File:Methylrot_Probe_methyl_red_test.jpg (Licensed under CC BY-SA)

Figure 8.2
"Indole Test Positive" by Microrao, retrieved from https://commons.wikimedia.org/wiki/File:Indole_test_positive.jpg (Available under Public Domain)

Figure 8.3
"Urease Test" by Centers for Disease Control and Prevention, retrieved from http://phil.cdc.gov/phil/details.asp?pid=6711 (Available under Public Domain)

Figure 8.4
"Negative and Positive Catalase Test Results" by Centers for Disease Control and Prevention, retrieved from http://www.cdc.gov/meningitis/lab-manual/chpt08-id-characterization-streppneumo.html (Available under Public Domain)

Exercise 9
Microorganisms in the Production of Foods: Fermentations

Objectives:

- To differentiate between fermentation pathways used to produce food
- To produce an alcoholic fermentation using yeast
- To produce a homolactic acid fermentation making yogurt
- To examine heterolactic acid fermentation in fermented vegetables

Fermentation Pathways

Heterotrophic microorganisms may break down carbohydrates to obtain energy by fermentation. Fermentation has four basic features:

a. it occurs in the absence of oxygen
b. energy producing electron transport is absent
c. it is much less energy efficient than respiration
d. metabolic intermediates or fermentation end products are produced

Fermentation of glucose occurs in two stages. The first involves the splitting of the molecule and the removal of two pairs of hydrogen atoms, resulting in the formation of carbon compounds more oxidized than glucose.

In the second, or reductive part of fermentation, the hydrogen atoms removed in the first stage reduce the oxidized compounds. Since an oxidation cannot proceed without an equivalent reduction, the number of hydrogen atoms is removed in the first part of a fermentation is always equal to the number used in the second.

The two major pathways used for bacterial fermentations are the Embden-Meyerhof-Parnas (EMP) scheme and the hexose monophosphate (HMP) scheme. In either pathway, pyruvic acid is always one of the compounds formed in the first stage of bacterial fermentation. The end products characteristic of the various bacterial fermentations are derived from the pyruvic acid and the hydrogen atoms produced in the first (or oxidation) stage of fermentation.
Among the bacteria, several different pathways for the fermentation of carbohydrates have been identified. Each is associated with a specific set of end products and each is characteristic of a particular group of bacteria.

The following table summarizes the seven most common pathways:

Type	Example	Source of Pyruvate
1. Alcoholic	*Saccharomyces cerevisiae* (yeast) glucose → ethanol + CO2 wine, beer, etc	EMP
2. Homolactic	*Strepococcus* sp. and some *Lactobacillus* sp. glucose → lactic acid yogurt	EMP
3. Heterolactic	*Leuconostoc* sp. and some *Lactobacillus* sp. Glucose →lactic acid, ethanol, C02 Sauerkraut	HMP
4. Propionic acid	*Propionibacterium* sp. Glucose (or lactate) → propionic acid, acetic acid, C02 Swiss and Tilsit cheese	EMP
5. Mixed acid	*E. coli* and many other enteric bacteria glucose →lactic acid, ethanol, C02, acetic acid, succinic acid, H2 methyl red positive organisms	EMP
6. Butanediol	*Enterobacter* and some other enteric bacteria glucose →2, 3-butanediol, ethanol, lactic acid, succinic acid, acetic acid, H2, C02 Voges Proskauer positive organisms	EMP
7. Butyric acid	*Clostridium* sp. Glucose →butanol, butyric acid, isopropanol, acetate, ethanol, acetone, H2, CO2 food spoilage in air-tight cans	EMP

A number of industrial processes make use of the end products of bacterial and fungal fermentation. Several of these fermentations will be studied in this lab.

9.1 Alcoholic Fermentation

In non-dairy fermentations such as the production of wine, yeast uses sucrose to produce ethanol (alcohol) and carbon dioxide in anaerobic conditions (no oxygen). Pyruvic acid is reduced to alcohol and carbon dioxide. Example: Brewer's yeast, *Saccharomyces cerevisiae.*
If oxygen is present, the yeast will grow aerobically releasing carbon dioxide and water as end products.

Figure 9.1 Wine made from the fermentation of grapes.

Procedure: (Per bench)

1. Using a scalpel slice 30 fresh grapes into three or four pieces and then add the fruit to two Erlenmeyer flasks. Add enough distilled water to cover the material plus an additional 1 cm.

2. Pasteurize one of the flasks in the 80°C water bath for ten minutes; allow to cool in the ice bath, then inoculate with 100/uL of the suspension of S. *cerevisiae.* Do not heat or inoculate the second flask. In this way, a comparison between the "old" method of making wine and the "new" method will be observed. The "old" method relies on the natural yeast found in the grapes to ferment the sugars. However, undesirable fungi, wild yeast and bacteria are also likely to be present which may result in an unsatisfactory product. The "new" method will result in a consistent product since only the inoculated yeasts will ferment the grape juice.

3. Incubate for one to two weeks at room temperature (about 25°C).

4. When the fermentation is complete, note the presence or absence of ethyl alcohol by its characteristic odor.

5. Prepare a Gram stain of samples from the pasteurized and unpasteurized flasks. Record the Gram reaction of each & sketch cellular morphology.

9.2 Homolactic fermentation

In dairy fermentations, microorganisms use lactose and produce lactic acid without using oxygen. Pyruvic acid is reduced to lactic acid only with the production of 2 moles of ATP. Organisms used in the production of yogurt include *Streptococcus* sp. and some *Lactobacillus* sp., such as *S. salivarius* subsp. *thermophilus*, and *L. delbrueckii.*

Figure 9.2 Yogurt is produced from the bacterial fermentation of milk.

Procedure: (Per pair)

1. Add 3 g of nonfat dry milk power to 100 ml pre-sterilized milk in a labeled drinking cup. Stir. Inoculate with 1-2 teaspoonfuls of commercial (non-pasteurized… Why?). Take a sample and gram stain your starting culture.

2. Record its pH. Record your observations of the characteristics of the substance (color, consistency, smell, etc…).

3. Incubate at 45°C for 24 hours. Make observations of the yogurt produced.

4. Determine the pH of the yogurt. Gram stain it. What do you observe. Do you notice any differences in organisms of the starting material before and after the yogurt was produced? What are they? Why?

9.3 Heterolactic fermentation

Organisms isolated from a variety of sources, including wines, sauerkraut, silage, and spoiled tomato products ferment hexoses with the production of ethanol, and carbon dioxide in addition to lactic acid and with the production of only 1 mole of ATP (e.g. *Leuconostoc* species and some *Lactobacillus* species)

Sauerkraut is produced by a sequential fermentation of cabbage by members of the genera *Leuconostoc* and *Lactobacillus.* These organisms are normally found on the leaves of the cabbage plant and are halotolerant and aciduric. Cabbage that is shredded or chopped contains about 2% carbohydrates. It is firmly packed into a container with about 2-3% salt. Weights may be applied to ensure that conditions are anaerobic. It is allowed to ferment for about 2-3 weeks. The initial fermentation is started by *Leuconostoc mesenteroides* that converts sugars to lactic acid, ethyl alcohol, and carbon dioxide. The organism is killed when the titratable acidity reaches 1.0%. *Lactobacillus plantarum* and *Lactobacillus pentoaceticus* continue the fermentation, the former species producing lactic acid from glucose; the latter producing the same products as *Leuconostoc*

mesenteroides. The lactobacilli raise the titratable acidity to a final 1.5% to 2.5%. The organic acids produced during fermentation act as a preservative.

In order to identify the organisms in the sauerkraut, selective and differential media will be inoculated with a Gram stain will be prepared. A sample of the juice will be tested to determine pH.

Figure 9.3 Fermented vegetables: sauerkraut, kimchi, pickles.

Procedure: (Per pair)

1. Examine the selection of fermented foods provided. They can include kimchi, sauerkraut, pickles and other fermented vegetables.

2. What food material(s) is each of these fermented foods made from? Take the pH of the material. What is it? What does this indicate?

3. What organisms are responsible for this fermentation. Choose one of the types of fermented vegetable products. Each bench should choose two different foods.

   Per pair: Inoculated 1 Rogosa SL media and sucrose plates a loopful of the material. Incubate plates at 30°C for 48 hours or until growth appears.

   Rogosa SL medium is selective for *Lactobacillus* sp. which are aciduric organisms. Growth of *Streptococcus* sp. is suppressed in this medium although some *Leuconostoc* sp. may grow.

   Sucrose agar is used for growth of some dextran producing organisms. Streptococci are suppressed in this medium although some *Leuconostoc* sp.may grow. *Leuconostoc* species when using sucrose as the carbon source produce copious quantities of dextran, a polymer of glucose. This results in *Streptococcus* sp. and *Lactobacillus* sp. do not produce dextran from sucrose and consequently the colonies are discrete. Incubate plates at 30°C for 48 hours or until growth appears.

4. Next lab make observations and Prepare a Gram stain of the colonies growing on the plate.

Sources

Figure 9.1
"Grapes, Nature, Food, Fruits" by SofiaPapageorge, retrieved from https://pixabay.com/ en/grapes-nature-food-fruits-1400727/ (Available under Public Domain)

"White Wine, Red Wine, Wine Glasses" by Didgeman, retrieved from https://pixabay.com/en/white-wine-red-wine-wine-glasses-848268/ (Available under Public Domain)

Figure 9.2
"Turkish Strained Yogurt" by Takeaway, retrieved from https://commons.wikimedia.org /wiki/File:Turkish_strained_yogurt.jpg (Licensed under CC BY-SA)

Figure 9.3
"Saurekraut-2" by Jules, retrieved from https://www.flickr.com/photos/stone-soup/14852679547 (Licensed under CC BY)

"Korean cuisine-Kimchi-08" by Jeremy Keith, retrieved from https://commons.wikimedia.org/wiki/File:Korean_cuisine-Kimchi-08.jpg (Licensed under CC BY)

"Dill Pickle Filled Jars" by Rebecca Siegel, retrieved from https://www.flickr.com/ photos/grongar/6019667931 (Licensed under CC BY)

Exercise 10
Food Microbiology – Contamination of Foods by Microorganisms

Objectives:

- To prepare serial dilutions from raw and cooked hamburger and to plate out appropriate dilutions.
- After incubation, to determine the number of bacteria isolated in the meat samples.
- To isolate and identify one colony from the hamburger sample and to determine if this colony could cause food poisoning.

