The Neotia University

Laboratory Manual

Microbial Physiology

Course Code:

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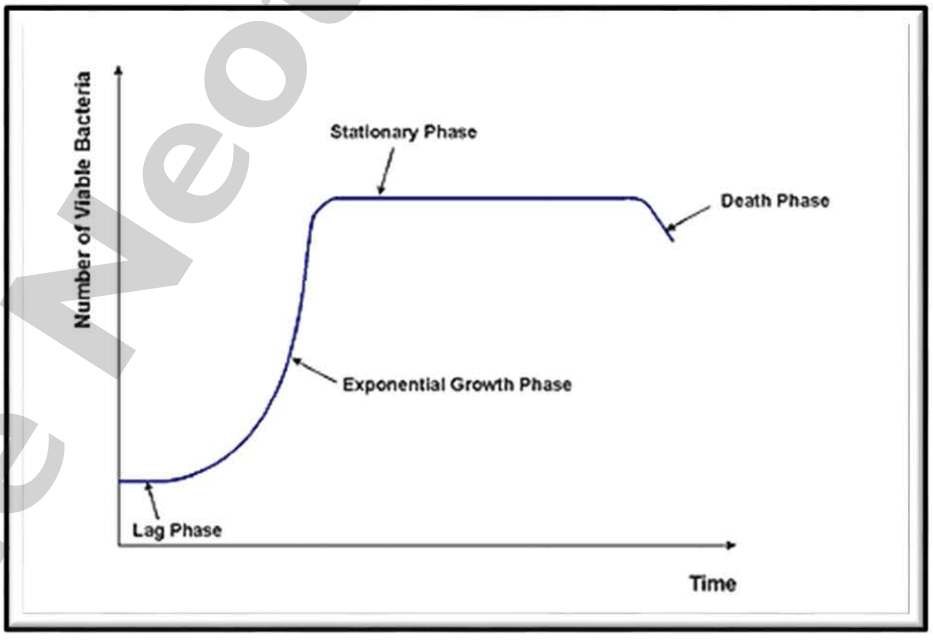
Practical I: Study and plot the growth curve of *Escherichia coli* by turbidometry and standard plate count methods & calculations of generation time and specific growth rate of bacteria from the graph plotted with the given data

Aim: To study and plot the growth curve of *Escherichia coli* by turbidometry and standard plate count methods & calculations of generation time and specific growth rate of bacteria from the graph plotted with the given data

Principle: Bacteria are as interesting as they are diverse. Though tiny, these unicellular life forms make huge contributions to many systems and cycles. From helping break down food in your intestine; to making the molecular assist in all three of the carbon, phosphorus, and nitrogen cycles- these little bacteria can accomplish big things. Unsurprisingly, bacteria are model organisms for research. Not only because of their diversity, but also because they are easily contained and reproduce quickly. When using bacteria for research, it is important to understand and track rates of bacterial growth within a sample. Inoculant bacteria in a typical laboratory setting tend to proceed through four distinct growth phases:

1. **Lag Phase**
2. **Exponential Phase**
3. **Stationary Phase**
4. **Death Phase**



The lapse of these phases provides data that can be compiled into a bacterial growth curve. During the lag phase, freshly cultured bacteria adjust to the media they've been placed in or

on. As they get a feel for their surroundings and nutritional options, the cells increase enzyme production and cell size accordingly. The cells have entered the exponential phase when they begin to grow and divide at a constant pace. This phase is also called the logarithmic phase because growth occurs very rapidly and the cell count can reach into the millions and billions. As such, the number of cells is calculated using logarithmic functions. Metabolic processes also occur at a constant rate and are influenced by conditions such as pH, temperature, and properties of the medium. The stationary phase is marked by a plateau in growth. Cell death and proliferation are roughly equal. Nutrients are increasingly depleted, toxins accumulate, and cell viability decreases. The death phase occurs when these conditions cause a greater rate of cell mortality than cell proliferation. The population begins to decline at a once again exponential rate.

