LAB SAFETY RULES AND PROCEDURES

In order to make the most of the laboratory experience, it is essential that safety be a top priority. Students are expected to follow all lab safety rules and procedures as outlined here as well as any additional instructions, written or verbal, provided by the instructor. Students should consult with their Physician should they have any questions regarding their health (pregnancy, immune condition, etc).

1. Perform the experiments as directed. Do not do anything which is not part of an approved experimental procedure. Follow all instructions given by your instructor.

2. Be properly prepared to do the experiment. Read the written procedures in advance and understand what you are going to do. Know the hazards before you do the experiment.

3. Never work without instructor supervision.

4. Wear appropriate protective equipment as directed by the instructor. Gloves and eye protection are required for dissection of preserved materials. Disposable lab aprons are available for student use upon request.

5. Learn the locations and operation of emergency equipment. This includes eyewash, fire extinguisher, fire blanket, broken glass containers, sinks, and first aid supplies. Know what to do in case of emergency.

6. Act in a responsible manner at all times. No horseplay in the laboratory.

7. Long hair, loose clothing and dangling jewelry must be restrained. Open-toed shoes are not permitted.

8. Never taste a chemical. Check odors only if instructed to do so, by gently wafting some of the vapor towards your nose with your hand. Keep fingers, pencils and pens out of your mouth.

9. Turn off your Bunsen burner or hot plate whenever you are not using it. Never let it operate unattended.

10. Treat burns immediately by putting the burned area under cold water for at least 15 minutes. Cold water markedly reduces the subsequent pain and blisters.

11. Smoking, eating, drinking or applying cosmetics in the lab are not permitted in the laboratory.

12. Report all accidents, injuries, and close calls to your instructor immediately.

13. Dispose of chemicals and used dissection specimens properly. Broken glass goes in special receptacles.
14. To help avoid spills, containers should be kept away from the edge of workbench. Report all spills, including water, to the instructor. Anything spilled on a person must be washed off immediately with plenty of water. Note that many chemicals will stain clothing and tarnish jewelry.

15. Treat all chemicals with the respect they deserve. Know the hazards before you handle the material.

16. Never take specimens, models, chemicals, supplies, or equipment out of the laboratory.

17. Only approved WCJC personnel or enrolled students are permitted in the lab.

18. Notify your instructor of any allergies or medical conditions that he/she should be made aware.

19. Work area must be cleaned at the end of every laboratory period. Disinfectant is required for cleanup after laboratories involving dissection of preserved materials. Put away all equipment and reagents, and wash your hands at the end of each lab.

**Additional measures applicable to Microbiology:**

20. Thoroughly wash hands with antibacterial soap and disinfect tables at the beginning and end of each lab session.

21. All books, backpacks, purses, etc. should be placed in a designated location. The only items allowed at the work area are lab papers and pencils.

22. Place all used culture media or trash that has come in contact with live bacteria or body fluids into the biohazard waste container. Place all other trash in the regular wastebaskets.
Live Bacterial Cultures for BIOL 2420 - Microbiology

**Alcaligenes faecalis**

**Bacillus cereus**

**Bacillus subtilis**

**Escherichia coli**

**Enterobacter aerogenes**

**Micrococcus luteus (Kocuria rhizophila)**

**Proteus vulgaris**

**Pseudomonas aeruginosa**

**Staphylococcus epidermidis**
Aseptic Technique Lab

A. Growth Medium
- contains nutrients that support microbial growth
- uses agar as a solidifying agent
- can be made selective due to nutrients, pH, antibiotics, etc.
1. Nutrient Agar
   - contains nutrients that support growth of a wide variety of bacteria
   - pH of 6.8
2. SDA – Sabouraud Dextrose Agar
   - higher dextrose content: 4%
   - lower pH: 5.6
   - favors fungal growth over bacterial growth
3. MacConkey Agar (used later in semester)
   - selective for Gram-negatives (Gram-positives inhibited by bile salts and dye)
   - differential: lactose fermenters turn agar pink; non-fermenters do not

B. Exercise
1. Obtain 1 NA plate and 1 SDA plate as well as 4 sterile swabs. You will be using the swabs to sample the environment. Samples may come from skin, nails, floor, shoes, money, cell phone, etc., but NO samples from bodily orifices/secretions or from the toilet!!!!
2. Label the bottom of each plate with your name, the date, the type of agar (NA or SDA), and the source of your specimen (doorknob, shoe, etc...). Select 2 different sources, one for each side of the plate.
3. Moisten a swab in the tube of sterile water provided at your table and proceed to roll the swab on the surface of your first source to obtain a sample.
4. Transfer the sample to one side of your NA plate by gently rolling the swab in a zig-zag pattern across the surface of the plate. Be careful not to gouge the agar. Dispose of the swab in the trash.
5. Continue obtaining samples and transferring them onto the agar plates until you have 2 different samples on your NA plate and the same 2 samples on your SDA plate.