10.1 Food Bacteriology

Contaminated food is another major route of transmission of infectious disease. Meat products such as beef, pork, and chicken often become contaminated during the slaughtering process, and individuals become infected when the meat is not properly cooked.

Some of the primary bacterial pathogens associated with contaminated meat include *Escherichia coli* and other fecal coliforms, *Campylobacter*, *Salmonella* and *Shigella*. In today's lab, we will be testing chicken and ground meat from the supermarket for the presence of gram-negative bacterial contaminants using a variety of differential and selective media including *Salmonella-Shigella* agar plates.

Salmonella-Shigella agar plates contain lactose, bile salts, ferric citrate, and neutral red. The bile salts in agar make the media selective for gram-negative enteric bacteria by inhibiting the growth of gram-positive organisms. The lactose in the media is a carbohydrate substrate which allows the differentiation between lactose fermenters and non-lactose fermenters. If lactose is fermented, the acid end-products will react with the neutral red pH indicator, giving the bacterial growth a pinkish-red color. The ferric citrate in the media acts as an indicator of hydrogen sulfide production, producing a black precipitate when H_2S is present.

10.2 Plate Counts for the Determination of Numbers of Bacteria in Foods

Food spoilage can result from microbial growth in foods. Spoilage can be from many different types of organisms, gram positives, gram negatives, and fungi. Plate counts are performed on foods by public health agencies and food processing companies. Heterotrophic plate counts are used to determine the total number of viable bacteria in a food sample. Presence of huge numbers of bacteria is undesirable in food because it increases the possibility that pathogens will be present and that food will spoil.

Plate Counts: Determination of Colony Forming Units

In heterotrophic plate counts, the number of CFU/ml is determined. One of the major ways to do this is to perform **viable plate counts**, in which bacterial cells from a liquid culture are spread onto an agar plate. The plate is incubated, the number of colonies that grow on the plate are counted, and the number of original bacterial cells in the culture is determined. In most cases, however, the liquid culture being quantified contains too many cells to be directly plated onto agar plates – there would be so much growth that it would be impossible to count individual colonies! Therefore, the liquid culture needs to be diluted, often 1-million-fold, before it can be plated.

When such a large dilution is required, an accurate dilution cannot be made in a single dilution step and it is necessary to make **serial dilutions**. Serial dilutions are a step-wise set of dilutions which sequentially dilute the bacterial culture. One or more of the dilutions are then plated on the agar plates to determine the number of colonies present in the original culture. Only plates containing between 30 and 300 colonies are counted to ensure statistically significant data.

Colony forming units/g or mL of sample = average **number of colonies**
Dilution of sample x amount plated out

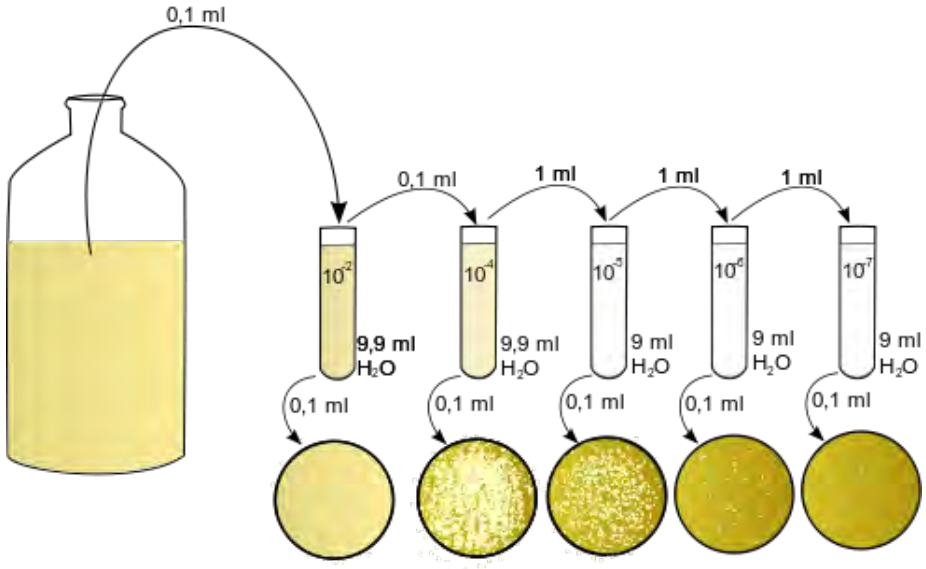

Figure 10.1 This is an example of a serial dilution. Serial dilutions can be performed in a variety of ways.

Examination of Bacteria in Raw Hamburger & Raw Chicken using SS Media

Materials: (per Bench)

- *Salmonella-Shigella (SS)* agar plates (2)
- Raw Chicken parts
- Raw Ground beef

Procedure: (please work in pairs)

One pair per bench will test raw chicken. The other pair will test raw hamburger.

1. Label SS plate.

2. Swab over the chicken or ground meat with a sterile cotton-tipped swab and rub over the SS plate.

3. Incubate the plates at 37° C for 24-48 hours.

4. Look up SS media. Is it differential or selective or both? Why?
   What are the differential and selective ingredients in the media? What are the indicators?
   What reactions would you expect to see next week? Why?

Next Laboratory Session:

Observe SS plates for bacterial growth. Determine which bacteria are growing on the plate using the following criteria:

- *Escherichia* and other fecal coliforms such as *K. pneumoniae*: ferment lactose and produce reddish pink colonies on the agar.
- *Salmonella*: do not ferment lactose, but do produce hydrogen sulfide. Colonies will either be black, or clear with a black dot in the center.
- *Shigella*: do not ferment lactose, and do not produce hydrogen sulfide. Colonies will be clear.

Determination of Heterotrophic Plate Count on Raw Hamburger Meat or Frozen Vegetables.

Materials: (Per Pair)

- Raw hamburger or Frozen thawed vegetables (see procedure)
- Dilution blanks – 9 ml – 1
- Dilution blanks – 99 ml – 2
- Micropipettes & tips
- Spreaders
- Alcohol and beakers for flame sterilizing spreaders
- BHI plates (6)

Procedure:

1. One pair at the bench choose hamburger, one chooses frozen vegetables. Label all plates and dilution blanks before starting. Read through the entire procedure first to see what you should label.

2. Weigh out 1g of food using the top loading balance and sterile spatula and weigh boat.

3. Transfer 1g food into a 9 ml dilution blank. Label the tube 1/10 dilution. Mix on a vortex mixer.

4. Transfer 1 ml of 1/10 dilution into a 99 ml dilution blank. Label 10^{-3} dilution. Cap tightly. Shake the dilution bottle 20 times as demonstrated.

5. Transfer 1 ml from the 10^{-3} dilution blank to a 99 ml dilution blank labeled 10^{-5}. Shake as before.

6. Label 2 BHI plates with dilutions 10^{-2}, 2 BHI plates with dilutions 10^{-4} and the final 2 BHI plates with dilutions 10^{-6}. Pipette 0.1 ml of the appropriate dilution onto each of the two labeled plates as demonstrated. Using an alcohol flamed spreader, evenly spread the diluted food over the surface of the BHI medium.

7. Invert plates and incubate at 37°C for 24-48 hours.

Next Laboratory Session:

- Perform plate counts on plates as demonstrated.
- Why should you not count plates with >300 colonies?
- Is it possible to include counts of plates with <30 colonies in counts? When?
- What does CFU stand for? What is a CFU?
- What is the formula for calculating CFU/ml of a substance?
- Determine the CFU/ml of the hamburger and frozen vegetables.
- What do these numbers mean?

Sources

Food Bacteriology and Plate Counts sections
"Microbiology Lab Manual: Biol2421L" by Donna Cain, Hershell Hanks, Donna Cain, Mary Weis, Carroll Bottoms, & Jonathan Lawson, retrieved from https://microcosmorflores.wikispaces.com/file/history/Microbiology+Lab+Manual+--+Revised+Spring+2013.pdf (Licensed under CC BY-SA)

Figure 10.1
"Verdünnungsreihe mit Ausplattieren" by Leberechtc, retrieved from https://commons.wikimedia.org/wiki/File:Verd%C3%BCnnungsreihe_mit_Ausplattieren.svg (Licensed under CC BY-SA)

Exercise 11
Microbiology of Water

A major route of transmission of infectious disease is contaminated water. Water contaminated with organic waste materials is a source of many microorganisms, both infectious and non-infectious. Water testing is performed worldwide for the presence of fecal coliforms. The presence of fecal coliforms in water is indicative of human &/or animal feces in the water. Total and fecal coliforms are members of the Enterobacteriaceae and most of them originate in the intestine. They are therefore useful indicators of sewage contamination and **indicate** the presence of more serious enteric pathogens such as *Vibrio cholerae*, *Escherichia coli, Salmonella* and *Shigella sp*. as well as enteric viral pathogens such as Norwalk virus, enterovirus, rotavirus and other.

Water testing is often useful to obtain an estimate of the total number of **heterotrophic bacteria** in a water sample as a general indicator of water quality. Unfortunately, there is no such thing as a universal growth medium so various media have been devised to grow as many environmental bacteria as possible. Heterotrophic plate counts usually underestimate the number of bacteria in water because they do not count dead or metabolically inactive bacteria, autotrophs and chemolithotrophs will not grow on them, and many environmental bacteria do not grow well in rich media without a period of adaptation. Nevertheless, the heterotrophic plate or **standard plate count** is still commonly employed. The Canadian Drinking Water Guideline specifies that drinking water should not contain more than 500 HPC bacteria/mL, no more than 10 total coliforms/100 mL and 0 fecal coliforms.