Bacterial growth curves are important for calculating generation time. Generation time is the time it takes for two new cells to arise from an original cell. If starting with an inoculum with a known number of bacteria, periodic sampling can be done to calculate the generation time using the equation:

$$GT = \frac{t}{3.3\log(\frac{b}{B})}$$

- GT is generation time
- t is the time interval between measurements b and B
- B is the initial population
- b is the population after time t

The two most common classroom methods to determine bacterial growth are the Standard Plate Count (SPC) technique and turbidimetric measurement. Examples of other methods include: microscopic count, membrane filter count, nitrogen determination, cellular weight determination, and biochemical activity measurement.

Material Required:

- Bacterial culture (*Escherichia coli),* Broth (Luria Bertani (LB) Broth, Nutrient Broth)
- Glass wares: Conical flasks, Measuring cylinder, Sterile test tubes, Sterile Petriplates
- Reagents: Distilled water

- Other requirements: Incubator, Shaker, Spectrophotometer, Micropipettes, Tips, Sterile Loops

Procedure:

Day 1:

1. Using sterile loop, streak a loopful of bacterial culture onto the agar plate.
2. Incubate at 37°C for 18-24 hours.

Day 2:

1. Pick up a single colony of each strain from the agar plate and inoculate it into a test tube containing 10 ml of autoclaved broth.
2. Incubate the test tube overnight at 37°C.

Day 3:

1. Take 250 ml of autoclaved broth in a sterile 500 ml conical flask.
2. Inoculate 5 ml of the overnight grown culture in above flask.
3. Take OD at zero hour. Incubate the flask at 37°C.
4. Aliquot 1 ml of the culture suspension at an interval of every 30 minutes and take the optical density (OD) at a wavelength of 600 nm using spectrophotometer, till the reading becomes static.

Alternatively, 50-100 µl of formaldehyde can be added to all the 1 ml aliquots of culture suspension taken after every 30 minutes. Optical density of all the aliquots can be taken at the end of the experiment.

5. At the end of experiment, plot a graph of time in minutes on X axis versus optical density at 600nm on Y axis to obtain a growth curve of bacteria.

Result:

Discussion:

Conclusion:

Practical II: Effect of pH on the growth of *Escherichia coli*

Aim: To study the effect of pH on the growth of *Escherichia coli.*

Principle: Yogurt, pickles, sauerkraut, and lime-seasoned dishes all owe their tangy taste to a high acid content. Recall that acidity is a function of the concentration of hydrogen ions [H^+] and is measured as pH. Environments with pH values below 7.0 are considered acidic, whereas those with pH values above 7.0 are considered basic. Extreme pH affects the structure of all macromolecules. The hydrogen bonds holding together strands of DNA break up at high pH. Lipids are hydrolyzed by an extremely basic pH. The proton motive force responsible for production of ATP in cellular respiration depends on the concentration gradient of H^+ across the plasma membrane. If H^+ ions are neutralized by hydroxide ions, the concentration gradient collapses and impairs energy production. But the component most sensitive to pH in the cell is its workhorse, the protein. Moderate changes in pH modify the ionization of amino-acid functional groups and disrupt hydrogen bonding, which, in turn, promotes changes in the folding of the molecule, promoting denaturation and destroying activity.