C. Growth Results
1. Distinguish between bacterial and fungal colonies (describe each).
2. Distinguish between types of growth on NA vs SDA medium.
3. Based on growth results, are microbes ubiquitous?

D. Aseptic Technique
1. Why is aseptic technique necessary/important?
2. Identify ways to practice aseptic technique.
Basic Microscopy

A. Types of Microscopes
1. Light Microscopes
   - use visible light as source of illumination
   a. Simple Microscope
      - contains a single magnifying lens
      - magnifies up to ~ 300x
   b. Compound Microscope
      - contains more than one magnifying lens
      - total magnification = ocular lens power x objective lens power
      - magnifies up to 1,000x
2. Electron Microscopes
   - use an electron beam as source of illumination
   - use magnets to focus the beam of e-s
   - cannot be used to view living specimens
   a. Transmission Electron Microscope
      - magnifies up to 1 million x
      - useful for studying interior of cell
   b. Scanning Electron Microscope
      - gives extremely detailed 3-D view of objects

B. Structure & Function of the Parts of the Microscope
1. Ocular (eyepiece) – magnifies 10x
2. Objectives: scanning – magnifies 4x
   - low power – magnifies 10x
   - high power – magnifies 40x
   - oil immersion – magnifies 100x
3. Revolving nosepiece – supports objective lenses and rotates them into position
4. Stage – holds slide
5. Mechanical stage control – slide holder and knobs that position slide
6. Stage aperture – opening in stage that allows light to illuminate specimen
7. Diaphragm – controls amount of light entering stage aperture
8. Condenser – concentrates light source into a cone of light that passes through diaphragm
9. Coarse-adjustment knob – brings specimen into focus quickly
10. Fine-adjustment knob – slowly brings specimen into sharper focus
11. Arm – supports coarse- and fine-adjustment knobs; holds head and stage
12. Head – holds oculars and houses mirrors
13. Base – houses light source, supports and stabilizes entire microscope
C. Use & Care of the Microscope – Proper Microscopy Technique

1. Carry the microscope with one hand grasping the arm and the other hand underneath the base.
2. Use lens paper to clean the lenses.
3. Place a slide on the stage, secure with stage clips, and move the stage all the way up.
4. Turn on the light source and center the specimen in the beam of light.
5. View the specimen on scanning power. Use the coarse adjustment and then fine adjustment to focus.
6. Be sure to always center the specimen in your field of view before rotating the nosepiece to change objectives.
7. Under low power, again use both the coarse and fine adjustment knobs to focus.
8. Use ONLY fine adjustment to focus when using high power or oil. (Never use the coarse adjustment when viewing a specimen under high power or oil because it may damage the slide and lens.)
9. After examining the specimen, turn off the light source and allow the bulb to cool before moving.
10. Put the scanning objective back in position, coil up the cord properly, and cover with the dust cover before storing.

D. Oil Immersion Procedure for 1,000x magnification

1. Focus on specimen under high power.
2. Rotate between high power and oil immersion objective.
3. Place a drop of immersion oil on the slide. (Do not touch tip of the oil dispenser to the slide.)
4. Rotate the oil immersion objective into place. (The lens will dip into the oil.)
5. Slowly use fine adjustment to bring into clear view.
6. Never return to a lower objective with oil on the slide!
7. When finished viewing, remove and dispose of slide.
8. Clean objective lens by putting lens cleaner on lens paper!!

E. Exercise

1. View each of the following slides using proper microscopy technique:
   a. prepared slide of human blood smear
   b. other prepared slides of microorganisms as time allows
2. Sketch the specimens at different magnifications: 40x, 100x, and 400x. Observe the difference in appearance as you progress from lower to higher magnification. Use oil immersion only if directed to do so by the instructor.
Media Inoculation

A. Culturing Microbes

1. Inoculation
   - a sample is transferred to the growth medium - provides ample nutrients and space; may be solid or liquid
   - use sterile swabs, inoculating loops, or inoculating needles

2. Incubation
   - allow to grow at optimum T: usually 20-40°C (68-104°F)
   - may be at room temperature or inside incubator
   - cell number increases rapidly

3. Isolation
   - some inoculation techniques separate bacterial cells to form individual colonies – contain a single type of bacteria
   - subculturing will eventually achieve a pure culture – a single species present in the medium
   - we will use the streak plate technique to achieve isolation

4. Inspection
   - look at microbe’s characteristics both macro- and microscopically
   - macro: colony morphology, color, consistency, (smell),...
   - micro: shape, arrangement, stain results, ...