In this exercise you will use one medium (Standard Plate Count Agar) to count heterotrophic bacteria by serial dilution and spread plating and two methods for estimating coliform organisms, **Membrane Filtration** and the **Most Probable Number** method. Heterotrophic plate count data are reported as Colony Forming Units (CFU) per mL but coliform data are reported as coliforms/100 mL because that volume is usually filtered before incubation. The Most Probable Number method has also been formulated so that results are reported /100 mL

Coliform organisms are enumerated as either total or fecal coliforms. Total Coliforms are defined as facultative anaerobic, Gram negative, non-spore forming rods that ferment lactose at 35 °C within 24 h and possess the enzyme p-galactosidase in their cell wall. These include many members of the Enterobacteriaceae, some of which occur naturally such as *Klebsiella* and *Enterobacter* spp. The presence of total coliforms suggests fecal
contamination but only *Escherichia coli* is diagnostic.

For this reason, two different media and growth conditions are employed to measure the two groups separately. Fecal Coliforms meet the criteria listed above and, in addition, ferment lactose at 44.5 ± 0.5 °C and contain the enzyme p-glucouronidase in their cell wall. The classic media for total and fecal Coliforms are m-Endo LES and M-FC respectively. A 100 mL aliquot of water is filtered through a 0.45 μm membrane and the membrane is placed on a small agar plate containing the appropriate medium. Red colonies are counted on m-Endo LES medium and blue colonies on M-FC medium.

Biotechnology has produced more advanced media such as Colilert broth for the simultaneous determination of both groups in a single presence/absence medium. We have adapted this medium to the Most Probable Number method so that quantification is added. The test is based upon growing coliform bacteria at 37 °C in a defined medium that contains two substrates, o-nitrophenol-beta-D galactopyranoside (ONPG) and 4-methyl-umbelliferyl glucuronic acid (MUG). Coliforms all possess the enzyme p-galactosidase which splits the ONPG molecule into p-galactopyranoside and o-nitrophenol which is yellow, p-glucouronidase on the *Escherichia coli* cell wall also splits MUG into p-glucouronide and 4-methyl-umbelliferone which fluoresces blue under ultraviolet light. Positive coliforms are indicated by the medium changing from colorless to yellow and positive fecal coliform is revealed by blue fluorescence under ultraviolet light.

The most probably number (MPN) method is widely applied to make positive reactions quantitative. The method can be used with either soil or water and is based on several dilutions with replicates. In our experiments, three dilutions and five replicates of each dilution will be prepared and the number of bacteria in the sample can be estimated from the number of tubes showing growth. The final number of bacteria is determined from a set of tables which has been prepared by running this experiment with a large number of samples, and then checking the actual number of coliforms.

Objectives

- To estimate the number of heterotrophic bacteria in a water sample.
- To estimate the total and fecal coliform organisms in a water sample using the membrane filtration, most probable number and MUG presence/absence tests.
- To identify bacteria isolated on membrane filtration plates using API 20E.

Procedure (per bench):

Obtain the water sample assigned by your instructor. Each bench will test a different water sample. Record the source of your water sample, location and date sampled.

Part A: Inoculations

1. **Standard (Heterotrophic) Plate Count**
    1. Prepare serial dilutions of the sample provided to 10^{-1}, 10^{-3} and 10^{-5}.
    2. Spread 100 μl of each dilution on 2 plates of Standard Plate Count Agar using flame/alcohol sterilization and a spreading tool. You will need 6 plates per sample.

2. **Total and Fecal Coliforms by Membrane Filtration**
    1. Aseptically place a 0.45 */um* filter with the grid up on the filter support of a filtration tower. Use alcohol-flame sterilized forceps.
    2. Screw on the filtration tower and connect a hand vacuum pump.
    3. Add 100 mL of the sample to the tower and apply vacuum.
    4. After all the sample has been passed through, aseptically (as above) remove the membrane and transfer it to a small Petri dish containing m-Endo LES agar.
    5. Repeat but transfer the second membrane to M-FC agar.

3. **Most Probable Number Determination of Total and Fecal Coliforms**
    1. Using the contaminated water provided, set up a presumptive coliform count using the most probable number method. Using a serological pipette, add a 10 mL sample of water to each of 5 tubes containing 10 mL of double strength Colilert broth, a 1 mL sample to each of 5 tubes of single strength Colilert broth, and 0.1 mL to each of 5 further tubes of single strength medium.
    2. Incubate at 37 °C. Use aseptic technique when pipetting.

4. **Coliscan Test kits.**
    - In addition to these tests, coliscan kits will be used to sample your water. Inoculate your water sample into the coliscan test kit as demonstrated by our instructor. Incubate plates as instructed.

Part B: Interpretation of results after incubation

1. **Heterotrophic Plate Count**
    - Perform plate counts. Report your results as CFU/mL.

2. **Total Coliforms by Membrane Filtration**
    - Take the top off the small Petri dish containing the m-Endo LES medium and examine it. Count all the colonies that are pink to dark red in color and have a metallic surface sheen. The sheen area may vary in size from a small pinhead to complete coverage of the colony surface. Report your results in CFU/100 mL.

3. **Fecal Coliforms by Membrane Filtration**
    - Repeat as for Total Coliforms using the membranes on M-FC Agar. This time, count only blue colonies (any shade of blue). Non-fecal coliforms are gray to cream colored. Report your results in CFU/100 mL.

4. **Identification of Oxidase Negative Bacteria by API 20E**
    1. Streak selected colonies from the m-Endo LES and M-FC plates using a stab onto MacConkey agar plates.
    2. After these have grown up, test selected colonies for oxidase using oxidase test sticks.
    3. Transfer several oxidase negative colonies to 5 mL BHI tubes and incubate for 24 hours. Choose one of these organism to transfer to API test strips.
    4. Use a sterile Pasteur pipette to fill the wells of the API test strip with the broth from 3. Note that both the tube and capsule needs to be filled for CIT, VP and GEL and that sterile mineral oil should be added to the capsule of ADH, LDH, ODC, H2S and URE to create anaerobic conditions. Fill the tube only for all other wells.
    5. Place about 25 drops of water in the bottom of the plastic trays provided, put in the strip and then seal with the top of the tray. Incubate for 24 h.
    6. Score the tests according to the information provided by your instructor and the paper strips provided. Within each group on the paper strips, sum the values associated with positive tests to produce a code which can be looked up in the Analytical Profile Index Code Book. If your code doesn't appear, look up the closest one and use the most probable identification of your isolate.

5. **Colilert Medium/Most Probable Number**

- Count the number of tubes that have turned yellow and record the results as the number of positives (out of five tubes) at each dilution. This will generate a three- digit code such as 4-3-2 (4 positive tubes at 10 mL; 3 positive tubes at 1 mL; 2 positive tubes at 0.1 mL). Now shine the ultraviolet light on the rack of tubes and record the number of blue-fluorescing tubes at each dilution to generate a new three- digit code. Look up the Most Probable Number for both total and fecal coliforms using the table provided by your instructor.

Number of Test Tubes Showing a Positive Reaction			MPN Index per 100ml	Number of Test Tubes Showing a Positive Reaction			MPN Index per 100ml	Number of Test Tubes Showing a Positive Reaction			MPN Index per 100ml	Number of Test Tubes Showing a Positive Reaction			MPN Index per 100ml	Number of Test Tubes Showing a Positive Reaction			MPN Index per 100ml
10 ml	1 ml	0.1 ml		10 ml	1 ml	0.1 ml		10 ml	1 ml	0.1 ml		10 ml	1 ml	0.1 ml		10 ml	1 ml	0.1 ml	
0	0	0	0	1	0	0	2	2	0	0	4.5	3	0	0	7.8	4	0	0	1	5	0	0	23
0	0	1	1.8	1	0	1	4	2	0	1	6.8	3	0	1	11	4	0	1	17	5	0	1	31
0	0	2	3.6	1	0	2	6	2	0	2	9.1	3	0	2	13	4	0	2	21	5	0	2	43
0	0	3	5.4	1	0	3	8	2	0	3	12	3	0	3	16	4	0	3	25	5	0	3	58
0	0	4	7.2	1	0	4	10	2	0	4	14	3	0	4	20	4	0	4	30	5	0	4	76
0	0	5	9	1	0	5	12	2	0	5	16	3	0	5	23	4	0	5	36	5	0	5	95
0	1	0	1.8	1	1	0	4	2	1	0	6.8	3	1	0	11	4	1	0	17	5	1	0	33
0	1	1	3.6	1	1	1	6.1	2	1	1	9.2	3	1	1	14	4	1	1	21	5	1	1	46
0	1	2	5.5	1	1	2	8.2	2	1	2	12	3	1	2	17	4	1	2	26	5	1	2	64
0	1	3	7.3	1	1	3	10	2	1	3	14	3	1	3	20	4	1	3	31	5	1	3	84
0	1	4	9.1	1	1	4	12	2	1	4	17	3	1	4	23	4	1	4	36	5	1	4	110
0	1	5	11	1	1	5	14	2	1	5	19	3	1	5	27	4	1	5	42	5	1	5	130
0	2	0	3.7	1	2	0	6.1	2	2	0	9.3	3	2	0	14	4	2	0	22	5	2	0	49
0	2	1	5.5	1	2	1	8.2	2	2	1	12	3	2	1	17	4	2	1	26	5	2	1	70
0	2	2	7.4	1	2	2	12	2	2	2	14	3	2	2	20	4	2	2	32	5	2	2	96
0	2	3	9.2	1	2	3	12	2	2	3	17	3	2	3	24	4	2	3	38	5	2	3	120
0	2	4	11	1	2	4	13	2	2	4	19	3	2	4	27	4	2	4	44	5	2	4	150
0	2	5	13	1	2	5	17	2	2	5	22	3	2	5	31	4	2	5	50	5	2	5	180
0	3	0	3.6	1	3	0	0.3	2	3	0	12	3	3	0	17	4	3	0	27	5	3	0	79
0	3	1	7.4	1	3	1	10	2	3	1	14	3	3	1	21	4	3	1	33	5	3	1	110
0	3	2	9.3	1	3	2	13	2	3	2	17	3	3	2	24	4	3	2	39	5	3	2	140
0	3	3	11	1	3	3	15	2	3	3	20	3	3	3	28	4	3	3	45	5	3	3	180
0	3	4	13	1	3	4	17	2	3	4	22	3	3	4	31	4	3	4	52	5	3	4	210
0	3	5	15	1	3	5	19	2	3	5	25	3	3	5	35	4	3	5	59	5	3	5	250
0	4	0	7.5	1	4	0	11	2	4	0	15	3	4	0	21	4	4	0	34	5	4	0	130
0	4	1	9.4	1	4	1	13	2	4	1	17	3	4	1	24	4	4	1	40	5	4	1	140
0	4	2	11	1	4	2	16	2	4	2	20	3	4	2	28	4	4	2	47	5	4	2	220
0	4	3	13	1	4	3	17	2	4	3	23	3	4	3	32	4	4	3	54	5	4	3	280
0	4	4	15	1	4	4	19	2	4	4	25	3	4	4	36	4	4	4	62	5	4	4	350
0	4	5	17	1	4	5	22	2	4	5	2	3	4	5	40	4	4	5	69	5	4	5	430
0	5	0	9.4	1	5	0	13	2	5	0	17	3	5	0	25	4	5	0	41	5	5	0	210
0	5	1	11	1	5	1	15	2	5	1	20	3	5	1	29	4	5	1	48	5	5	1	350
0	5	2	13	1	5	2	17	2	5	2	23	3	5	2	32	4	5	2	56	5	5	2	540
0	5	3	15	1	5	3	19	2	5	3	26	3	5	3	37	4	5	3	64	5	5	3	920
0	5	4	17	1	5	4	22	2	5	4	29	3	5	4	41	4	5	4	72	5	5	4	1000
0	5	5	19	1	5	5	24	2	5	5	32	3	5	5	45	4	5	5	81	5	5	5	2400+