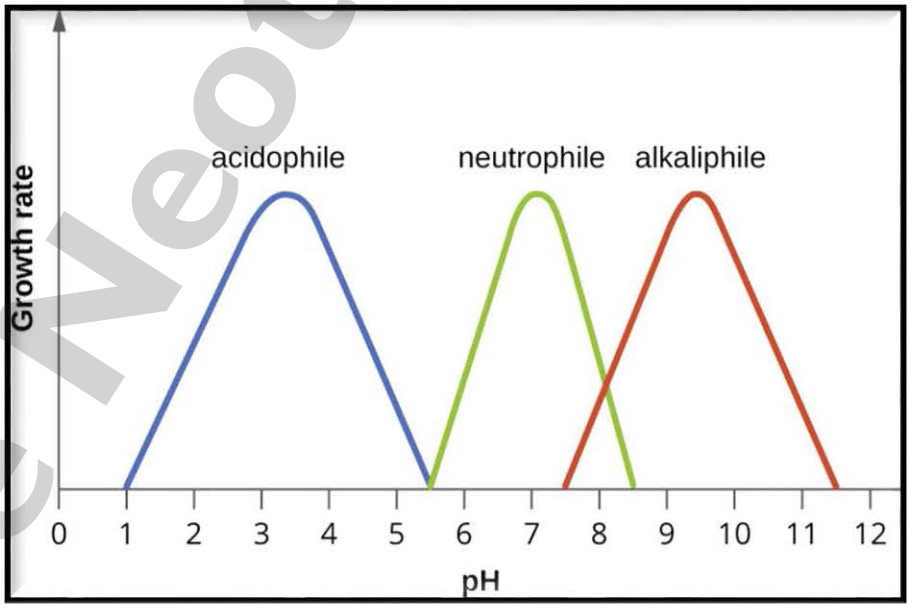
The optimum growth pH is the most favorable pH for the growth of an organism. The lowest pH value that an organism can tolerate is called the minimum growth pH and the highest pH is the maximum growth pH. These values can cover a wide range, which is important for the preservation of food and to microorganisms' survival in the stomach. For example, the optimum growth pH of Salmonella spp. is 7.0-7.5, but the minimum growth pH is closer to 4.2.

Most bacteria are neutrophiles, meaning they grow optimally at a pH within one or two pH units of the neutral pH of 7. Most familiar bacteria, like Escherichia coli, staphylococci, and Salmonella spp. are neutrophiles and do not fare well in the acidic pH of the stomach. However, there are pathogenic strains of *E. coli*, *S. typhi*, and other species of intestinal pathogens that are much more resistant to stomach acid. In comparison, fungi thrive at slightly acidic pH values of 5.0-6.0.

Microorganisms that grow optimally at pH less than 5.55 are called acidophiles. For example, the sulfur-oxidizing Sulfolobus spp. isolated from sulfur mud fields and hot springs in Yellowstone National Park are extreme acidophiles. These archaea survive at pH values of 2.5-3.5. Species of the archaean genus Ferroplasma live in acid mine drainage at pH values of 0-2.9. Lactobacillus bacteria, which are an important part of the normal microbiota of the vagina, can tolerate acidic environments at pH values 3.5-6.8 and also contribute to the acidity of the

vagina (pH of 4, except at the onset of menstruation) through their metabolic production of lactic acid. The vagina's acidity plays an important role in inhibiting other microbes that are less tolerant of acidity. Acidophilic microorganisms display a number of adaptations to survive in strong acidic environments. For example, proteins show increased negative surface charge that stabilizes them at low pH. Pumps actively eject H^+ ions out of the cells. The changes in the composition of membrane phospholipids probably reflect the need to maintain membrane fluidity at low pH.

At the other end of the spectrum are alkaliphiles, microorganisms that grow best at pH between 8.0 and 10.5. Vibrio cholerae, the pathogenic agent of cholera, grows best at the slightly basic pH of 8.0; it can survive pH values of 11.0 but is inactivated by the acid of the stomach. When it comes to survival at high pH, the bright pink archaean *Natronobacterium*, found in the soda lakes of the African Rift Valley, may hold the record at a pH of 10.5. Extreme alkaliphiles have adapted to their harsh environment through evolutionary modification of lipid and protein structure and compensatory mechanisms to maintain the proton motive force in an alkaline environment. For example, the alkaliphile *Bacillus firmus* derives the energy for transport reactions and motility from a Na^+ ion gradient rather than a proton motive force. Many enzymes from alkaliphiles have a higher isoelectric point, due to an increase in the number of basic amino acids, than homologous enzymes from neutrophiles.