5. Information Gathering
   - additional tests for function and characteristics
   - biochemical tests, immunological tests, genetic typing, ...

6. Identification
   - use analysis and results of inspection and information gathering to identify species
B. Media Inoculation Technique

Always label tubes and plates to be inoculated.

Always practice aseptic technique when inoculating:
1. Wash hands and disinfect countertops.
2. Sterilize loop or needle by holding in open flame. Also flame the neck as well as the needle tip or loop when inoculating tubes. Be sure to allow tool to cool before touching culture.
3. Keep lids on plates and tubes when not in use.
4. Hold tubes at an upward angle.
5. Flame the opening of tubes.

**Broth**
- used to grow large numbers quickly
  1. Use sterile loop to transfer sample from broth culture into sterile broth.

**Plate**
- used for isolating and determining colony morphology
  1. Perform the streak technique as demonstrated by instructor.
  2. Be sure to sterilize the loop between each successive section.

**Slant**
- same use as plate, but conserves material and space
  1. Use sterile loop to obtain sample from broth culture.
  2. Position loop at base of slant surface; zig-zag upward over surface.

**Deep**
- may be solid or semi-solid; used to determine $O_2$ requirements, motility
  1. Use sterile needle to obtain sample from broth culture.
  2. Perform stab by inserting needle into center of deep and extending downward almost to the bottom.
  3. Withdraw needle up through same pathway.
Simple Morphological Negative Stains

Simple Morphological Stains
- use only 1 dye; for determining size, shape, and arrangement
- include positive (direct) stains and negative stains

A. Positive (Direct) Stain
- positively-charged (basic) dye binds to negatively-charged cell surface
- positive dyes: methylene blue, crystal violet, safranin

Procedure
1. Work in pairs, one partner staining a bacillus and one staining a coccus.
2. Smear Preparation:
   a. Place a loopful of distilled water onto the slide and then emulsify a loopful of bacteria into the water. Use the loop to spread the mixture out in a wide rectangular area.
   b. Allow the slide to air dry.
   c. Heat-fix the slide by gently passing it over the Bunsen burner flame a few times. This will cause the bacteria to adhere to the slide and not be rinsed off as easily during the staining process.
3. Apply a few drops of stain to the smear and allow to sit for 1 minute. Be sure to cover the entire smear with stain.
4. Gently rinse the stain with distilled water. Spray the water at the top of the slide and allow it to run over the smear rather than spraying directly onto the smear.
5. Blot the slide with bibulous paper.
6. Examine with the microscope and sketch observations. Sketch the bacillus at 400x and the coccus at 1,000x.

B. Negative Stain
- negatively-charged (acidic) dye is repelled by negatively-charged cell surface
- no heat fixation required, so no distortion of shape
- negative dyes: nigrosin, India ink, Congo red

Procedure
1. Work in pairs, one partner staining a bacillus and one staining a coccus.
2. Place a small drop of stain near the end of a clean slide.
3. Use a sterile inoculating loop to add a loopful of distilled water to the stain.
4. Use the loop to transfer a small amount of bacteria into the water/stain mixture.
5. Emulsify by using the loop to stir the mixture.
6. Use the end edge of another glass slide to spread the mixture along the slide.
7. Allow the smear to air dry.
8. Observe with the microscope and sketch observations. Sketch the bacillus at 400x and the coccus at 1,000x.
Motility

A. Bacterial Motility
- true motility is achieved by the rotation of flagella
- motion due to collision with molecules of surrounding medium is *Brownian* movement and not true motility
- flagella are too thin to be seen with the compound light microscope

1. Flagella Stain
- special staining process leaves a precipitate around flagella so that they appear larger
  a. Observe and sketch prepared slides of flagella stains:
     1) *Proteus vulgaris*
     2) *Spirillum volutans*
  b. Label the flagellar arrangement of each specimen as amphitrichous or peritrichous.