Table 11.1 Most Probable Number (MPN) index per 100 ml test sample using five 10 ml samples, five 1 ml samples, and five 0.1 ml samples.
Adapted from *Handbook of Microbiology*, by M. B. Jacobs & M. J. Gerstein.

Exercise 12
Isolation of Bacteriophage from Sewage

Bacterial viruses, or **bacteriophages** as they are commonly called, are found in the environment which is rich with the bacteria that they parasitize. Viruses are much smaller than bacteria being non cellular particles composed only of RNA or DNA surrounded by a protein envelope. The viruses lack any metabolic machinery and therefore must exist as parasites on other living cells using their hosts' enzymes to reproduce.

Bacteriophages exist in many sizes and shapes, but many of the including the *E. coli* bacteriophage, have a head-like structure made up of a protein coat which is called the capsid, and a tail-like structure. The extreme end of the tail has the ability to become attached to a specific receptor site on the surface of phage-sensitive bacteria. Once the tail of the virus attaches to the bacterial cell, the nucleic acid of the virus is injected through the cell wall and membrane of the host. With the invasion of the bacterial cell by the viral nucleic acid, either lysis or lysogeny will eventually occur. This experiment will demonstrate **lysis** of *E. coli* cells by bacteriophage isolated from raw sewage.

Bacteriophages are too small to be seen with the light microscope. They also cannot be cultured on an agar plate in the usual manner. Therefore, in order to visualize the *E. coli* bacteriophage, it is necessary to inoculate the virus onto an agar plate where *E. coli* bacteriophage will infect a single *E. coli* cell. It will reproduce itself using the synthetic machinery of the host cell and then lyse the bacterial cell wall liberating infectious phage particles that are capable of infecting new susceptible host cells. This starts the cycle over again. Therefore, if a **lawn** of bacteria covers the entire surface of the agar plate, the area of the plate will lack growth where the bacteriophage have infected and bacterial cells and caused them to lyse. This appears as a clear spot on the lawn which is called a **plaque**. It is assumed that each plaque has originated from one virus particle. By counting the number of plaques formed on the agar plate, it is possible to determine the number of phage particles in the original suspension. This is called the **titre** of the bacteriophage.

In this experiment the host bacteria to be used is *E. coli.* The *E. coli* bacteriophage has been isolated from a sample of sewage water obtained from the Medicine Hat Sewage Treatment Plant. Normally, the numbers of bacteriophage in any sample are not high, and one of the first steps in their recovery is an enrichment procedure in which the sewage is incubated with a culture of host cells to increase the number of bacteriophage. As the bacterial cells grow, viral particles are formed; the cells eventually disrupt and release the virions into the medium. Following this enrichment procedure, the sample containing the increased bacteriophage population is centrifuged to remove coarse material and then passed through a bacterial filter to remove bacterial contaminants. The clear liquid should contain bacteriophage. You can demonstrate this by mixing some of the liquid with a young culture of

uninfected host cells and layering the mixture on the surface of an agar plate. Following incubation, the film of bacterial growth will be mottled by clear circular areas called **plaques.** These plaques represent areas of phage reproduction and lysis of the population of infected bacterial cells in the area. In this exercise you will attempt to isolate a bacterial virus for *Escherichia coli* since bacteriophage for this bacterium are relatively common.

Objective:

- To become familiar with techniques for cultivating, isolating and enumerating bacteriophage using E. coli as the host cell and raw sewage as the source of viruses.

Materials: (Per pair)

- Sewage filtrate produced from sewage collected from Medicine Hat Sewage Treatment Plant
- 24-hour nutrient broth culture of *E. coli*
- BHI agar plate (3)
- Molten overlay tubes of nutrient agar in 45°C water bath (3)
- micropipettes and tips
- Vortex mixer

NOTE: ASEPTIC TECHNIQUES MUST BE USED THROUGHOUT THE ENTIRE PROCEDURE.

Procedure:

1. Add 45 ml of raw sewage to 5 ml of concentrated broth. (Note: In this concentrated broth - deca strength broth - ingredients are present at about ten times the normal concentration to provide the proper level of nutrients upon dilution with the sewage.)

2. Inoculate the mixture with 5 ml of a 24-hour culture of *E. coli* and incubate at 37°C for 24 h. This step is essentially an enrichment procedure intended to increase the number of phage.

3. Clarify 10 ml of the sewage culture by centrifuging at 2500 rpm for 10 minutes.

4. Pass the centrifuged liquid through a bacteriological filter. (This removes organisms that would overgrow the plates as the exercise continues.) The clear liquid (Sewage Culture Filtrate) can now be tested for phage.

   NOTE: Steps 1-4 may be performed for you prior to the lab session. Your instructor will notify you if this is the case.

5. Add 0.1 ml (100µL) of a 24-hour *E. coli* culture to **each** of three tubes of soft agar (0.7%) while they remain in the 45°C water bath.

6. To the first tube of soft agar (inoculated with *E. coli*), add one drop the Sewage Culture Filtrate (from step 4), mix on the Vortex Mixer, and pour over the surface of a BHI plate. **Label as "Sewage Culture Filtrate - 1 drop"**.

7. To the second tube of soft agar (inoculated with *E. coli*), add 5 drops of the sewage culture filtrate, mix on the Vortex Mixer, and pour over the surface a second BHI plate. **Label as "Sewage Culture Filtrate - 5 drops"**.

8. As a **CONTROL**, mix then pour the contents of the third tube of soft agar (inoculated with *E. coli*), over the surface of a third BHI plate. **Label as "CONTROL".**

9. Allow the soft agar to solidify and then invert and incubate the plates at 37°C until plaques are visible (6-24 hours).

Next Laboratory Session:

- Examine the plates seeded with the sewage cultures for plaques (round clear areas). These are areas in which phage particles have attacked *E. coli* cells causing lysis.

Record your results below:

	# of Plaques
Control	
Filtrate – 1 Drop	
Filtrate – 5 Drops	
Determine the phage titre of your sewage sample as follows:	
PFU/ml = $\frac{\text{\# of plaques}}{\text{(dilution of original sample) x (volume of sample used)}}$.	

Exercise 13
Transformation of E. coli

In this experiment you will transform a strain of *E. coli* with no antibiotic resistance to ampicillin and produce *E. coli* cells that are ampicillin resistant.

Objectives:

- To perform transformation in bacterial cells using plasmids.
- To use heat shock and chemical methods to transform bacteria
- To use fluorescent proteins as indicators of transformation
- To transform bacteria with no antibiotic resistance to bacteria with antibiotic resistance
- To calculate transformation efficiency

Bacterial Transformation:
Bacterial transformation is the ability of bacterial cells to incorporate naked DNA from the environment into their genome. This results in changes to their their genome that are passed to daughter cells. Not all prokaryotes are transformable in nature.

Competence:
Bacterial cells that are transformable are called 'competent'. This means that their cell walls are permeable to macromolecules including DNA. The capacity to take up DNA is genetically determined. In nature the source of DNA for transformation comes from other cells that have died. Their cells walls lyse releasing DNA molecules into the surrounding medium. Competency occurs naturally in some strains of Streptococcus, *Haemophilus, Pseudomonas* and *Bacillus* when nutrient and oxygen levels are low. Genera of bacteria that aren't naturally competent can be treated in the lab to become artificially competent. Electrical, chemical or temperature changes can affect the structure and permeability of the cell wall and membrane allowing DNA molecules to pass through the prokaryotic cell wall.

In this experiment we will perform transformation on *E. coli* by subjecting it to calcium chloride and heat shock treatments.

