Exercise 24-A
MICROBIAL CONTROL METHODS
(Effects Of Temperature, Ultra Violet Light, Disinfectants And Antiseptics)

Introduction

Microorganisms, like all other life forms, are greatly influenced by the environments they inhabit. Environmental effects can be divided into three general categories: **physical** (the effects of temperature, radiation, pressure, etc.), **chemical** (the effects of nutrient availability, toxic substances present, etc.) and **biological** (influences exerted by other organisms coexisting in the area). Biological influences are frequently chemical in nature, but are exerted by living organisms.

There is an optimum set of environmental conditions under which each microbial species grows and reproduces best; but there is a wider range of tolerance among microbes in general than there is among "higher" organisms (such as people), and many microorganisms tolerate environmental extremes. Bacteria are especially well adapted to cope with environmental extremes since they may temporarily stop their enzymatic activities without actually dying. When conditions become more favorable, the cells become active again. Even bacteria, however, cannot cope with environmental factors such as extreme heat and high concentrations of chemical toxins. Both of these forces inactivate enzymes and/or other proteins, and so stop life processes.

The experiments associated with this exercise are designed to demonstrate some of the responses of microorganisms to changes in environmental conditions such as temperature, radiation and chemical composition. Because these factors impact microbial function, they can be and often are employed as microbial control methods.

A. Effects of Temperature on Microbial Function:

Various bacteria have distinct requirements as to the temperature at which they will grow. Maximum growth occurs within a rather limited range known as the **optimum temperature**. This is generally correlated with the temperature of the normal environment of the organisms, or where they are usually found. A change in temperature above a maximum, or below a minimum often results in limited or halted growth.

Temperature exerts its effect through the activity of bacterial **enzymes**. Since bacteria lack mechanisms to conserve or dissipate the heat generated by metabolism, and because of their small size, their enzyme systems are influenced directly by changes in **ambient** (outside) temperature. At optimum temperatures, the bacterial enzymes function at full speed, but above or below the optimum range, enzyme activity is decreased or in some cases stopped.

At temperatures near or below freezing, essentially all metabolic activities cease because there is no liquid water available in the cell, and because enzyme activity is stopped. When temperatures exceed a maximum, enzymes and proteins stop functioning due to **denaturation**, i.e., they experience changes in their molecular structure (secondary, tertiary and/or quaternary). Very high temperatures result in permanent damage and ultimately the death of bacteria, while very low temperatures often stop function on only a temporary basis (most bacteria are **psychroduric**, so can endure considerable exposure to cold without being harmed). Bacteria collections are maintained frozen (-70 to -90^{o} C) and samples from such collections grow readily when provided appropriate media at optimum temperatures.

In the first section of this exercise we will observe the effects of temperature on enzyme activity by observing two bacterial features, pigment production, and cell viability. We will also review some effects of temperature as demonstrated in previous lab exercises.

A-1. Effect of Temperature on Pigment Production

The red variety of *Serratia marcescens* produces a red-colored, non-water soluble pigment called **prodigiosin** (prodigious = extraordinary) that is usually evident when these organisms are grown in vitro at room temperature. Colonies may be totally red, or show a red center, red margin, or red sectors. The exact color is variable and is dependent upon growth conditions (amino acids available, etc.). Some of the enzymes involved in pigment production are temperature sensitive, with their range for optimum activity being between 12 and 36^{o} C. Prodigiosin production is common among environmental isolates of *Serratia marcencens* and the pigment may serve to reduce the number of competitive microbes as it displays antibacterial, antifungal and antiprotozoal activity.

Procedure: (Students may work in pairs or in groups of three.)

1. Obtain two tryptic soy agar plates, and streak each one with the culture of *Serratia marcescens* provided. For best results, streak your plates to obtain well-isolated colonies.

2. Incubate one plate at 37^{o} C (in the incubator) and the other at 25^{o} C (in your laboratory drawer).

3. During the next laboratory period, or after 24-48 hours of growth, compare the colonies present on the two agar plates. Record the variation in morphology (pigment production) evident, and share this information with other members of the class.

A-2. Effect of Temperature on Cell Viability in Water

Clean water is a precious commodity often taken for granted by humans living in this area. Many residents of Placer and Nevada County receive water from Lake Spalding through surface canals, and use this for irrigation, drinking, bathing, etc. Lakes such as Folsom, Nimbus, Tahoe and others serve as recreational areas, and rivers are frequented by summer visitors (rafters, swimmers, etc.). Small streams are used for fishing, and as sources of drinking water for hikers and backpackers. Although these local water sources typically appear clean, they actually contain large numbers of microorganisms, some of which can be pathogens. In this exercise, students will use fresh samples of locally obtained water to test the effects of temperature on cell viability and will gain an appreciation for the number and variety of bacteria in untreated water.