2. Semi-solid Media
   a. Inoculate motility medium by performing a stab.
   b. Incubate until the following week.
   c. Observe growth pattern:
      1) growth only along stab line → non-motile
      2) growth spreading through medium → motile

3. Hanging Drop Procedure
   a. Place a small dab of petroleum jelly at each corner of a cover slip.
   b. If using broth culture, place a loopful of broth onto center of cover slip. If using a plate culture, place 2 loopfuls of distilled water onto center of cover slip and stir in a small sample of bacterial culture.
   c. Place depression slide on top of cover slip so that concavity covers drop.
   d. Invert slide so that cover slip is on top and the drop is hanging into the concavity before placing slide on microscope.
   e. Allow instructor to view on low or scanning before proceeding to high power. Observe movement at 400x on several different slides throughout the class.
Culture Characteristics of Bacteria

A. Culture Characteristics
- unique growth pattern of a particular bacterial species exhibited on different forms of media such as broth, deep, plate
- used to help distinguish between types of bacteria and to identify species
- must be combined with other characteristics (staining, biochemical) to completely identify bacterial species

1. Inoculation
   a. Work in groups of three to five people.
   b. You will receive 5 broth cultures of bacteria: Bacillus, Micrococcus, Pseudomonas, Staphylococcus, and an Unknown.
   c. Pre-label 1 sterile broth, 1 deep, 1 motility tube, and 1 plate for each of the bacterial broth cultures.
   d. Use aseptic technique to properly inoculate each of the pre-labeled forms of media.
   e. Incubate until next week.

2. Inspection & Identification
   - inspect each form of media of each bacterial culture and record observations in the Culture Characterization Table

   a. Broth (a species may have multiple broth characteristics)
      - turbid: cloudy
      - sediment: collection of cells at bottom of tube
      - pellicle: thin film across surface
      - ring: residue around perimeter of surface
      - flocculent: containing small masses or flakes

   b. Deep – shows O₂ requirements
      - obligate aerobe: growth only on surface
      - facultative anaerobe: growth throughout with more at surface

c. Motility Tube
   - motile: growth spreading through semi-solid agar
   - non-motile: growth only along stab line; no spreading

d. Plate - shows colony morphology
   - pigmentation: color
   - appearance: shiny or dull
   - texture: smooth or rough
   - size: pinpoint, small, medium, large
   - shape: circular, irregular, filamentous

   - use your findings to determine the species of your Unknown
<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Broth</th>
<th>Deep</th>
<th>Motility Tube</th>
<th>Colony Morphology (Plate)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacillus</strong></td>
<td></td>
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<tr>
<td><strong>Micrococcus</strong></td>
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<tr>
<td><strong>Pseudomonas</strong></td>
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<tr>
<td><strong>Staphylococcus</strong></td>
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<tr>
<td><strong>Unknown</strong></td>
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</tbody>
</table>

What is the genus of your Unknown? __________________________
A. Kingdom Fungi
- includes both macroscopic and microscopic members
- microscopic members include yeasts and molds

<table>
<thead>
<tr>
<th>Yeasts</th>
<th>vs.</th>
<th>Molds</th>
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</thead>
<tbody>
<tr>
<td>single-celled</td>
<td>-</td>
<td>multicellular</td>
</tr>
<tr>
<td>oval shape</td>
<td>-</td>
<td>consist of filamentous hyphae</td>
</tr>
<tr>
<td>reproduce by budding</td>
<td>-</td>
<td>reproduce by spore formation</td>
</tr>
</tbody>
</table>

B. Observations
- observe and sketch prepared slides of:

1. **Penicillium** mold
   - produces penicillin
   - sketch on high power and label **conidia** – identify as naked or covered, sexual or asexual

2. **Rhizopus** mold
   - black bread mold
   - sketch on low power
   - label **sporangiospores** enclosed w/in **sporangia**
   - label **zygospores** (on different area of slide)
   - identify structures as sexual or asexual

3. **Candida albicans** yeast
   - cause of yeast infections
   - note **dimorphism**
   - sketch on high power; label **yeast form** and **hyphae**

4. **Saccharomyces** yeast
   - used in production of bread and beer (baker’s yeast)
   - sketch on high power and label **buds** – identify as sexual or asexual
   - if time allows, prepare a wet mount of budding yeast cells:
     a. use a pipette to put a small drop of yeast culture on slide
     b. cover with cover slip
     c. adjust diaphragm to reduce light for improved contrast
Direct Count of Yeast Cells

1. Place a disposable counting chamber on the microscope stage. Using the scanning objective, find the ruled area and note the size and arrangements of the squares making up the grid.

2. Pipette 1mL of the yeast suspension provided by the instructor into a test tube containing 1 mL of water in your test tube rack. Replace the lid on the test tube. (You now have a 1:2 dilution of the yeast suspension.)