Plasmids:
Plasmids are used as vectors in transformation. They are self-replicating extra chromosomal, double stranded circular DNA molecules found in many strains of bacteria. Plasmids are often engineered to contain two things such as (i) genes for antibiotic resistance to allow selection of transformed cells and (ii) a site where genes of interest can be inserted.

Fluorescent Green & Blue Protein Markers:
In this experiment, you will express fluorescent proteins: green fluorescent protein (GFP) and blue fluorescent protein (BFP) in transformed bacterial cells.

Objective:

- In this experiment you will transform a strain of competent *E. coli* with no ampicillin resistance with plasmid DNA which has a gene for ampicillin resistance. Two plasmids are used for transformation. The plasmid that produces the green fluorescent protein contains the GFP gene is pFluoroGreen. The plasmid producing the blue fluorescent protein is pFluoroBlue. Both pFluoroGreen and pFluoroBlue also possess the gene for ampicillin (amp) resistance. Bacterial cells will be selected for the presence of plasmid by plating them onto agar medium containing ampicillin. Only bacterial cells that take up the plasmids will survive selection on ampicillin agar plates. When using pFluoroGreen, green fluorescent colonies will be visible under long UV light. When using pFluoroBlue, colonies will appear as fluorescent blue colonies. The transformation efficiency will then be estimated.

Transformation Efficiency:
Transformation efficiency is a measure of how successful a transformation experiment is. It is the number of transformants obtained per microgram of DNA.

Overview of Experiment:

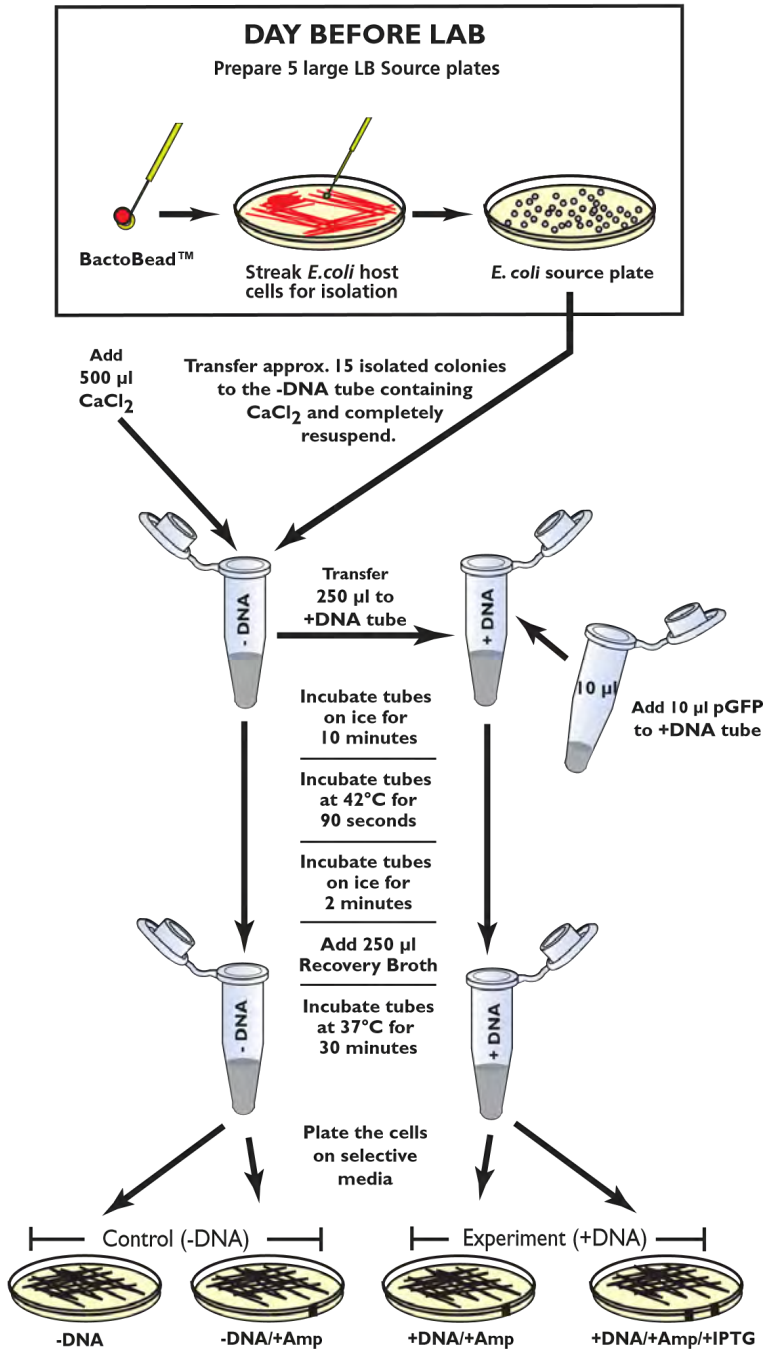

Figure 13.1 – Transformation Experiment Overview

NOTE: Longwave UV light is required to observe fluorescent colonies. Ensure that you are wearing

protective goggles when observing your plates.

Procedure: *(from Edvotek, The Biotechnology Education Company, EDVO-Kit # 222)*

CAUTION: This experiment involves use of the antibiotic AMPICILLIN. If you are allergic to antibiotics, please contact your instructor before proceeding with this experiment. USE GLOVES to proceed.

Setting Up Transformation Experiment:

1. Label one micro centrifuge tube "+DNA". This will be the transformation tube with one or both plasmid DNA's. Place tube in foam block.

2. Label a second micro centrifuge tube "-DNA". This will be the experimental CONTROL tube (no plasmid DNA). Place tube in foam block.

3. Add 250 µl ice cold $CaCl_2$ to each tube.

4. Pick colonies from the source plate of *E. coli.*

   Use the following method for each of the test tubes labeled "+DNA "and "-DNA":
   a. Use a sterile loop to transfer several colonies (2-4 large colonies) from the source plate to the micro centrifuge tube. Between your fingers, twist the loop vigorously and up and down in the cold $CaCl_2$ solution to dislodge the cells. AVOID scraping up agar when transferring the cells from the source plate to the tubes with calcium chloride solution. It is important that the cells are re-suspended in the calcium chloride solution and are not left on the loop or on the wall of the tube.

5. Suspend the cells completely in both tubes by tapping or vortexing.

6. To the tube labeled "+DNA" add one of the following options:
   a. 10 µl pFluoroGreen (from tube labeled pFG)
   b. 10 µl pFluoroBlue (from tube labeled pFB)
   c. 5 µl of each pFG and pFB for a total volume of 10 µl

   Proper experimental technique is critical. The plasmid DNA (pFG or pFB) must be added directly into the cell suspension.

7. Incubate the two tubes on ice for 15 minutes.

8. Place both transformation tubes at 42°C for 90 seconds. This heat shock step facilitates the entry of DNA into the bacterial cells.

9. Return both tubes immediately to the ice bucket and incubate for 2 minutes.

10. Add 250 μl Luria Recovery Broth to each tube and mix.

11. Incubate the cells for 30 minutes in a 37°C water bath for a recovery period.

12. While the tubes are incubating, label 4 agar plates with your name and the following information as indicated below:
    a. label one unstriped plate: LB –
    b. label one unstriped plate: LB + (pFG, pFB, or both plasmids)
    c. label one striped plate: LB/amp
    d. label one striped plate: LB/amp +(pFG, pFB, or both plasmids)

13. After the recovery period, remove the tubes from the water bath and place them on the lab bench. Proceed to plate the cells for incubation.

Plating the cells:

14. Plating Control plates (-DNA):
    a. Use a micropipette to transfer recovered cells from the tube labeled "-DNA" to the middle of the following plates:
        i. 250 μl to plate labeled LB-
        ii. 250 μl to plate labeled LB/amp-

    b. Use a sterile inoculating loop to spread cells in one direction back and forth across the surface of the plate. Then turn the plate and spread cells 90° to the first direction. Cover both plates and allow liquid to be absorbed into the agar surface.

15. Plating Cells from Test Plates (+DNA):
    c. Use a micropipette to transfer recovered cells from the tube labeled "+DNA" to the middle of the following plates:
        i. 250 μl to plate labeled LB+ (pFG, pFB, or both plasmids)
        ii. 250 μl to plate labeled LB/AMP+ (pFG, pFB or both plasmids)

    d. Spread cells with sterile inoculating loop as in step # 14.

    e. Allow plates to sit upright (covered) for liquid to be absorbed – about 15-20 minutes drying time.

    f. Incubate plates inverted @ 37°C overnight.

Viewing plates after incubation:

- Darken room and use a long wave UV light to examine transformed cells that will glow as green or blue if green or blue fluorescent proteins are expressed.

Laboratory Results:

1. Before performing the experiment, write a hypothesis that reflects the outcome of the experiment.

2. Answer these questions before analyzing your results:
    a. On which plate(s) would you expect to find bacteria most like the original non-transformed E. coli cells? Explain.
    b. On which plate(s) would you find only genetically transformed bacterial cells? Explain.
    c. Why would one compare LB/amp- and LB/amp+ plates?

3. **DATA**:
   Observe results obtained on your transformation and control plates.

Control Plates (-DNA)	**Transformation Plates (+DNA):**
-DNA	**+DNA/+Amp**
-DNA/+Amp	**+DNA/+Amp/+IPTG**

   For each plate record:
    a. how much bacterial growth do you observe? Count colonies.
    b. what color are the colonies?

4. Calculate **TRANSFORMATION EFFICIENCY:**
    a. Count number of colonies on plate with ampicillin labeled: LB/amp+ (green, blue, or both)
    b. Determine transformation efficiency using formula:

$$\text{number of transformants per } \mu g = \frac{\text{number of transformants}}{\mu g \text{ DNA}} \times \frac{\text{final volume at recovery (ml)}}{\text{volume plated (ml)}}$$

NOTE: **0.05 μg DNA is used**
Final volume at recovery is 0.50 ml
Volume plated is 0.25 ml

Questions:

1. Naked DNA does not passively enter E. coli cells that are not competent. How did you make your E. coli cells competent?