Procedure: (Students may work in pairs or in groups of three.)

1. Obtain three nutrient agar plates, a 100-1000μL pipette (blue tips), and a 400mL beaker. Fill the beaker about half full with water and place it on a ring stand above a lit burner.

2. Obtain a sterile culture tube and fill it 1/3 full with one of the water samples provided (surface water collected from a local water source in a sterile container), and label your agar plates with the name of this source water and the treatment applied in each case as follows (one per plate):
   a. Fresh water – no treatment
   b. Boiled 1 minute – Pasteurized
   c. Boiled 5 minutes - sanitized

3. Using aseptic technique, transfer 500μL of water from your culture tube to the plate labeled "no treatment".

4. Place the tube of water in the boiling water bath for one minute and then, using a new, sterile pipette tip, transfer 500μL of Pasteurized water to the plate labeled "boiled 1 minute".

5. Return the tube to the boiling water bath for an additional 4 minutes and then, using a new, sterile pipette tip, transfer 500μL of sanitized water to the plate labeled "boiled 5 minutes".

6. Obtain or prepare a glass, spreading rod, sterilize it in the burner flame, and then allow it to cool.

7. Starting with the sanitized water plate, use the glass spreader to spread the inoculum evenly over the entire agar surface, then do the same with the Pasteurized water plate and then the untreated water plate. **Do not reverse the plate order during this procedure!**

8. Tape the three plates together in a stack, and then invert them (quick flip) so that the agar side is up. Place the plates in the 37^{o} C incubator until the next lab session.

9. During the next lab session, observe the three plates and compare the growth visible on their surfaces. Estimate how many different types of bacteria are present on each plate and compare their overall numbers to determine the effect of temperature on cell viability. Remember that colony-forming units (cfu) are often single cells.

Review Information

The effect of temperature on microbial growth was demonstrated in an earlier laboratory exercise on the Culture of Selected Bacteria from the Environment. By boiling a soil suspension for one minute we selected for spore-forming organisms. Most vegetative cells in the soil sample (*Pseudomonas*, *Azotobacter*, *Streptomyces*, etc.) were killed by exposure to high temperatures. We also saw that air organisms (e.g., *Micrococcus*) grow best at room temperature rather than at 37^{o} C (incubator temperature). Keep this information in mind when working with unknown samples.

B. Effect of Temperature and Pressure on Microbial Growth:

Moist heat (steam) under pressure is a common method used in the control of microorganisms and is the method used in this laboratory to sterilize most materials prior to use. The instrument used for this method is called an **autoclave**, and is located in the media preparation facility. Most media, glassware, and used materials are autoclaved at 120^{o} C (250^{o} F) for 15 minutes using steam under 30 pounds pressure. This treatment effectively kills all microorganisms likely to contaminate our materials.

C. Effect of Osmotic Pressure and pH on Microbial Growth:

The growth of microorganisms can be greatly affected by the osmotic pressure and/or pH of the environment. When the medium surrounding organisms is **hypotonic** (low in solute content) there is a tendency for water to flow into cells through osmosis. Except for some marine forms (those living in salt water) this is not harmful to most bacteria because their rigid cell walls prevent rupture. Cells placed in a **hypertonic** (high in solute content) environment tend to loose water, and this may greatly influence metabolic processes. Depending on the particular organism type, the **tonicity** (effective osmotic pressure) of the environment, and the nature of the solute involved, inhibition due to osmotic pressure may be temporary, or may cause irreversible damage to the cells present.

The **pH** (power of hydrogen, or -log of the hydrogen ion concentration) influences bacterial growth by acting on enzymes involved in the production of new protoplasm. Microorganisms in general have pH optimums at which they grow best, but these are somewhat variable depending on other factors such as media type, temperature, and osmotic pressure.

We will not complete a lab activity specifically designed to demonstrate the effects of pH and osmotic pressure, but students should be aware of the following as demonstrated during past exercises.

1. Bacteria and fungi have different pH requirements for growth, and this is a primary factor in selectively growing these organisms. The potato dextrose agar and other media used to culture fungi have a pH of 5.6 at 25^{o} C, while nutrient agar (and most other bacteriological media) has a pH of 6.8 at 25^{o} C.