3. Use the vortex stirrer in the room to shake your test tube of yeast mixture for a few seconds in order to distribute the cells evenly. Be sure the lid is on the test tube when you do this!

4. Take the counting chamber from the microscope (without changing the focus on the 4x objective) and use a pipette to transfer some of the yeast mixture from your test tube into a well opening of the counting chamber. Note: the tip of the pipette needs to touch the well opening to prevent air bubbles. Capillary action will occur to fill the chamber and move the yeast suspension from the opening of the well into the grid area. If you do not observe capillary action occurring within 3 minutes, repeat the process with the other counting chamber on the same slide.

5. Once you see the capillary action occurring, use a paper towel to gently dab any excess yeast mixture from the surface of the slide. Then carefully place the counting chamber back onto the microscope stage and observe the cells under scanning power. You may need to reduce the amount of light by closing the diaphragm.

6. Work up to the high power objective to count the number of yeast cells in a B square (see image on next page). To avoid counting the same cell twice: if it falls on a line, include in your count those cells on the top and left-hand lines and exclude those on the bottom and the right-hand lines. (If the yeast cells are too dense to count, dilute your sample as in step 2 above and start again.) Each person will select 2 B squares to count and average. Record your numbers below and compare results with your partner.
   - Number of cells in first B square selected
   - Number of cells in second B square selected
   - Total number of cells in the two B squares selected
   - Average number of cells per B square

7. Now calculate the number of yeast cells per milliliter by dividing the average number of cells per B square by the volume of each B square. (The volume of each B square is 4x10^-6 milliliters.)

   \[
   \text{Concentration of cells} = \frac{\text{Average number of cells from step 6}}{\text{Volume of each counting square}} = \frac{\text{Average number of cells from step 6}}{4x10^{-6} \text{ milliliters}} = \]

8. If you diluted the sample you must also multiply your results by the dilution factor to determine the concentration of yeast cells in the original sample. (You know you have a dilution factor of at least 2 from step 2 above).

   \[
   \text{Concentration of yeast cells in original sample} = \frac{\text{Average number of cells from step 6}}{\text{Volume of each counting square}} \times \frac{\text{Volume of each counting square}}{\text{Dilution factor}} = \]

   (cells/mL) (dilution factor)
Counting Chamber Slide

This is a B-square.
1 B-square = 4 x 10^{-6} ml
Protozoa & Helminths

A. Protozoa
   - single-celled, heterotrophic protists
   1. Amoeba
      - agent of amebic dysentery
      - observe and sketch prepared slide on low power
      - label nucleus and pseudopodia
   2. Trypanosoma
      - blood parasite (Chaga’s disease; African sleeping sickness)
      - observe and sketch prepared slide on high power
      - label erythrocytes and flagella
   3. Paramecium
      - free-living
      - observe and sketch prepared slide high power
      - label cilia
   4. Plasmodium
      - non-motile agent of malaria; vector is a mosquito
      - observe and sketch prepared slide at high power
      - label erythrocytes and ring stage

B. Helminths
   - multicellular worms: roundworms vs. flatworms
   1. Ascaris
      - intestinal roundworm parasite
      - observe and sketch adult specimens in jars and mounts
      - distinguish between male and female; label spicule of male
   2. Enterobius
      - pinworm; intestinal roundworm parasite
      - observe and sketch prepared slide of adult female on low power
      - note pointed female tail for depositing eggs
   3. Trichinella
      - roundworm parasite of final host; larvae encyst in muscle
      - observe and sketch prepared slide of larvae on low power
      - label larvae and muscle tissue
   4. Fasciola
      - fluke: observe adult specimen in jar, noting flat body
   5. Taenia
      - tapeworm; intestinal parasite of carnivores
      - observe prepared slide of adult on low power
      - note: slide has 4 sections – start with scolex and progress to more mature proglottids as you move toward opposite side
      - sketch scolex and label hooks and suckers
      - observe immature proglottids (not filled with eggs)
      - observe gravid / mature proglottids (filled with eggs)
Biochemical Tests

A. Biochemical Tests
- used to evaluate metabolic properties of specific bacteria
- used in conjunction with staining and culture characterization to definitively identify bacteria

1. Catalase Test
The catalase enzyme catalyzes the breakdown of $\text{H}_2\text{O}_2$ (hydrogen peroxide) into $\text{H}_2\text{O}$ and $\text{O}_2$. If bacteria have the catalase enzyme, bubbling will occur from the breakdown of $\text{H}_2\text{O}_2$.
   