2. What is the function of IPTG?
3. What evidence do you have that transformation was or was not successful?
4. What are some reasons why transformation may not be successful?
5. What is the source of the fluorescence?
6. Differentiate between the following types of horizontal gene transfer?
    a. Transformation
    b. Transduction
    c. conjugation

Source

Figure 13.1

Edvotek. (2014). *Transformation with green fluorescent protein (GFP), EDVO-Kit #223.* Retrieved from http://www.edvotek.com/site/pdf/223.pdf

Exercise 14
Ames Test

The conventional way to determine whether or not a chemical substance is carcinogenic is to inject the material into animals and look for the development of tumors. If tumors develop, it is presumed that the substance can cause cancer. Although this method works well, it is costly, time-consuming, and cumbersome.

The fact that carcinogenic compounds induce increased rates of mutation in bacteria has led to the use of bacteria for screening chemical compounds for possible carcinogenesis. The Ames Test, developed by Dr. Bruce Ames at the University of California, Berkley, has been widely used for this purpose. The correlation between carcinogenesis and mutagenicity is between 85 - 90%. Scientists are now using the Ames test to screen many chemicals quickly and inexpensively to determine which are mutagenic and therefore, potentially carcinogenic.

The standard way to test chemicals for mutagenesis has been to measure the rate of back mutations in strains of auxotrophic bacteria. In the Ames test, a strain of *Salmonella* that is auxotrophic for the amino acid histidine and lacks DNA repair enzymes which prevents the correlation of DNA injury, is exposed to a chemical agent. After chemical exposure and incubation on histidine-deficient medium, the rate of reversion (back mutation) to prototrophy is determined by counting the number of colonies that are seen on the histidine-deficient medium. Each colony represents $his^- \rightarrow his^+$ revertant. A positive result, indicating mutagenicity, is obtained when an obvious increase in the number of colonies is evident when compared to the number of spontaneous revertants on the negative control plate.

Keep in mind, when making your observations and conclusions that this is a simplified version of the Ames test. Only one tester strain of *Salmonella* is being used in this experiment. Normally, several other strains would be used in order to accommodate different kinds of chemical compounds. While one chemical agent may be mutagenic on one tester strain, it may produce a negative result on another strain. Also, normally the test chemical agents are treated with a mammalian liver enzyme preparation. There is evidence that liver enzymes convert many noncarcinogenic chemical agents to carcinogenic ones.

Objectives:

- To determine why the Ames test is effective in screening for potentially carcinogenic chemicals.

Procedure: (work in pairs)

1. **Preparation of Agar Overlays:**
   Prepare agar overlays as follows onto three plates of minimal glucose agar plates:

   a. Pipette 100 μl of *Salmonella* mutant TA1538 into 1 tube of soft agar that is obtained from the 50°C water bath. Quickly pour the contents of the tube over a plate of minimal glucose medium. Tip the plate enough to spread the agar over the surface. Allow to harden.

   b. Repeat the above steps two more times using two new soft agar tubes and minimal glucose plates.

2. **Preparation of Test Plates**:
   a. **Negative Control**
   Label the first plate '**negative control'**. Dip a sterile disc, using sterile forceps, into the sterile water. Drain any excess water by touching to the side of the container. Place the disc in the centre of the plate and press gently so it will adhere to the surface of the agar.

   b. **Positive Control**
   Label the second plate **'positive control'.** Dip a sterile disc into a known mutagen (instructor will advise you on which one to use). Place disc in center of plate and touch gently so that it adheres to the agar surface.

   c. **Unknown Test Compound**
   Label the third plate '**unknown'**. Using the same technique as above, moisten a disc with the solution to be tested. Affix it to the agar surface. You will test this compound for mutagenicity.

3. Place the plates for incubation in the holder on the front bench.

4. Incubate the plates for 48 hours at 30°C.

Results:

The soft molten agar is prepared with a trace of histidine to allow the bacteria to undergo several cell divisions. This is necessary for mutation to occur. This early growth of bacterial cells produces a faint background lawn of growth that is barely visible. The test chemical diffuses outward from the disc forming a concentration gradient. If the test chemical is a mutagen, then some of the bacterial mutants revert and form colonies on the plate. These mutants are called revertants or back mutations because this second mutation returns the mutant to the prototrophic state. The reversion frequently depends on both the mutagen's concentration and its potency as a mutagen.

Count the number of colonies present on each plate and record on the chart below. Ignore the barely visible background lawn of bacterial growth.

Determine and record the number of chemically induced mutations by subtracting the number of colonies on the negative control plate, representative of spontaneous mutations, from the number of colonies on the positive control plate
and the unknown test plate.

Determine and record the relative mutagenicity of the test compounds on the basis of the number of induced mutations:

If <10 (-); if >10(+); if >100 (++); and if >500 (+++).

Chemical Tested	Number of Colonies/Plates	Number of Induced Mutations	Degree pf Mutagenicity
Negative Control:			
Positive Control:			
Unkown:			

Discussion Questions:

1. What are the advantages of using bacterial systems instead of mammalian systems to test for chemical carcinogenicity?
2. What are the disadvantages of using bacterial systems instead of mammalian systems to test for chemical carcinogenicity?

Appendix 1
Media and Methods used for Isolating and Cultivating Bacteria

Preparation of Media

Bacteria can rarely be identified only from a study of their cellular morphology and it is necessary therefore to obtain pure cultures of these organisms growing in the laboratory. Bacteria display many variations for the major nutritional requirements and so the utilizable sources of energy also vary. However, media for the cultivation of bacteria must contain a) water, b) source of energy, usually in the form of a carbon compound, c) essential elements including N, P, and S, d) metallic elements such as K, Mg, Ca, Fe, Mn, Cu, and Zn in varying proportions and e) organic nutrients. These are known as growth factors, compounds that an organism requires as a precursor of its organic cell material but which it cannot synthesize from sampler carbon sources. They include: i) amino acids, ii) purines and pyrimidines and iii) vitamins.

In prepared media, chemicals of defined composition are to be preferred to those of undefined composition. Since the nutritional patterns of many bacteria are not sufficiently well known, it is often necessary to add substances of complex organic composition e.g. peptone. In addition, media are often solidified with agar, a complex polysaccharide extracted from red algae. It is essential therefore, to use the same source of these materials for any comparative experiments. Most chemicals should be dissolved in distilled water. Tap water may contain toxic elements, particularly copper.
Most bacteria can only grow within a restricted pH range. The reaction of the medium must be adjusted so that the final pH, after sterilization is between 6.8 and 7.2, unless a different reaction is needed for some special purpose.

Buffers, which prevent large changes in pH, are often required to facilitate growth. This is particularly true of media composed of simple compounds or in which acid-producing bacteria are cultivated. Mixtures of sodium and potassium phosphates are often employed. In complex media, buffering is provided by the peptides and amino acids.

To determine pH changes during growth, indicators may be included in the medium. Where critical experiments are being performed it is often advisable to add the indicator to a sample tube only as a control, and to a small quantity of the actual culture fluid at the **end** of the experiment. Otherwise it is possible that the indicator or the alcohol it is dissolved in might act as a substrate for bacterial growth.

Types of Media for the Cultivation of Bacteria

The nutritional classification of organisms is based on three parameters: the energy source, the principal carbon source and the source of reducing power. With respect to energy source, phototrophs are photosynthetic organisms that use light as their energy source and chemotrophs are organims that depend on a chemical energy source. Organisms able to use C02 as a principal carbon source are autotrophs. Heterotrophs depend on an organic carbon source. To designate the source of reducing power, the term lithotroph or organotroph is applied. Lithotrophs use inorganic compounds as their source of reducing power, and organotrophs use organic compounds as their source of reducing power. The terms litho and organotrophs are not in common usage.

To summarize:

	Energy Source	Carbon source	Source of Reducing Power
photoautotroph (photolithotroph)	Light	C02	Inorganic oxidizable substrate
photoheterotroph (photoorganotroph)	Light	Organic	Organic
chemoautotroph (chemolithotroph)*	Chemical – Oxidation of reducing inorganic compounds e.g. NH_3 ,NO_2^- , and H_2	C02	Inorganic
chemoheterotroph (chemoorganotroph)	Chemical	Organic	Organic

*AII chemoautotrophs are chemolithotrophs, but not all lithotrophs are autotrophic.

For example, the methylotrophic bacteria can use organic carbon as their carbon source.

Media for Autotrophic Bacteria

Autotrophic bacteria are cultivated in solutions of mineral salts without organic additives (except in those species requiring growth factors). The media will be variable depending on whether the organism to be grown is a phototroph or a chemotroph.

Media for Heterotrophic Bacteria

Many different types of media have been perfected for the cultivation of heterotrophic microorganism

depending on their metabolic processes and nutritional requirements. Those organisms with complex requirements are said to be nutritionally exacting and include several of the pathogenic organisms and certain soil species. They are usually grown on complex natural media while the less exacting types (and the autotrophs) are grown on synthetic media of known composition.

Recipes for various types of media can be found in the appendices of most microbiology textbooks and in the Difco Manual online.

The Cultivation of Anaerobic Bacteria

Many species of bacteria are facultative aerobes, i.e. they can grow under aerobic or anaerobic conditions, the latter ability being dependent upon the presence of some substance that can be utilized as a hydrogen acceptor by the species concerned. Generally, facultative organisms prefer to grow aerobically rather than anaerobically. Aerobically, a larger amount of ATP and a larger amount of all mass is produced. Some bacteria are obligate aerobes, unable to use anything but oxygen that are able to grow in the presence of oxygen. Others are obligate anaerobes which cannot use oxygen as an electron acceptor. A few bacteria are somewhat intermediate, growing best in low oxygen tensions. These are called microaerophilic bacteria.

