ENVIRONMENTAL PARAMETERS OF GROWTH

The growth and survival of microorganisms are affected by the chemical and physical conditions of the external environment. Environmental factors which have significant effects on microbial growth and activity include temperature, oxygen, pH and osmotic pressure (water availability). In this exercise, the effect of temperature and pH will be studied. **NOTE:** Although we will attempt to vary only one parameter at a time, you should be aware that changes in one parameter can affect the other parameters. In a natural environment, an organism must contend with all of the interactive changes that occur.

TEMPERATURE

The rate of microbial growth depends on temperature and how temperature affects the activity of enzymes. Unlike homeotherms, bacteria lack mechanisms which conserve or dissipate heat generated by metabolism, and consequently their enzyme systems are directly affected by environmental temperatures. Bacteria, as a group, grow in a broad temperature range that extends from approximately -20°C to above 122°C. Each species, however, requires a narrower range that is determined by the heat sensitivity of enzyme systems. Specific temperature ranges consist of three cardinal temperature points:

1. **Minimum growth temperature:** The lowest temperature at which growth will occur. Below this temperature, enzyme activity is inhibited and the cells are metabolically inactive, so that growth is negligible or absent.

2. **Maximum growth temperature:** The highest temperature at which growth will occur. Above this temperature, most enzymes are denatured and the organism dies.

3. **Optimum growth temperature:** The temperature at which the rate of growth is most rapid; however, it is not necessarily optimum for all enzymatic activities of the cell.

All bacteria can be classified into one of three major groups: **psychrophiles** that are able to grow at temperatures between 0°C and 5°C; **mesophiles** that grow within a temperature range of 20°C to 45°C; and **thermophiles** that grow at temperature greater than 45°C.

In this experiment, we will determine the approximate optimum growth temperatures for four microorganisms. To do this, we will incubate cultures at four different temperatures and record turbidity after ~48 hours.

pH

Each bacterial species has the ability to grow within a specific pH range, which may be broad or limited, with the most rapid growth occurring within a narrow optimum range. Most natural environments have pH values between 5 and 9, and organisms with pH optima in this range are most common. The pH optimum for most bacteria is between 6.5 and 7.5, making them **neutrophilic**. Fungi, molds, and yeast prefer an acidic environment, with optimum activities at a pH of 4 to 6. Only a few species can grow at pH values of less than 2 or greater than 10. Organisms that live at low pH are called **acidophiles**; organisms with high pH optima are **alkaliphilic**.

FIRST PERIOD

Material:

1. Sixteen 9-ml dilution tubes of nutrient broth
2. Three tubes of nutrient broth, pH 3.0
3. Three tubes of nutrient broth, pH 5.0
4. Three tubes of nutrient broth, pH 6.0
5. Three tubes of nutrient broth, pH 7.0
6. Three tubes of nutrient broth, pH 10.0
7. Broth cultures of: *Escherichia coli*
Staphylococcus aureus
Pseudomonas aeruginosa
Micrococcus roseus
Geobacillus stearothermophilus

Procedure: (work in groups of 4)

A. Effects of Temperature on Microbial Growth

1. Inoculate four nutrient broths with one of the four bacterial cultures (*E. coli; Geobacillus stearothermophilus; Pseudomonas aeruginosa; Micrococcus roseus*).

2. Repeat the procedure for each of the remaining three cultures

3. Incubate one tube of each "set" for 24 to 48 hours at **4°C**, one at **25°C**, one at **37°C**, and one at **50°C**.

B. Effects of pH on Microbial Growth

1. Inoculate the series of pH-adjusted nutrient broths with *E. coli*.

2. Repeat step **1** with *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

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

SECOND PERIOD

Observe the growth in the tubes. Measure and record the growth of these cultures using the turbidimetric method.

1. Once again, using the spectrophotometer, determine the optical density (OD) of each culture under the array of temperature and pH conditions. Make sure to blank with the different broth media types. Note: If serial dilutions are necessary, use dilutions by a factor of two (i.e., 1/2, 1/4, 1/8) from each broth culture to get your best estimate from between 0.1 and 1.0 OD_{600} units.

2. Record your results. Graph your data accordingly using 2-cycle log paper (or similar). Plot temperature and pH on separate graphs. Make sure you know the difference between to abscissa (x-axis) and the ordinate (y-axis).

Record OD_{600} data:

Temperature	*E. coli*	*Geobacillus stearo.*	*Pseudomonas aeruginosa*	*Micrococcus roseus*
4°C				
25°C				
37°C				
45°C				

pH	*E. coli*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
3			
5			
6			
7			
10			

ENVIRONMENTAL PARAMETERS OF GROWTH

OXYGEN

The oxygen requirements of bacteria range from the **strict** (obligate) **aerobes** that cannot exist in the absence of oxygen to the **strict** (obligate) **anaerobes** that cannot exist in the presence of oxygen. All bacteria can be classified into one of four groups:

1. **aerobes -** bacteria that grow in the presence of oxygen;
2. **anaerobes -** bacteria that grow in the absence of oxygen;
3. **microaerophiles -** organisms that require free oxygen, but only in limited amounts (at levels lower than atmospheric O_2 concentrations);
4. **facultative -** organisms that grow either in the presence or absence of free oxygen.

In this experiment, we will inoculate liquid thioglycollate media with several organisms that have different oxygen requirements. The test organisms will be obligate aerobes, microaerophiles, or facultative anaerobes; we do not have strict anaerobes in our culture collection.