   Negative result: no bubbling
   Positive result: bubbling $\text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{O}_2$ (bubbles)

2. Denitrification Test
In denitrification, the substance potassium nitrate is converted to nitrogen gas. If bacteria have the nitrate reductase enzyme to break down the nitrate ($\text{NO}_3$) in nitrate broth, there will be nitrogen gas ($\text{N}_2$) bubbles produced. Note the small inverted tube in the bottom of the nitrate broth medium. This is called a durham tube and collects gases generated by the culture.
   
   Negative result: no bubbles in durham tube
   Positive result: bubbles in durham tube $\text{NO}_3 \rightarrow \text{N}_2$ (bubbles)

3. Hydrogen Sulfide (H₂S) Production
Sulfide Indole Motility (SIM) media contains the amino acid cysteine as well as iron. If bacteria have the cysteine desulfurase enzyme to catalyze the breakdown of cysteine to produce H₂S, the H₂S will combine with the iron to produce iron sulfate which blackens the medium.
   
   Negative result: no color change
   Positive result: blackening of the medium $\text{cysteine} \rightarrow \text{H}_2\text{S}$
   $\text{H}_2\text{S} + \text{Fe} \rightarrow$ blackening

4. Indole Production
SIM media also contains the amino acid tryptophan. If bacteria have the tryptophanase enzyme that catalyzes the breakdown of tryptophan to indole, the indole will react with Kovac’s reagent to form a red color.
   
   Negative result: no color change
   Positive result: red color $\text{tryptophan} \rightarrow \text{indole}$
   $\text{indole} + \text{Kovac's} \rightarrow$ red
5. **Methyl Red Test**
Methyl red pH indicator is a yellow to orange color at slightly acidic levels (5-6), but will turn red at more acidic levels (below 4.4). If bacteria have multiple enzymes that break down sugars and produce enough acid to drop the pH to about 4, the pH indicator will turn red. If bacteria do not have multiple enzymes for the breakdown of sugar, fewer acids will be produced and the pH indicator will remain yellow.

- Negative result: yellow glucose → pyruvic acid only
- Positive result: persistent red color glucose → pyruvic, acetic, formic

6. **Oxidation-Fermentation Test**
The Oxidation-Fermentation test determines oxygen requirements for the breakdown of sugar. O-F media contains sugar as well as a color indicator that is green at neutral pH and turns yellow when the pH drops below 6. If bacteria have the enzyme to catalyze the breakdown of sugar, acid will be produced and the medium will turn yellow. The addition of oil to a tube creates an anaerobic environment; the absence of oil renders the environment aerobic.

- Inert to the medium (O$_2$ is irrelevant): both tubes stay green
- Obligate aerobe: tube w/ oil remains green; tube w/out oil turns yellow
- Facultative anaerobe: both tubes turn yellow

### B. Session 1 – Inoculation
Work in groups of 3-5. Label all biochemical media tubes with the genus with which it is to be inoculated as indicated below.

1. **Catalase Test**
   Inoculate 1 plate with *Staphylococcus*.

2. **Denitrification Test**
   Inoculate 1 nitrate broth tube with *Alcaligenes* and 1 with *Pseudomonas*.

3. **H$_2$S Production**
   Inoculate 1 SIM tube with *E. coli* and 1 SIM tube with *Proteus*.

4. **Indole Production**
   Inoculate the remaining SIM tube with *Enterobacter*.

5. **Methyl Red Test**
   Inoculate 1 MR-VP tube with *Enterobacter* and the other with *E. coli*.

6. **Oxidation-Fermentation Test**
   Inoculate 2 O-F tubes with *E. coli* and 2 with *Pseudomonas*.
   Place a dropper of mineral oil in 1 of the *E. coli* and also in 1 of the *Pseudomonas*. 
C. Session 2 – Results

1. **Catalase Test**
   Place 2 drops of hydrogen peroxide on a clean slide. Emulsify a loopful of *Staphylococcus* into the peroxide.
   Expected results: *Staphylococcus* – catalase-positive

2. **Denitrification Test**
   Examine the durham tubes in the nitrate broth for bubbles.
   Expected results: *Pseudomonas* – denitrification-positive
                  *Alcaligenes* – denitrification-negative

3. **H₂S (hydrogen sulfide) Production**
   Examine the SIM tubes inoculated with *Proteus* and *E. coli* for color change.
   Expected results: *Proteus* – H₂S-positive
                   *E. coli* - H₂S-negative

4. **Indole Production**
   Place 5 drops of Kovac’s reagent into the SIM tubes that were inoculated with *E. coli* (also used previously in test 3) and *Enterobacter*.
   Expected results: *E. coli* – indole-positive
                 *Enterobacter* – indole-negative

5. **Methyl Red Test**
   Place 5 drops of methyl red pH indicator in each MR-VP tube. Gently swirl the tube and examine for color change.
   Expected results: *E. coli* – methyl-red-positive
                  *Enterobacter* – methyl-red-negative

6. **Oxidation-Fermentation Test**
   Examine the O-F tubes for color changes. Be sure to note whether or not oil is in each tube.
   Expected results: *Pseudomonas* – obligate aerobe
                    *E. coli* – facultative anaerobe
<table>
<thead>
<tr>
<th>Biochemical Test</th>
<th>Reagent</th>
<th>Observations</th>
<th>Results</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Catalase Test:</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Staphylococcus</td>
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<tr>
<td>2 Denitrification:</td>
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<td></td>
<td>Pseudomonas</td>
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<tr>
<td></td>
<td>Alcaligenes</td>
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<tr>
<td>3 H₂S Production:</td>
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<td></td>
<td>Proteus</td>
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<tr>
<td></td>
<td>E. coli</td>
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<td>4 Indole Production:</td>
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<tr>
<td></td>
<td>E. coli</td>
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<td></td>
<td>Enterobacter</td>
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<tr>
<td>5 Methyl Red Test:</td>
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<td></td>
<td>E. coli</td>
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<tr>
<td></td>
<td>Enterobacter</td>
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<tr>
<td>6 Oxidation-Fermentation</td>
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<td></td>
<td>E. Coli</td>
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<tr>
<td></td>
<td>Pseudomonas</td>
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</table>
Differential Stains: Capsule, Endospore, and Gram Stain

Differential Stains
- use 2 different dyes
- differentiate between cell types or cell structures

A. Capsule Stain
- differentiates between capsule, cell, and background
  1. Observe a prepared slide of a capsule stain.
  2. Sketch on high power, noting the capsule.

B. Endospore Stain
- differentiates between spores and vegetative cells
  1. Observe prepared slides of spore stains.
  2. Sketch on high power, noting whether the endospore has a central or terminal location within the vegetative cell. Also note any free spores.

C. Gram Stain
- differentiates chemical composition of cell wall:

<table>
<thead>
<tr>
<th>Gram-positive</th>
<th>vs.</th>
<th>Gram-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>- thick peptidoglycan</td>
<td>- thin peptidoglycan</td>
<td></td>
</tr>
<tr>
<td>- no outer membrane</td>
<td>- outer membrane (highly negative)</td>
<td></td>
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<tr>
<td>- stain purple</td>
<td>- stain pink</td>
<td></td>
</tr>
</tbody>
</table>

Components of Gram Stain
Primary stain: crystal violet – stains all cells purple
Mordant: Gram’s iodine – fixes crystal violet into cell wall of G+ cells
Decolorizer: ethyl alcohol – removes crystal violet from G- cells
Counterstain: safranin – stains G- cells pink
Gram Stain Procedure

Work in groups of 3-4: 1 will stain a Gram-positive control, 1 will stain a Gram-negative control, and the other(s) will stain a mixed culture of both.

1. Prepare a smear of the bacteria.
2. Apply crystal violet to the smear. Allow to sit for 1 minute and then rinse with distilled water.
3. Apply Gram’s iodine and allow to sit for one minute. Rinse with distilled water.
4. Rinse gently with ethyl alcohol. Do not over-rinse - stop rinsing after a few seconds. Rinse with distilled water.
5. Apply safranin and allow to sit for 1 minute. Rinse with distilled water.
6. Blot dry with bibulous paper.
7. Observe on high power. Continue on to oil immersion as necessary.
8. Sketch and label the Gram-positive control, the Gram-negative control, and a mixed sample.

Purple indicates Gram-positive while pink indicates Gram-negative. Remember that the Gram-positive/Gram-negative designation is important for describing cell wall structure and thus appropriate treatment measures.
Identification of Unknown

Week 1
1. Record broth characteristics.
2. Inoculate TSA and MacConkey agar plates using quadrant streak technique.
3. Use broth culture sample to perform a Gram stain and record cell shape, cell arrangement, and Gram result.
4. Use the dichotomous key to determine which biochemical test should be run and set it up before leaving.

Week 2
1. Record colony characteristics from TSA streak plate.
2. Double check your Gram result from the Gram stain last week with today’s MacConkey agar plate results. (Gram-positives will not grow on MacConkey agar; only Gram-negatives will grow on MacConkey.)
3. Record biochemical test results from the previous week.
4. Double check your O-F result with the MacConkey plate. (Lactose-fermenters will turn the agar pinkish/red: this indicates a facultative anaerobe. Aerobes will not ferment the lactose and will not turn the agar pinkish/red.)
5. Use the dichotomous key to determine which biochemical test(s) should be run next and set it up before leaving.

Week 3
1. Record biochemical test results from previous week.
2. Use the dichotomous key to identify unknown by genus name.
3. Turn in Laboratory Report and Dichotomous Key (traced out).
   - be sure your name and student ID are on each paper
# Laboratory Report

## Identification of a Nonclinical Bacterial Unknown

**Unknown:** __________

**Name:** ________________  **Student ID #:** __________  **Date:** __________

<table>
<thead>
<tr>
<th>Culture Characteristics</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broth Characteristics</td>
<td></td>
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<tr>
<td>Colony Characteristics</td>
<td></td>
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<tr>
<td>from TSA plate</td>
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<tr>
<td>MacConkey plate</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Staining Characteristics</th>
<th>Observations</th>
<th>Results</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Shape</td>
<td>xxxxxxxxxxx</td>
<td>xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx</td>
<td></td>
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<tr>
<td>Cell Arrangement</td>
<td>xxxxxxxxxx</td>
<td>xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx</td>
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<tr>
<td>Gram Stain</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Biochemical Characteristics</th>
<th>Reagents Added</th>
<th>Observations</th>
<th>Results</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase Test</td>
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<tr>
<td>Denitrification Test</td>
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<tr>
<td>H₂S Production</td>
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<tr>
<td>Indole Production</td>
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<tr>
<td>Methyl Red Test</td>
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<tr>
<td>Oxidation-Fermentation Test</td>
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</tbody>
</table>

## Conclusion

I conclude that my unknown is ________________________________
Dichotomous Key for Identification of Unknown

Gram Stain

<table>
<thead>
<tr>
<th>Coccus</th>
<th>Bacillus</th>
</tr>
</thead>
<tbody>
<tr>
<td>G (-)</td>
<td>G (+)</td>
</tr>
</tbody>
</table>

**Neisseria**
- (+) Catalase (-)
- (OF) glucose

**Enterococcus**
- aerobe
- facultative anaerobe

**Micrococcus**
- aerobe

**Staphylococcus**

**Pseudomonas**
- facultative anaerobe (lactose-fermenter)
- indole (-)
- H₂S (-)

**Enterobacter**
- indole (-)
- H₂S (-)

**E. coli**
- indole (+)
- H₂S (+)

**Alcaligenes**
- denitrify (+)

**Mycobacterium**
- denitrify (-)
- (-) spores (+)

**Bacillus**
- aerobic
Microbial Control

A. Chemical Control
   1. Antiseptics
      - Active ingredient:
      Antibacterial hand soap
         - active ingredient: __________
      Hand gel sanitizer
         - active ingredient: __________
   2. Disinfectants
      - Active ingredient:
      Brand: __________
         - active ingredient: __________
      Brand: __________
         - active ingredient: __________
      Brand: __________
         - active ingredient: __________
      Brand: __________
         - active ingredient: __________

B. Antibiotics
   - Terms: Kirby-Bauer Method, Mueller-Hinton agar, bacti-spread, lawn, zone of inhibition, narrow spectrum vs. broad spectrum antibiotics, resistant vs. susceptible bacteria

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Bacillus (G+ rod)</th>
<th>Staphylococcus (G+ coccus)</th>
<th>E. coli (G- rod)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (Am)</td>
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<tr>
<td>Bacitracin (B)</td>
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<td>Penicillin (P)</td>
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<tr>
<td>Tetracycline (T)</td>
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<td>Vancomycin (Va)</td>
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</table>
A. Yogurt
- prepared by the action of fermentative bacteria (i.e., *Streptococcus thermophilus*, *Lactobacillus acidophilus*) on milk
- the lactic acid produces a tart taste and causes the milk protein (casein) to precipitate, thickening the product

B. Procedure
1. Pipet 1 mL of distilled water into test tube.
2. Add a small sample (1 loop) of yogurt to the tube and mix.
3. Replace cap and gently shake for 5 to 10 seconds.
4. Transfer 2 loopfuls of tube mixture to slide and prepare a smear (including air drying and heat fixing).
5. Proceed with Gram stain procedure.
6. Observe on high power. Check with instructor before proceeding to oil.

C. Results
1. Sketch the 2 types of bacteria observed in the yogurt.
2. Label cell shape and cell arrangement.
3. What is the Gram reaction of these bacteria? Describe their cell wall.