Materials Required:

- Bacterial culture (*Escherichia coli),* Broth (Luria Bertani (LB) Broth, Nutrient Broth), MES, HEPES
- Glass wares: Conical flasks, Measuring cylinder, Sterile test tubes, Sterile Petriplates
- Reagents: Distilled water
- Other requirements: Incubator, Shaker, pH meter, Micropipettes, spectrophotometer, Tips, Sterile Loops

Procedure:

- Five set of 5 mL samples of Nutrient broth containing *Escherichia coli* and buffer it with acetic acid at a pH of 4 and 5, with MES at pH 6 and HEPES at pH 7 and 8.
- Then from each flask the sample will be taken at a time interval of 0, 0.5, 1, 2, 3 and 4 hours.
- Then the bacterial population in each sample will be measured by using spectrophotometer at 600 nm.
- Finally, a growth curve will be drawn.

Result:

Discussion:

Conclusion:

Practical III: Effect of temperature on the growth of *Escherichia coli*

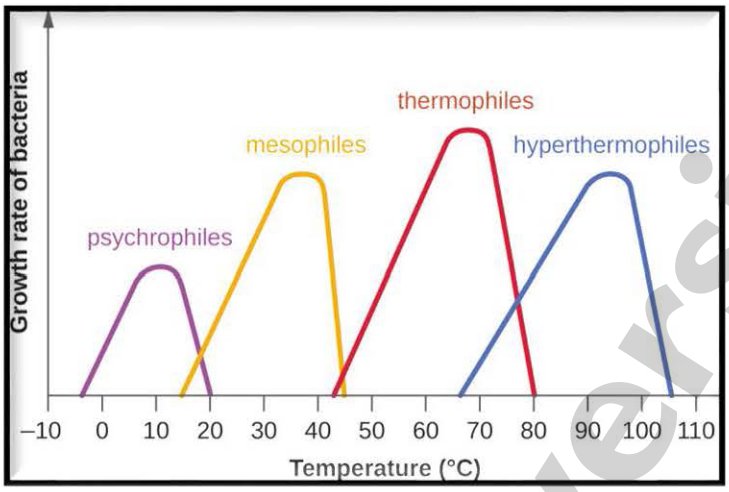
Aim: To study the effect of temperature on the growth of *Escherichia coli*

Principle: When the exploration of Lake Whillans started in Antarctica, researchers did not expect to find much life. Constant subzero temperatures and lack of obvious sources of nutrients did not seem to be conditions that would support a thriving ecosystem. To their surprise, the samples retrieved from the lake showed abundant microbial life. In a different but equally harsh setting, bacteria grow at the bottom of the ocean in sea vents (Figure 8.3.18.3.1), where temperatures can reach 340 °C (700 °F).

Microbes can be roughly classified according to the range of temperature at which they can grow. The growth rates are the highest at the optimum growth temperature for the organism. The lowest temperature at which the organism can survive and replicate is its minimum growth temperature. The highest temperature at which growth can occur is its maximum growth temperature. The following ranges of permissive growth temperatures are approximate only and can vary according to other environmental factors.

Organisms categorized as mesophiles ("middle loving") are adapted to moderate temperatures, with optimal growth temperatures ranging from room temperature (about 20 °C) to about 45 °C. As would be expected from the core temperature of the human body, 37 °C (98.6 °F), normal human microbiota and pathogens (e.g., *E. coli*, *Salmonella* spp., and *Lactobacillus* spp.) are mesophiles.

Organisms called psychrotrophs, also known as psychrotolerant, prefer cooler environments, from a high temperature of 25 °C to refrigeration temperature about 4 °C. They are found in many natural environments in temperate climates. They are also responsible for the spoilage of refrigerated food.



Materials Required:

- Bacterial culture (*Escherichia coli),* Broth (Luria Bertani (LB) Broth, Nutrient Broth)
- Glass wares: Conical flasks, Measuring cylinder, Sterile test tubes, Sterile Petriplates
- Reagents: Distilled water
- Other requirements: Incubator, Shaker, water-bath, Spectrophotometer, Micropipettes, Tips, Sterile Loops

Procedure:

- 1 mL of inoculant will be serially diluted and further inoculated into different test tubes containing 5 mL of Nutrient broth.
- Then the test tubes will be exposed to different temperature starting from 0 to 70 ℃.
- After 24 hour of incubation the colony forming unit will be measured and growth curve will be drawn with obtaining data by using spectrophotometer.