Thioglycollate medium is a rich medium that supports the growth of both aerobic and anaerobic bacteria. It contains sodium thioglycollate, which is a strong reducing agent that reacts with O_2 and reduces it to H_2O. The medium also contains a redox indicator dye called resazurin. Resazurin in the reduced form is colorless. In the presence of oxygen the dye becomes pink. Since the oxygen tension is always higher near the surface of the medium, the medium will be pink at the top and colorless in the middle and bottom.

After sodium glycollate reacts with oxygen throughout the tube, oxygen penetrates only near the top where the medium contacts air. Obligate aerobes grow only at the surface. Facultative organisms grow throughout the tube, but growth is better where oxygen is available. Microaerophiles grow near the top, but not in the most oxic zone. Anaerobes grow only near the bottom of the tube, where oxygen cannot penetrate.

FIRST PERIOD

Material:

1. Three tubes of thioglycollate broth
2. Broth cultures of: *Escherichia coli*
Micrococcus roseus
Pseudomonas aeruginosa

Procedure: (work in groups of 4)

1. Inoculate individual thioglycollate broths with *Escherichia coli*, *Micrococcus roseus*, and *Pseudomonas aeruginosa*. **NOTE:** Inoculate the **bottom** portion of the thioglycollate broth with a loop of bacteria obtained from the **bottom** of the broth culture. Avoid shaking the thioglycollate medium as oxygen will be introduced!

2. Incubate the tube with *M. roseus* at 25°C for 24-48 hours. Incubate all other tubes at 37°C for 24-48 hours.

SECOND PERIOD

Observe the amount of growth and the area of growth (top, bottom, throughout) in the tubes. Classify the test organisms as aerobes, facultative organisms, microaerophiles, or anaerobes.

Oxygen Utilization	Classification Type
Escherichia coli	
Micrococcus roseus	
Pseudomonas aeruginosa	

SOIL AS A SOURCE OF ANTIBIOTIC-PRODUCING *STREPTOMYCES* SPECIES

The genus *Streptomyces* belongs to the order Actinomycetales, a large group of Gram-positive, aerobic, filamentous bacteria, whose members are found primarily in soil. Extensive branching of the filaments results in the formation of a mycelial network, analogous to the mycelium formed by filamentous fungi. This matrix of filaments causes *Streptomyces* colonies to be dusty in appearance and compact in nature. However, characteristic aerial filaments can be formed as the colony ages, and a distinctive feature of various *Streptomyces* groups is the presence of specialized reproductive spores called conidia at the tips of the aerial filaments.

Streptomycetes are noted for production of extracellular metabolites which have a variety of functions. Geosmins are one example of the metabolites which can be produced. Such compounds are responsible in large part for the characteristic earthy odor of soil. But perhaps the most important extracellular metabolites of the streptomycetes are antibiotics. Except for a few antibiotics that are produced by fungi, e.g., penicillin and cephalosporin, and a few that are produced by other bacteria, e.g., bacitracin and polymyxin, all other antibiotics that are medically useful and have a wide application are synthesized by actinomycetes. Over 500 distinct antibiotic substances, including tetracycline, erythromycin and chloramphenicol, are produced by species of *Streptomyces*.

The serial dilution and plating techniques which have been described in previous exercises are frequently used for the isolation of soil actinomycetes. With most soils, suitable plates can be obtained from dilutions in the range of 10^{-2} to 10^{-6}. Many different types of media have been suggested to encourage the growth of *Streptomyces* rather than the growth of other soil microorganisms. Generally, the best carbon sources are glycerol, starch and chitin, with casein, asparagine and arginine as organic nitrogen sources. When attempting to isolate *Streptomyces* from soils, antifungal antibiotics are sometimes used to prevent or reduce fungal growth. Nystatin and cycloheximide inhibit most soil fungi, while having no deleterious effect on actinomycetes.

In this exercise, you will attempt to isolate streptomycetes from soil samples, using a dilution and spread plate technique. Isolated strains of *Streptomyces* will then be tested for antibiotic production. Susceptibility of both Gram-positive and Gram-negative bacteria to the antibiotic substances produced by the *Streptomyces* will also be determined.

FIRST PERIOD

Material:

1. Dried garden, compost, forest, etc., soil samples
2. Three 4.5-ml dilution tubes of sterile water
3. 9.5 ml sterile water in a 125-ml Erlenmeyer flask
3. Four plates of yeast malt agar
4. 1.0 and 0.1 ml pipets
5. Glass spreader
6. 95% ethyl alcohol in glass beaker

Procedure: (work in pairs)

1. Weigh 0.5 g of soil and transfer into the 9.5-ml dilution flask. Mix the solution completely, then allow to settle for 10 minutes. Prepare serial dilutions (10^{-2}, 10^{-3} and 10^{-4}) by transferring **0.5** ml at each step.

2. Transfer 0.1 ml of each dilution, including the original diluted soil suspension, to a yeast malt agar plate. Spread and let dry.

3. Invert the four plates and incubate in special "*Streptomyces*" (room temperature) incubator until the next lab period.

SECOND PERIOD

Material:

1. Yeast malt agar plates

Procedure: (work in pairs)

1. Choose three well-isolated colonies of "suspect" *Streptomyces* and prepare a Gram stain. **HINT**: Many *Streptomyces* strains will produce black colonies on yeast malt agar; they can also be easily identified by their opaque, rough and nonspreading morphology.

2. If the Gram stain reveals Gram-positive filamentous cells, transfer the organisms, using streak plate methods, to another yeast malt agar plate.

3. Invert the plates and incubate in special "*Streptomyces*" (room temperature) incubator for 2-5 days.