Anaerobic forms occur in several families of bacteria such as *Bacteroidaceae* and *Peptococcaceae.* Other anaerobic organisms include the Genus *Clostridium,* an endospore forming organism, certain autotrophic bacteria e.g. the purple sulfur bacteria, and some other soil or water bacteria e.g. *Desulphovibrio.*

During growth aerobic bacteria tend to utilize all available oxygen and so reduce the medium. Thus, in mixed cultures the oxidation-reduction potential (Eh) of the medium may become low enough to allow anaerobes to develop.

Methods Involving Removal of Oxygen

Stab and shake cultures
Many anaerobes can grow in deep stab or shake cultures in glucose agar. The method is particularly useful for microaerophilic species. A seal of liquid paraffin or Vaseline is sometimes advocated to help maintain anaerobic conditions, e.g. Hugh and Leifson fermentation test. This method is not entirely reliable and some evidence has been collected showing that oxygen diffuses through paraffin quite quickly.

Anaerobe Jar Culture
If surface cultures are required, the plates or slopes should be placed in an anaerobe jar. This is a heavy metal or glass jar with a lid that can be clamped firmly down to form an effective seal. Inlet and outlet valves allow the air in the jar to be replaced with hydrogen, after slow and careful evacuation of most of the oxygen. There are Gas Pak systems that replace the oxygen in the tube. Methylene blue strips are used as an indicator.

Thioglycollate Medium
This is prepared by adding 0.1% (up to 0.4%) thioglycollic acid to nutrient broth before adjusting the pH. 1% glucose must also be added. Although the medium may be solidified with agar, it is more usual to use a semi-solid medium, i.e. 0.5% agar. The increased viscosity of the medium prevents the distribution of oxygen (dissolved at the exposed surface), by convection currents. Methylene blue is added to act as an indicator of reducing conditions. It is colorless when reduced (i.e. between EH + 0.12 and 0.3 volts, which will allow most anaerobes to develop). A blue-green layer at the surface shows the depth to which oxygen has diffused into the medium. Inoculum should be introduced carefully by means of a fine pipette, at the bottom of the tube.

Methods of Isolating Bacteria

Isolation from Mixed Culture

Progressive dilution is the basis of isolation techniques. This can be applied to suspensions containing two or more bacterial species as follows:

Pour Plate Method
Melt and cool to 45°C two tubes of an agar medium. Inoculate one tube with one loopful of the suspension and mix well by rotating it between the hands. Flame the loop thoroughly and transfer one loopful of the mixture from the first tube to the second. Mix again. Pour the contents of both tubes into sterile Petri dishes and allow to set. Take care that the agar does not set before it is poured. The second plate should show well separated colonies after incubation. Distinct colonies can then be picked off and examined. If the suspension is very heavy initial dilution in sterile saline may have to be made.

Streak Plate Method – Multistreak Technique
Prepare a streak plate for single colony isolates using a multistreak technique. After incubation, well separated colonies should be found along the streak marks. Before deciding that a culture is pure by either method, colonies should be picked off, growth and then re-separated, until all colonies are the same. Morphological variants can upset strict application of this principle. Staining can be used as a check on the purity of the final isolations.

Isolation from the Environment/Natural Sources

The object of many viable counting techniques is to estimate the number of all live organisms in a given sample of material. To do this, a medium satisfying the nutritional requirements of as many of the bacteria in the sample as possible, is required. Thus, in soil bacteriology, soil extract media have been developed as most resembling an ideal non-selective medium. Other types of viable counts and all isolation techniques embody the reverse principles. Here it is necessary to pick out and encourage one type of organism and to prevent or depress the development of other types. In natural habitats

the organisms which one is isolating may be present only in small numbers.

The first step in such cases is thus to obtain an enrichment culture by one or more of three methods:

1. by using selective media
2. by using selective conditions of incubation
3. by selective pretreatment of the material

Several generations of sub-cultures on liquid, or solid media may be necessary, but the final step will consist of plating out the organisms by one of the methods described earlier.

Selective Media

A completely selective medium allowing the growth of only a single species is not attainable in practice, but media can be obtained which will discourage all but the required species. Some selective media are also **differential.** Certain organisms when grown on them exhibit distinctive biochemical or morphological characters which enable them to be recognized easily. This may be very important from the medical point of view, where the range of species concerned is small. Where a large range of species is involved, e.g. in soil, differential media cannot be used to separate taxonomic groups, but are useful to distinguish groups concerned in some biochemical process, e.g. cellulose decomposition.

Selectivity may be achieved in three ways:

1. **Addition of Substances to a Medium**
   A classic example of selective media is MacConkey bile-salt lactose broth. Bile salts discourage the growth of most bacteria except those of intestinal origin. The presence of 1 % lactose and a pH indicator make the medium differential as well. Coliform organisms ferment the lactose to produce acid and gas from the medium indicating fecal pollution in water and milk, etc. The same medium solidified with agar is used to isolate the pathogenic *Salmonella/Shigella* group of bacteria. While the coliforms produce acid and therefore a color change, the *Salmonella* and *Shigella* group are not able to ferment lactose and no color change occurs.

2. **Alteration of the pH of a Medium**
   Organisms tolerating a high pH, e.g. *Vibrio* and *Pseudomonas* sp., can be encouraged by adjusting the reaction to pH 9-10, e.g. Dieudonne's blood-alkali agar for isolating *Vibrio cholerae.* Acid tolerant bacteria can be more readily isolated if the pH is reduced to 5 or lower. Thus, *Lactobacilli* are isolated on media containing 0.5% acetic acid. After isolation, the organisms should be grown on normal media.

3. **Omission of One of More Substances**
   Media without organic substances are used to isolate and grow autotrophic microorganisms. Several transfers are usually needed. Although the first cultures show much growth, some organic matter is inevitably introduced with the inoculum causing contamination. Mannitol phosphate media used for nitrogen fixing organisms is an example of this method of selection.

Using Selective Incubation

Selective incubation of cultures can select quite different parts of the microbial population. Two methods will be considered here, temperature and aeration.

1. **Temperature**

   Microorganisms have more or less definite temperature requirements, with maximum temperatures above which they fail to grow and minimum temperatures below which they will fail to grow. These limits can be altered by various cultural conditions but are sufficiently precise to be used in isolation procedures.

   The temperature range of a given microorganism is relatively easy to establish. One culture of a given species, **uniformly inoculated,** should be incubated over a range of temperatures, e.g. 50°, 45°, 37°, 25°, 18°, 10°, 2°C. Tubes incubated at high temperatures must be enclosed in polythene bags to prevent excessive desiccation. The amount of growth occurring after one-two days - two weeks' incubation should be noted visually and recorded as a scale of plus signs i.e., +, ++, +++, or ++++. The method may be made more precise by employing stringent inoculation procedures and using an absorptiometry to assess the turbidity of a suspension.

   The results obtained from this experiment will vary, **thermophiles** growing at 45°C - 50°C and above, **mesophiles** rarely growing above 40°C, body organisms having an optimum at 37°C, soil organisms growing at about 10°C-25°C. Organisms growing at low temperatures are usually termed **psychrophiles.** These temperature distinctions are made for convenience and the precise numbers should not be taken as absolute.

   Thermophiles occur widely in soil and are important in the production of silage and compost, and in the canning and sugar-refining industries. To isolate such organisms from soil, a moist sample should be incubated at 45°C-60°C or higher for three days. A suspension of this material should then be made in sterile Ringer's solution and heated in a water bath at 100°C for twenty minutes. Add 1 ml of this suspension to 15 ml of nutrient agar melted and cooled to 45°C. Pour into a sterile petri dish. Incubate at 45°C-60°C with the dishes in a polythene bag to prevent drying. Thermophiles are thermoduric. However, a similar sample should be incubated at 25°C to demonstrate that some thermoduric organisms are not thermophiles. The distinction between mseophiles and thermophiles may be imprecise. Extreme thermophiles have been found in hot springs such temperatures at the boiling point, in steam vents (fumaroles) where temperatures may reach 150°C - 500°C and in geothermal vents at the bottom of the ocean which have temperatures of 350°C and higher.

2. **Aeration**

   Bacteria have a definite relationship to aeration conditions. Some will only grow with a supply of oxygen, the obligate aerobes. Others will only grow in the absence of oxygen, the obligate anaerobes. The great bulk of organisms will grow in either state to a certain degree. These are known as facultative.

Appendix II:
Microbiological Techniques

Staining:

Acid-fast Stain

1. Place the dried fixed film on the heating rack. Flood the smear with Ziehl's carbol fuchsin for 5 min. Steam gently but don't let the slide dry out. Add more carbol fuchsin as required. Wash with tap water and remove excess. Place slide on regular staining rack in sink.
2. Treat the smear with 20% sulfuric acid until no more stain comes out. Wash with tap water and remove excess.
3. Counterstain with 1% aqueous methylene blue for 1 min.

Acid fast organisms are RED; all others are BLUE.

Capsule Stain

1. Remove a loop of culture from the agar plate of the organism to be studied.
2. Add the bacteria to a drop of nigrosin on a slide and mix until all the bacteria have been transferred to the slide.
3. Using the end of a second slide or a cover slip draw the nigrosin to the opposite end of the slide. The nigrosin should form a thin translucent layer on the slide. Do not heat fix, but allow to air dry. Observe with the oil immersion objective.

Cells are COLORLESS/CLEAR (with capsules that resemble halos) and the background is BLACK.

Endospore Stain

Generally, the organism is grown on a sporulation agar plate for exactly 48 hours before doing an endospore stain.

1. Heat fix bacterial smear. Add enough 5% aqueous malachite green to saturate the paper. Heat the slide on the heating rack until steam begins to appear. Allow to sit for 15-20 minutes. Wash with water.
2. Counterstain with safranin for 60 seconds.
3. Wash and blot dry and examine with the oil immersion lens.

The endospores will stain GREEN and the rest of the cell RED/PINK.