Result:

Discussion:

Conclusion:

Practical IV: Effect of salt concentration on the growth of *Escherichia coli*

Aim: To study the effect of salt concentration on the growth of *Escherichia coli*

Principle: Generally, bacteria belonging to the family Enterobacteriaceae, such as *E. coli* and *Salmonella*, do not tolerate high salt levels. However, certain strains of *E. coli* are halo-tolerant and are able to survive and grow in high salt concentrations. This high osmotic strength seems to be due to the production of proline in the cells. When enteric and pathogenic bacteria are released from their hosts into natural environments, they are often challenged by various environmental stresses, such as nutrient starvation, osmotic shock, temperature variation, oxidative stress, etc. Greater understanding of the effects of environmental stresses on *E. coli* is urgently required. Transmission to a new host often involves a period of exposure to a hostile external environment. How *E. coli* cells cope with such exposure and the possible role of the new information concerning the influence of environmental factors on the physiology of *E. coli* cells should help increase our understanding of how these organisms survive and retain infectivity in natural environments and may also help in the development of improved methods for the resuscitation and recovery of environmentally stressed cells.

Materials Required:

- Bacterial culture (*Escherichia coli),* Broth (Luria Bertani (LB) Broth, Nutrient Broth)
- Glass wares: Conical flasks, Measuring cylinder, Sterile test tubes, Sterile Petriplates
- Reagents: Distilled water
- Other requirements: Incubator, Shaker, Spectrophotometer, Micropipettes, Tips, Sterile Loops
- **Media modifications:** The compositions of media were modified by addition of sodium chloride, potassium chloride and glucose in different concentrations as follows:

❖ 1. Modified Nutrient Broth (MNB) + 0.5% NaCl
❖ 2. MNB + 1.0% NaCl
❖ 3. MNB + 1.5% NaCl
❖ 4. MNB + 0.5% KCl

- 5. MNB + 1.0% KCl
- 6. MNB + 1.5% KCl
- 7. MNB + 70 mM NaCl
- 8. MNB + 70 mM KCl
- 9. MNB + 140 mM glucose

Procedure:

- Thirty bottles containing 10 ml each of medium will be used. Out of the 30 bottles 15 contained NaCl, 5 each with 0.5, 1.0 and 1.5% (w/v) NaCl and the other 15 bottles contained KCl, 5 each with the same concentration of KCl as in NaCl.
- The bottles will be inoculated with a loop full of *E. coli* culture and incubated at 37°C for 24 hours. The same procedure will be repeated and the incubation will be done at 44°C for 24 hours. The growth in each bottle will be measured by APC.
- A standard curve of *Escherichia coli* will be drawn at 37 °C.

Result:

Discussion:

Conclusion:

Practical V: Demonstration of alcoholic fermentation

Aim: To demonstrate the microbial alcoholic fermentation

Principle: Interest in fermentation, the breakdown of sugar to alcohol, dates far back in human history. Until 1860, it was believed to be a purely chemical process having nothing to do with living organisms. Then Louis Pasteur showed that fermentation involves a living process carried out by yeast and bacteria. Glucose is fermented according to the following net equation:

$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$

Many different intermediate products can be formed during this reaction and in various concentrations depending upon the conditions. In this demonstration, the production of ethyl alcohol and CO2 are illustrated. The use of the color indicator, resazurin, further helps to highlight the conditions present in the fermentation chamber.

Materials Required:

- Glucose (dextrose),
- 15 g Erlenmeyer flask,
- 250-mL iodine solution (several drops)
- Glass tube
- Phenol red solution, 0.01%
- Resazurin solution, 0.1%
- Sodium hydroxide solution, 1 M
- Yeast, active dry, 3 g Stopper
- Water, distilled or deionized Test tube

Procedure:

1. Fill a test tube ¾ full with 0.01% phenol red solution and place it in a test tube rack. \
2. Place 150 mL of distilled water into a 250-mL Erlenmeyer flask. (A larger flask can be used with proportional increases in other ingredients.)
3. Place 15 g of glucose (or dextrose) into the flask and swirl the contents until all the glucose dissolves.