2. In the exercise on food Microbiology (fermented foods) we learned that the production of Sauerkraut requires a gradual change in the types of organisms present due to pH change. It is also the acidic pH that prevents the growth of unwanted contaminants, thus preserving the food.

3. The selective property of mannitol salt agar (MSA) is due to the high solute content (MSA contains 7.5% NaCl). This is an example of growth restriction due to osmotic pressure.

D. Effect of Radiation (Ultra Violet Light) on Microbial Growth:

Different types of radiation (**electromagnetic waves** from the sun or other sources) vary considerably in wavelength and other properties. Some of the categories into which these radiation types can be divided include: Infrared rays (greater than 800 nm), visible rays (400-800 nm), ultra violet rays (roughly 4-400 nm), X-rays, Gamma rays, and cosmic rays. The form of radiation most readily used by microbiologists is ultra violet (UV light). Although ultra violet rays have very little penetrating power, they are strongly **microbicidal** (lethal to microbial cells), **mutagenic** (known to causes mutations), and **carcinogenic** (known to cause cancer) on direct contact. The wavelength found to be the most lethal and mutagenic to bacteria is 260-270 nm. This wavelength is strongly absorbed by the nucleic acids of cells (DNA absorption has a peak at 260 nm) and by some proteins (270-360 nm).

Procedure: (Students should work in pairs or groups of three.)

1. Obtain four agar plates and label them to indicate which microorganism type you are using for this portion of the exercise. Use nutrient agar if the bacteria are *Serratia* or *Bacillus* and Tryptic soy agar (TSA) if the bacteria used are *E. coli*, *Staphylococcus* or *Pseudomonas*. DO NOT use *Proteus* for this activity.

2. Inoculate each plate with one of the cultures provided using a sterile cotton swab dipped in broth culture. Do not use a variety of organisms, but inoculate all four plates with the same culture. To avoid dripping the bacteria on the work area, squeeze excess liquid from the cotton by rolling it gently against the inside of the tube before streaking your plate. Spread the inoculum over the entire agar surface so as to attain complete coverage (a "lawn" of growth). Remember; DO NOT use *Proteus* for this activity.

3. Expose two of your plates to the UV light source for one minute, one with the cover in place, and one with the cover removed.

4. Expose a third plate for 5 minutes after removing the cover, and placing a strip of cardboard (3x5 card) over the plate so that it covers 1/2 of the agar surface.

5. Use the remaining plate as a control, i.e., do not expose it to the ultra violet light.

6. Incubate all plates (agar side up) at 37^{o} C until the next laboratory period.

7. Observe the effects of the UV light, and record your results. Compare your results with those obtained by other members of the class.

E. Effect of Chemicals (Antiseptics & Disinfectants) on Microbial Growth:

An **antiseptic** is a chemical agent designed to control pathogens on **living** surfaces. Literally the word means "opposition to sepsis or decay". A **disinfectant** is a chemical agent used to control pathogens on **non-living** surfaces. An antiseptic could be defined as a disinfectant that can be used on living surfaces without causing damage to the tissues present, but most disinfectants are actually too strong to be used in this way. Certain chemicals can be categorized as either antiseptics or disinfectants depending on how they are used.

In order to test the effectiveness of various chemical agents, student groups will spread inoculum (bacterial culture) on an agar plate with a cotton swab, and then place filter paper discs soaked with various types of chemicals on the plate surface. After incubating the plates for 24-48 hours, they can be examined to determine the degree to which growth was inhibited.

Procedure: (Students should work in pairs or in groups of three.)

1. Select one of the cultures provided and the appropriate medium as indicated in section “D” above. Using a sterile cotton swab dipped in broth culture, spread the inoculum over the entire agar surface to create a "lawn" of growth.

2. Obtain five clean, sterile, filter paper discs and place them on a clean sheet of paper towel such that they are spaced at least two inches away from one another. Add one of the following types of chemicals to each disc:

    a. Iodine (halogen)
    b. Ethanol or isopropanol (alcohols)
    c. Mercurochrome, copper sulfate, or silver nitrate (metal ions)
    d. Laboratory disinfectant (surfactant)
    e. Carbol fuchsin (phenol derivative)

3. Mark the under side (agar side) of the plate with the letters representing each type of chemical (as listed above) to indicate where each disc will be placed.

4. With a pair of sterile forceps, place each type of disc onto the inoculated agar surface at the appropriate location. Tap each disc gently to secure it to the agar surface.

Incubate the plate for 24-48 hours and then examine it for **zones of inhibition**, i.e., zones around the discs where bacterial growth has been inhibited. Compare the zones on your plate with those obtained by other groups to roughly determine which chemicals are the most effective in controlling the various bacteria being tested (compare especially Gram-positive VS Gram-negative forms). Note – Because we do not know the concentrations of all the chemicals used, nor their diffusion rates, we cannot quantify the results obtained; however, by comparing the different plates we can obtain some information about the extent to which these chemicals affect microbial growth.

Questions:

1. What is the name of the red-colored pigment produced by *Serratia marcescens*?

2. What variation in pigment production was visible when *Serratia marcescens* was grown at 25^{o} C and at 37^{o} C?

3. What is the overall effect of high temperatures (heat) on bacteria suspended in fresh water? Does exposure time influence this effect?

4. The device used in this lab to sterilize media and glassware is called a/an _____ and uses what physical factors to kill microbes?

5. Name one test medium or experimental procedure that demonstrates the effects of (A) pH and (B) osmotic pressure on microbial growth.

6. What is Ultra Violet radiation? What wavelength of UV radiation is most effective in controlling microorganisms?

7. Were the chemical agents you tested more effective in the control of Gram-negative organisms or Gram-positive organisms? What explanation can you give for the variation in results obtained?

Name_____ Lab Section_____

WORKSHEET

Exercise 24A

Microbial Control Methods

Goals: _____

_____

_____

Materials & Methods:

A-1. Effect of Temperature on Pigment Production

Date: _____ Organism type used: _____

Medium used: _____

Incubation temperatures: _____ Duration of incubation: _____

A-2. Effect of Temperature on Cell Viability in Water

Date: _____ Source of water: _____

Medium used: _____

Incubation temperature: _____ Duration of incubation: _____

Time Exposed to Boiling Conditions:

"Untreated" = _____ "Pasteurized"= _____ "Sanitized" = _____

D. Effect of Ultra Violet Radiation

Date: _____ Organism type used: _____

Medium used: _____

Incubation temperature: _____ Duration of incubation: _____

UV Treatment:

Plate	Duration of UV Exposure	Lid Placement?
1		
2		
3		
4		

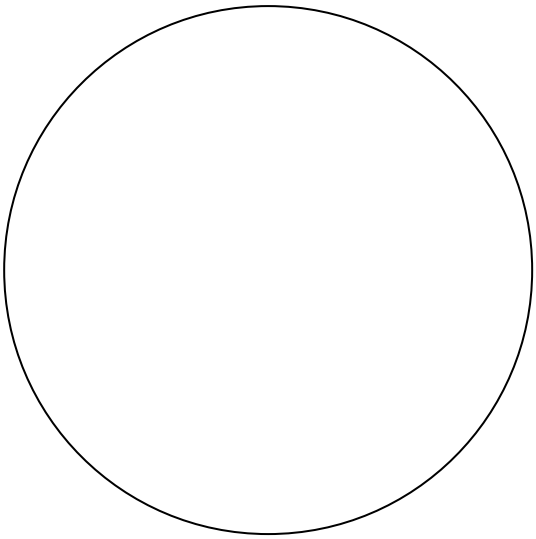
E. Effect of Antiseptics & Disinfectants

Date: _____ Organism type used: _____

Medium used: _____

Incubation temperature: _____ Duration of incubation: _____

Use the space below to indicate which chemical agents you used and where you placed each one.



Data & Results:

A-1. Effect of Temperature on Pigment Production

	Pigment?
Room Temperature Plate	
37°C plate	

A-2. Effect of Temperature on Cell Viability in Water

	Total Number of Colonies	Number of Colony Types
"Untreated"		
"Pasteurized"		
"Sanitized"		

D. Effects of Ultra Violet Radiation

Plate	Duration of UV Exposure	Lid Placement?	Number of Colonies
1			
2			
3			
4			

E. Effects of Antiseptics & Disinfectants

Chemical Agent	Zone of Inhibition (Visible/Size)

Additional Notes: _____
_____
_____

Conclusions:

Based on your data, do the words pasteurized and sanitized correctly describe what occurred? _____ Explain _____
_____
_____
_____

Did UV light affect the growth of the organism type tested? _____ Explain. _____
_____
_____
_____
_____

Which chemical agent(s) was your organism type sensitive to? _____
_____
_____

Which chemical agent(s) was your organism type resistant to? _____
_____
_____

NOTES, OBSERVATIONS & ADDITIONAL INFORMATION