Gram's stain

1. Prepare the smear. Heat fix.
2. Flood smears with crystal violet for 1 min. Wash with tap water.
3. Add Lugol's iodine to the slide for 1 min. Wash with tap water.
4. Decolorize with 95% ethanol. Drip alcohol down the slide for approximately 10 sec and rinse with water.
5. Counterstain with safranin for 1 min. Wash the slides with tap water, blot dry with paper towels, and examine under the oil immersion lens.

   Gram-positive organisms stain PURPLE
   Gram-negative organisms RED.

Cellular Morphology

Cellular morphology is generally obtained from a Gram stain slide. The cellular morphology includes:

- size - as determined by calibration of the microscope diameter (coccus)
- length x width (rod)
- shape
- arrangement of cells
- Gram reaction

A diagram drawn to scale usually accompanies the cellular morphology.

Colonial Morphology

The following characteristics are those most commonly used to describe colony morphology. A single ISOLATED colony is observed when describing colony morphology.

1. 1.Shape
2. Surface
3. Elevation
4. Size
5. Pigment
6. Opacity

Streak plate

The colony morphology is obtained from a plate culture. The agar plate must be streaked using a multistreak technique.

Incubation of Cultures

Individual tube and plate cultures should be labeled legibly with information including the

organism/source of inoculum, date, incubation temp, student name, name of media.
The plates are labelled on the bottom only (agar side) since the lids may get mixed up. Plates should be incubated agar side up so any condensation will fall on the lid. Tubes are labelled on the test tube, NOT on the lid.

Cultures should be incubated at the temperature most favorable to growth or the specific activity being studied. Human pathogens and commensal species grow best at body temperature, i.e. 37°C. Soil organisms and plant pathogens are normally incubated at 20-25°C. The optimum temperature is that temperature at which the growth rate is maximal for a particular organism. Cultures will usually grow at their optimum temperature in 24 hours. Some specific tests may take 48-96 hours of incubation to reach maximum growth.

Appendix III
Biochemical Tests

Catalase Test
Hydrogen peroxide is added to bacterial smear on a glass slide. Bubble of oxygen gas indicate the presence of the enzyme catalase. No bubbles indicate organism does not produce the enzyme catalase.

Carbohydrate Tests
Refer to Exercise 8 for results/discussion of Hugh & Liefson's glucose & lactose

H&L glucose & H&L lactose
NOTE: Other CHO fermentation tests include Phenol red glucose, lactose and mannitol stabs/broths.

Citrate test
Simmons citrate medium is a mineral medium with 0.3% sodium citrate as the sole carbon source. The indicator bromthymol blue is incorporated into the medium. It is green at pH 6.8 and blue at pH 7.6. The organism is streaked onto the surface of the slant and incubated. Organisms able to utilize the citrate grow on the surface of the medium. Due to oxidative formation of sodium carbonate, the medium becomes alkaline and changes color from green to blue.

Coagulase Test
The organism being tested is inoculated into a tube of 0.5 ml blood plasma which is prepared fresh daily. The inoculation should be done in the morning, so the tube is incubated for 6-8 hours. A solid clot indicates a positive result. The organism produces the enzyme coagulase which coagulates the blood plasma to form a clot.

Hydrogen Sulfide test
Hydrogen sulfide may be produced from sulfur containing amino acids, e.g. cysteine, present in peptone. The organism is inoculated into a tube of peptone water and a piece of lead acetate paper is suspended into the tube (not toughing the liquid) by the cap. After overnight incubation, if the organism breaks down cysteine and produces H2S, the lead acetate paper turns black due to the formation of lead sulfide. A negative result is indicated by the lead acetate paper remaining white.

Indole test

Indole is produced by some bacteria upon degradation of the amino acid tryptophan. The organism is inoculated into a tube of peptone water and incubated overnight. Several drops of Kovac's reagent are added after incubation. If the organism breaks down tryptophan to produce indole, the indole and the amyl alcohol present in the Kovac's reagent produce a cherry red color at the interface of the medium and the Kovac's reagent. A negative result is indicated by a brown color.

Litmus Milk

Litmus milk is composed of skim milk with sufficient litmus to produce a lilac color. There is a small amount of glucose present in the milk and a larger amount of lactose. Casein is the main protein in milk and gives the milk its opacity. Reactions occur due to the fermentation of the carbohydrates or utilization of the proteins. The pH indicator, litmus, changes color as a result of the reactions that occur. The organism is inoculated into the medium and incubated for 2-5 days. Litmus indicates changes in the pH of the media and also in the oxidation-reduction state of the media.

A wide variety of reactions may occur:

- **acid production** – litmus turns red. The glucose and/or lactose is used resulting in acidic end products.
- **alkaline reactions** – litmus turns dark blue. The proteins are used resulting in the production of ammonia, an alkaline end product.
- **reduction** – litmus is neither red nor blue, it becomes colorless (white). The litmus has been reduced by a reductase enzyme and the medium will become colorless in anaerobic conditions.
- **coagulation** – the milk solidifies. This occurs when a great deal of acid is produced. The acid coagulates the milk proteins.
- **peptonization** – the milk loses its opacity when the casein is digested by the enzyme casease and is converted to a translucent or watery fluid.

Mannitol Yeast Extract Congo Red

The organism is streaked onto a plate of mannitol yeast extract Congo red and incubated for 2-5 days. The organism grows on the mannitol yeast extract agar. If it can absorb the Congo red, the colonies are red. A negative result is indicated by growth of translucent colonies on the agar.

Methyl Red - Voges Proskauer (MR-VP) test

The Methyl Red (MR) test and the Voges-Proskauer (VP) test determines two possible end products of the fermentation of glucose. The organism is inoculated into two tubes of MR-VP broth and incubated overnight.

In the MR test, the organism ferments the glucose in the medium to pyruvate which is then

metabolized to the end products acetic, lactic and succinic acids, and ethanol, C02 and H2. This is called mixed-acid fermentation. This results in a high concentration of organic acids so that the pH becomes very acidic. The addition of methyl red, a pH indicator that is red at pH 4.4 and yellow at pH 6.2, indicates the amount of acid production. Several drops of methyl red are added to one tube. A red color will indicate that the organism has produced a large amount of acid and gives a positive result. A yellow color indicates that only a small amount of acid has been produced by the organism and the result is negative.

In the VP test, the organism ferments the glucose in the medium to pyruvate, but the main production of pyruvate metabolism is acetoin which is then further reduced to 2, 3-butylene glycol (2,3-butanediol fermentation). After incubation 1.5 ml of a-naphthol, which acts as a color intensifier, and 0.5 ml of 40% KOH, to provide an alkaline environment, are added to the second tube. The tube is shaken gently to expose the medium to oxygen in order to oxidize the acetoin, if present, to diacetyl. This then provides the red color and indicates a positive test. If there is no acetoin present, the reagents will turn a brown color.

Motility

A stab is used to inoculate the organism into the TTC motility medium. Stab carefully to obtain only a single stab line and incubate overnight. The triphenyl tetrazolium chloride is reduced when broken down by the organism and turns red. Therefore, the medium turns red where it is inoculated. If the organism is a facultative organism and motile it swims throughout the medium and the whole tube becomes red. If the organism is an aerobic organism, the stab line turns red and the entire top of the medium turns red where the organism swims in the presence of oxygen.

Ornithine Decarboxylase Test

Some amino acids can be utilized as a carbon source after decarboxylation. In this process, the carboxyl group is converted to C02 and the amino acid is converted to an amino in the presence of the coenzyme pyridoxal phosphate. The production of the amine results in a rise in pH which changes the color of the indicator in the media used. The amino acid decarboxylases are lysine, arginine and ornithine. This test will use the amino acid ornithine which s not found as a constituent of protein but does occur in the free form. The medium used is Moller's amino acid medium. It incorporates basal salts, glucose, the indicator bromcresol purple, as well as 1% L-ornithine.

A tube of ornithine decarboxylase TEST medium and a tube of decarboxylase CONTROL medium is inoculated with the organism, and approximately 1 ml of sterile mineral oil is poured into each tube. The tubes are incubated overnight.
The indicator, bromocresol purple is yellow at pH 5.2 and purple at pH 6.8. If the organism can utilize the glucose in the control tube, the medium turns yellow because of acid production. If the organism can utilize the glucose and the ornithine in the test tube, the medium first becomes yellow because of acid production from glucose, later if decarboxylation occurs, the medium turns alkaline and back to purple. A positive result is indicated by a yellow control tube and a purple test tube. A negative result is indicated by a yellow control and a yellow test tube.

Phenol Red Mannitol Broth

In the phenol red broth medium, the indicator is phenol red which is red at pH 8.0 and yellow at pH 6.6. Mannitol is added at a concentration of 1% and other nutrients such as peptone and beef extract are included in the medium to support growth. To detect gas production a Durham vial is placed in the tube in an inverted position. If the organism can use mannitol, acid is produced and the medium changes to yellow. If the organism can use mannitol and also has the enzyme to produce gas, the medium turns yellow and the bubble forms in the Durham vial. If the organism cannot use mannitol, the medium stays red.

Pigment Solubility Tests

The pigment produced by some microorganisms can be either a water soluble pigment or organic solvent soluble. To determine water solubility, observe the NA or BHI agar on which the organism is grown. Water-insoluble pigments remain confined within the cells; water-soluble pigments dissolve into the surrounding medium. To determine organic solvent solubility, add 10 drops of alcohol: acetone (1:1) mixture to a test tube. Add a loop of the cells grown on a NA or BHI agar plate. If the pigment is soluble, the liquid will be tinted the color of the pigment and will be transparent. If the pigment is insoluble, the liquid may appear to be tinted due to the suspended cells, but the suspension will be opaque.

Urease Test

A solution of urea is added aseptically to a sterile cooled basal medium in which phenol red is added as a pH indicator. Phenol red is red at pH 8.0 and yellow at pH 6.6. The organism is inoculated into the medium and incubated overnight. If the organism possesses the enzyme urease, it will break down the urea, ammonia is produced and a pink color results. A negative result is indicated by the medium remaining gold/peach.