4. Add 3 g of active dry yeast to the flask and swirl until the yeast is uniformly mixed in the solution.
5. Add three drops of resazurin solution to the solution in the flask.
6. Use a one-holed stopper, a glass tube, and flexible plastic or latex tubing to complete the fermentation setup
7. Note the initial color of the solution in both the flask and the test tube.
8. Let the fermentation apparatus sit undisturbed for an extended period of time (even overnight if necessary).
9. Record the color changes of the solutions in both the flask and test tube.
10. Save the solution in the flask for the optional test for alcohol.

Result:

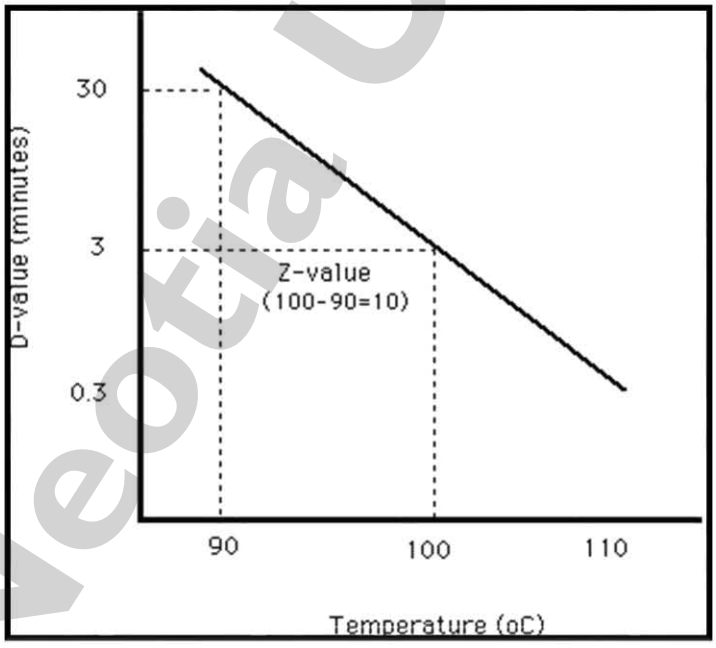
Discussion:

Conclusion:

Practical VI: Demonstration of the thermal death time and decimal reduction time of *Escherichia coli*

Aim: Demonstration of the thermal death time and decimal reduction time of *Escherichia coli*

Principle: Since heating methods are commonly used to control microbial growth, it is important to be able to define the effectiveness of a heating method for a particular bacterial species. One way to do this is to determine the thermal death point (TDP) and the TDT (thermal death time). The TDT is the minimum time it takes to kill a population of microbes at a specific temperature. The TDP is the lowest temperature that is required to kill a population of microbes when applied for a specific time.



Materials Required:

- Test tube rack
- sterile dropper pipettes
- 2 nutrient agar plates
- 1 broth culture of *Escherichia coli*
- 1 empty glass test tube

- 1 large beaker
- heavy duty tongs for lifting beaker
- hot plate thermometer
- test tube holder
- inoculating loops

Procedure:

- Using a sterile inoculating loop, transfer one loopful of the E. coli broth culture to the control sector of the *E. coli* agar plate
- Fill the large beaker about one-half full with tap water and set on the hot plate to heat. Insert a thermometer into a non-sterile test tube containing approximately 2 mL of tap water. Place the water filled test tube and thermometer into the water bath.
- Monitor the temperature, and when the thermometer reads the assigned temperature (either 63°C (± 1°C) or 72°C (± 1°C)), use the tongs to remove the beaker from the hot plate and set it on the bench top.
- Place the broth culture test tubes (*E. coli*) into the water bath and begin timing. At the specified times, remove one loopful of the broth and subculture to the appropriate sector on the appropriate agar plate. Be sure to monitor the thermometer to ensure that the water bath remains at the specified temperature for the entire 15 minute incubation period.
- When complete, incubate the two agar plates at 35°C for 18-24 hours. Discard the broth culture tubes into the biohazard container.

Result:

Discussion:

Conclusion: