DEPARTMENT OF MICROBIOLOGY
ST ALOYSIUS COLLEGE (AUTONOMOUS), MANGALURU

LABORATORY MANUAL

SECOND SEMESTER
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1. THE MICROSCOPIC MEASUREMENT OF MICROORGANISMS.

Principle

Determination of microbial size is not simple. Before an accurate measurement of cells can be made, the diameter of the microscopic field must be established by means of optic devices, namely, an ocular micrometer and a stage micrometer. The **ocular micrometer**, which is placed on a circular shelf inside the eyepiece, is a glass disc with graduations etched on its surface. The distance between these graduations will vary depending on the objective being used, which determines the size of the field. This distance is determined by using a **stage micrometer**, a special glass slide with etched graduations that are 0.01 mm, or 10 micrometers (μm), apart. The calibration procedure for the ocular micrometer requires that the graduations on both micrometers be superimposed on each other. This is accomplished by rotating the ocular lens. A determination is then made of the number of ocular divisions per known distance on the stage micrometer. Finally, the calibration factor for one ocular division is calculated as follows:

\[
\text{One division on ocular micrometer in mm} = \frac{\text{Known distance between two lines on stage micrometer}}{\text{Number of divisions on ocular micrometer}}
\]

**Example:** If 13 ocular divisions coincide with two stage divisions (2 × 0.01 mm = known distance of 0.02 mm), then:

\[
\text{One ocular division} = \frac{0.02 \text{mm}}{13} = 0.00154 \text{mm}, \text{or 1.54 \mu m}
\]

Once the ocular micrometer is calibrated, the size of a microorganism can easily be determined, first by counting the number of spaces occupied by the organism and second by multiplying this number by the calculated calibration factor for one ocular division.
**Example:** If an organism occupies five spaces on the ocular micrometer, then:
Length of organism = number of ocular divisions occupied \( \times \) calibration factor for one ocular division
\[ = 5 \times 1.54 \, \mu m = 7.70 \mu m \]

In this experiment, you will calibrate an ocular micrometer for the oil-immersion objective and determine the sizes of microorganisms such as bacteria, and yeast.

**Materials**

**Slides**
Prepared slides of yeast cells, protozoa, and bacterial cocci and bacilli.

**Equipment**
Ocular micrometer, stage micrometer, microscope, immersion oil, and lens paper.

**Procedure**
1. With the assistance of a laboratory instructor, carefully place the ocular micrometer into the eyepiece.
2. Place the stage micrometer on the microscope stage and center it over the illumination source.
3. With the stage micrometer in clear focus under the low-power objective, slowly rotate the eyepiece to superimpose the ocular micrometer graduations over those of the stage micrometer.
4. Add a drop of immersion oil to the stage micrometer, bring the oil-immersion objective into position, and focus, if necessary, with the *fine-adjustment knob only*.
5. Move the mechanical stage so that a line on the stage micrometer coincides with a line on the ocular micrometer at one end. Find another line on the ocular micrometer that coincides with a line on the stage micrometer. Determine the distance on the stage micrometer (number of divisions \( \times \) 0.01 mm) and the corresponding number of divisions on the ocular micrometer.
6. Determine the value of the calibration factor for the oil-immersion objective.
7. Remove the stage micrometer from the stage.
8. To determine the size of the cocci on the prepared slides under the oil-immersion objective, do the following:
   a. Calculate the number of ocular divisions occupied by each of three separate cocci.
   Record the data in the readings columns of the first observation chart in the Lab Report.
   b. Determine and record the average of the three measurements.
   c. Determine the size by multiplying the average by your calculated calibration factor, and record this value.
9. Determine and record the size of the other microorganisms by observing the remaining prepared slides under oil immersion as outlined in Step 8. Since these organisms are not round, both length and width measurements are required.

10. Check that your observations are complete in the Lab Report.

**Observations and Results**

1. Calibration of ocular micrometer for the oil-immersion objective:
   - Distance on stage micrometer ____________
   - Divisions on ocular micrometer ____________
   - Calibration factor for one ocular division ____________

2. Use the charts below to record your observations and measurements of the microorganisms studied.

   **WIDTH OF MICROORGANISMS IN MICROMETERS (μm)**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Readings</th>
<th>Average × Calibration Factor = Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocci</td>
<td>1  2  3</td>
<td></td>
</tr>
<tr>
<td>Bacilli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protozoa</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

   **LENGTH OF MICROORGANISMS IN MICROMETERS (μm)**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Readings</th>
<th>Average × Calibration Factor = Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacilli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protozoa</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2. THE HEMOCYTMETER (COUNTING CHAMBER)

The hemocytometer (or haemocytometer or counting chamber) is a specimen slide which is used to determine the concentration of cells in a liquid sample. It is frequently used to determine the concentration of blood cells (hence the name “hemo-“) but also the concentration of sperm cells in a sample. The hemocytometer (or haemocytometer or counting chamber) is a specimen slide which is used to determine the concentration of cells in a liquid sample. It is frequently used to determine the concentration of blood cells (hence the name “hemo-“) but also the concentration of sperm cells in a sample. The cover glass, which is placed on the sample, does not simply float on the liquid, but is held in place at a specified height (usually 0.1mm). Additionally, a grid is etched into the glass of the hemocytometer. This grid, an arrangement of squares of different sizes, allows for an easy counting of cells. This way it is possible to determine the number of cells in a specified volume.

For microbiology, cell culture and many of the applications that require use of cell suspensions, it is necessary to determine the concentration of cells. The device used for determining the number of cells per unit volume of a suspension is called a counting chamber. It is the most widely used type of chamber, since it was mainly designed for performing blood cell counts. It is now used to count other types of cells and other microscopic particles as well.

The hemocytometer was invented by Louis-Charles Malassez. It is a special type of microscope slide consisting of two chambers, which is divided into nine (1.0mm x 1.0mm) large squares which are separated from one another by triple lines. The area of each is 1mm². Cover glass is supported over the chambers at a height of 0.1mm. Because of that, the entire counting grid lies under the volume of 0.9 mm² on one side. The cell suspensions are introduced into the cover glass. The hemocytometer is placed on the microscope stage and the cell suspension is counted.

The glass microscope slide has a rectangular indentation that creates an 'H’ shaped chamber at the centre. This chamber is engraved with a laser-etched grid of perpendicular lines. Two counting areas with ruled
grids are separated by the horizontal groove of the ‘H’. There is also a very flat, reusable cover slip. The glass cover slip is held at 0.1 mm above the surface of the counting areas by ground glass ridges on either side of the vertical grooves of the H shape. The device is carefully crafted so that the area bounded by the depth and lines of the chamber is also known. Because the height is constant, the volume of fluid above each square of the grid is known with precision.

The hemocytometer is used by putting the cover slip on the device, and filling the space with a liquid containing the cells you want to count. There is a "V" or notch at either end which is the place where the cell suspension is loaded into the hemocytometer. The fluid is usually drawn into the space by capillary action. A cover glass, which is placed on the sample, does not simply float on the liquid, but is held in place at a specified height. In addition, the grid arrangement of squares of different sizes allows for an easy counting of cells. It is possible to identify the number of cells in a specified volume by this method.

The ruled area of the hemocytometer consists of several large 1 x 1 mm (1mm²) squares, which are subdivided in three ways; 0.25 x 0.25 mm (0.0625 mm²), 0.25 x 0.20 mm (0.05 mm²) and 0.20 x 0.20 mm (0.04 mm²). The central, 0.20 x 0.20 mm marked, 1 x 1 mm square is further subdivided into 0.05 x 0.05 mm (0.0025 mm²) squares. Hold the cover slip (0.1 mm) at the raised edges of hemocytometer, which gives each square a defined volume.

<table>
<thead>
<tr>
<th>Dimensions</th>
<th>Area</th>
<th>Volume at 0.1 mm depth</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x 1 mm</td>
<td>1 mm²</td>
<td>100 nl</td>
</tr>
<tr>
<td>0.25 x 0.25 mm (1/16)</td>
<td>0.0625 mm²</td>
<td>6.25 nl</td>
</tr>
<tr>
<td>0.25 x 0.20 mm (1/20)</td>
<td>0.05 mm²</td>
<td>5 nl</td>
</tr>
<tr>
<td>0.20 x 0.20 mm (1/25)</td>
<td>0.04 mm²</td>
<td>4 nl</td>
</tr>
<tr>
<td>0.05 x 0.05 mm (1/400)</td>
<td>0.0025 mm²</td>
<td>0.25 nl</td>
</tr>
</tbody>
</table>
A number of stains have been employed to distinguish between viable and nonviable cells. This is based on the principle that live cells contain intact cell membranes that eliminate certain dyes, like trypan blue, Eosin, or propidium. In dead cells, the stain enters the cytoplasm and the cells take on the stain. If more than 25% of the cells are stained, the cell suspension is most likely not a viable one.

To prepare the counting chamber, the mirror-like polished surface is carefully cleaned with 75% ethanol and the cover slip is also cleaned. The cover slips used for counting chambers are specially made, and are thicker than those cover slips used for conventional microscopy, since they must be heavy enough to overcome the surface tension of a drop of liquid. A cover slip is placed on the counting surface prior to putting on the cell suspension. Introduced any of the cell suspension into any of the V-shaped wells with a micropipette. The area under the cover slip fills by capillary action. Sufficient liquid should be introduced, so that the mirrored surface is just covered. The charged counting chamber is placed under the microscope stage and the counting grid is brought into focus at low power.

Materials Required

1. Hemocytometer plus a supply of cover slips.
2. Uniform cell suspension.
3. 0.4% Trypan Blue stain (fresh & filtered) in phosphate buffered saline.
4. Tally Counter.
5. Cell Suspension.
6. Micropipettes.

Procedure

1. **Obtain a uniform suspension of cells:** Follow the typsinization/trypsin neutralization protocol for the specific cell type. Place the cell suspension in a suitably-sized conical centrifuge tube. For an accurate cell count to be obtained, a uniform suspension containing single cells is necessary. Pipette the cell suspension up and down in the tube 5-7 times using a pipette with a small bore (5 ml or 10 ml pipette). For cells thawed from cryopreservation (in 1ml cryopreservation medium), pipette up and down 7-10 times using a one ml pipette.
2. **Prepare a 1:1 dilution of the cell suspension in trypan blue**: Approximately 10 microliters of cell suspension will be required to charge one chamber of the hemocytometer. In a conical microfuge tube, add 10 microliters of 0.4% trypan blue solution. Gently swirl (finger vortex) the cell suspension and remove 10 microliters of it using sterile technique. Combine the 10 microliters of cell suspension with the 10 microliters of trypan blue in the microfuge tube. Pipette up and down several times to ensure a uniform cell suspension using the same pipette tip and allow to stand for 5-15 minutes.

3. **Load the hemocytometer**: Moisten and affix cover slip to the hemocytometer. Ensure the cover slip and hemocytometer are clean and grease-free (use alcohol to clean). A small amount of trypan blue-cell suspension is transferred to one of the chambers of the hemocytometer by carefully touching the cover slip at its edge with the pipette tip and allowing each chamber to fill by capillary action. The chamber should not be overfilled or underfilled.

4. **Determine the number of cells (total and viable)**: View the cells under a microscope at 100x magnification. Under the microscope, you should see a grid of 9 squares. Focus the microscope on one of the 4 outer squares in the grid. The square should contain 16 smaller squares. Count all the cells in the four 1 mm corner squares. If there are too many or too few cells to count, repeat the procedure, either concentrating or diluting the original suspension as appropriate.

For an accurate determination, the total number of cells overlying one 1 mm$^2$ should be between 15 and 50. If the number of cells per 1 mm$^2$ exceeds 50, dilute the sample and count again. If the number of cells per 1 mm$^2$ is less than 15, use a less diluted sample. If less dilute samples are not available, count cells on both sides of the hemocytometer (8 x 1 mm$^2$ areas).

Keep a separate count of viable and non-viable cells. If more than 25% of cells are non-viable, the culture is not being maintained on the appropriate amount of media. Reincubate the culture and adjust the volume of media according to the confluency of the cells and the appearance of the media. Include cells on top and left touching middle line. The cells touching middle line at bottom and right are not counted.
Note:

i. Trypan Blue is the "vital stain"; excluded from live cells.

ii. Live cells appear colourless and bright (refractile) under phase contrast.

iii. Dead cells stain blue and are non-refractile.

1. Calculation:- Count 4 corner squares and calculate the average. Each large square of the hemocytometer, with cover slip in place, represents a total volume of 0.1 mm$^3$ (1.0mm X1.0mmX 0.1mm) or $10^{-4}$ cm$^3$. Since 1 cm$^3$ is equivalent to approximately 1 ml, the total number of cells per ml will be determined using the following calculations:

   - %Cell Viability = [Total Viable cells (Unstained) / Total cells (Viable +Dead)] X 100.
   - Viable Cells/ml = Average viable cell count per square x Dilution Factor x $10^4$ /
   - Average viable cell count per square = Total number of viable cells in 4 squares / 4.
   - Dilution Factor = Total Volume (Volume of sample + Volume of diluting liquid) / Volume of sample.
   - Total viable cells/Sample = Viable Cells/ml x The original volume of fluid from which the cell sample was removed.
   - Volume of media needed = (Number of cells needed/Total number of viable cells) x 1000.

Cleaning the hemocytometer:- Clean the instrument as soon as possible after use. Use protective clothing, gloves and eyewear. Trypan blue is a mutagen. Clean the instrument with dilute bleach solution followed by 70% isopropanol. Air dry. Dispose of trypan blue contaminated articles in biohazard waste.
3. TEASE MOUNT & STAINING TECHNIQUES FOR FUNGI

Identify a fungal unknown based on colonial morphology and microscopic appearance.

**Principle**
In this experiment, you will be provided with a number-coded pure culture of a representative fungal organism for cultivation and subsequent identification.

**Materials**

**Cultures**
Number-coded, 7-day Sabouraud broth spore suspensions of *Aspergillus, Mucor, Penicillium, Alternaria, Rhizopus, Cladosporium, Fusarium,* and *Yeast.*

**Media**
One Sabouraud agar plate per student.

**Reagent**
Lactophenol–cotton-blue solution.

**Equipment**
Bunsen burner, dissecting microscope, hand lens, sterile cotton swabs, glass slides, coverslips, inoculating loop, and glassware marking pencil.

**Procedure Lab One**
1. With a sterile inoculating loop, inoculate an appropriately labeled Sabouraud agar plate with one of the provided unknown cultures by placing one loopful in the center of the plate.
   *Note: Do not spread culture.*

2. Incubate the plates in a noninverted position for 1 week at 25°C in a moist incubator.

**Procedure Lab Two**
1. Observe mold cultures with a hand lens, noting and recording their colonial morphologies.

2. Prepare a wet mount by suspending some of the culture in a few drops of lactophenol–cotton-blue solution. Be gentle to avoid damaging the fungal structures.

3. Examine the preparation under high-power and low-power magnifications with the aid of a dissecting microscope and record your observations in the Lab Report.

**Observations and Results**

1. Draw representative microscopic fields of your culture below.
2. Using Figure given below, identify your unknown fungal organism.

a. Color pigmentation

b. Diameter (mm)

c. Texture (cottony, smooth, etc.)

d. Margin (entire, undulating, lobular, etc.)

e. Aerial hyphae (septate, nonseptate)
<table>
<thead>
<tr>
<th>DIAGRAM</th>
<th>COLONIAL MORPHOLOGY</th>
<th>MICROSCOPIC APPEARANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Molds</strong></td>
<td>Rapidly growing white-colored fungus swarms over entire plate; aerial mycelium cortical and fuzzy</td>
<td>Spores are oval, colorless, or brown; nonseptate mycelium gives rise to straight sporangiophores that terminate with black sporangium containing a columella; rootlike hyphae (rhizoids) penetrate the medium</td>
</tr>
<tr>
<td>- Sporangium</td>
<td></td>
<td>Spores are oval; nonseptate mycelium gives rise to single sporangiophores with globose sporangium containing a columella; there are no rhizoids</td>
</tr>
<tr>
<td>- Columnella</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Colliarette</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Sporangiphore</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Stolon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Mycelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Rhizoid</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Rhizopus; Black bread mold: common laboratory contaminant</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mucor; Food contaminant</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Sporangium</td>
<td>Resembles the colonies of Rhizopus except that it lacks rhizoids and colliarettes; sporangiophore arises directly from mycelial mat. Note: Branching sporangiophores may occur with Mucor.</td>
<td></td>
</tr>
<tr>
<td>- Columnella</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Colliarette</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Sporangiphore</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Mycelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Rhizoid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIAGRAM</td>
<td>COLONIAL MORPHOLOGY</td>
<td>MICROSCOPIC APPEARANCE</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td><strong>Molds (continued)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conidia</td>
<td>Grayish-green or black colonies with gray edges rapidly swelling over entire plate; aerial mycelium not very dense, appears grayish to white</td>
<td>Multicelled spores (conidia) are pear-shaped and attached to single conidiophores arising from a septate mycelium</td>
</tr>
<tr>
<td>Conidiophore</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Alternaria</strong></td>
<td>Normally found on plant materials; also found in house dust</td>
<td></td>
</tr>
<tr>
<td>Conidia</td>
<td>Wooly, white, fuzzy colonies changing color to pink, purple, or yellow</td>
<td>Spores (conidia) are oval or crescent-shaped and attached to conidiophores arising from a septate mycelium; some spores are single cells, some are multicelled</td>
</tr>
<tr>
<td>Conidiophore</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fusarium:</td>
<td>Found in soil; also likely in eye infections</td>
<td></td>
</tr>
<tr>
<td>Conidia</td>
<td>White colonies become greenish-blue, black, or brown as culture matures</td>
<td>Single-celled spores (conidia) in chains developing at the end of the sterigma arising from the terminal bulb of the conidiophore; the vesicle; long conidiophores arise from a septate mycelium</td>
</tr>
<tr>
<td>Conidiophore</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillus:</td>
<td>Plant and animal pathogens; some species used industrially</td>
<td></td>
</tr>
<tr>
<td>Conidia</td>
<td>Mature cultures usually greenish or blue-green</td>
<td>Single-celled spores (conidia) in chains develop at the end of the sterigma arising from the metula of the conidiophore; branching conidiophores arise from a septate mycelium</td>
</tr>
<tr>
<td>Sterigma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vesicle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conidiophore</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillium:</td>
<td>Antibiotic-producing citrus fruit contaminant; soil inhabitant</td>
<td></td>
</tr>
</tbody>
</table>
4. STUDY OF YEAST WET MOUNT AND STAINED SPECIMEN OBSERVATION.

Principle

Yeasts are nonfilamentous unicellular fungi. Yeast cultures resemble bacteria when grown on the surface of artificial laboratory media; however, they are 5 to 10 times larger than bacteria. Microscopically, yeast cells may be ellipsoidal, spherical, or in some cases, cylindrical. Unlike molds, yeast do not have aerial hyphae and supporting sporangia. Yeast reproduce asexually by budding or by fission. In budding, an outgrowth from the parent cell (a bud) pinches off, producing a daughter cell (Figures 37.3a and 37.4). Fission occurs in certain species of yeast, such as those in the genus Schizosaccharomyces. During fission, the parent cell elongates, its nucleus divides, and it splits evenly into two daughter cells. Some yeast may also undergo sexual reproduction when two sexual spores conjugate, giving rise to a zygote, or diploid cell. The nucleus of this cell divides by meiosis, producing four new haploid nuclei (sexual spores), called ascospores, contained within a structure called the ascus (Figure 37.3b). When the ascus ruptures, the ascospores are released and conjugate, starting the cycle again. Yeasts are important for many reasons. Saccharomyces cerevisiae is referred to as baker’s yeast and is used as the leavening agent in dough. Two major strains of yeast, Saccharomyces carlsbergensis and Saccharomyces cerevisiae, are used for brewing. The wine industry relies on wild yeast (present on the grape) for the fermentation of grape juice, which is supplemented with Saccharomyces ellipsoideus to begin the fermentation. Also, the high vitamin content of yeasts makes them particularly valuable as food supplements. As useful as some yeasts are, there are a few species that can create problems in the food industry or are harmful to humans. Undesired yeast must be excluded from the manufacture of fruit juices, such as grape juice or apple cider, to prevent the fermentation of fruit sugars to alcohol. The contamination of soft cheese by some forms of yeast will destroy the product. Finally, some yeast such as Candida albicans are pathogenic and responsible for urinary tract and vaginal infections known as moniliasis and infections of the mouth called thrush.

Materials

Cultures

7-day Sabouraud agar cultures of Saccharomyces cerevisiae,

Morphological Characteristics

Prepare a wet mount of each yeast culture in the following manner:

1. Suspend a loopful of yeast culture in a few drops of lactophenol–cotton-blue solution on a microscope slide and cover with a coverslip.
2. Examine all yeast wet-mount slide preparations under low and high power, noting the shape and the presence or absence of budding. Record your observations in the Lab Report.

Observations and Results

Morphological Characteristics

Draw a representative field for each organism in the chart below. Note the shape and presence or absence of budding (+ or -).

Saccharomyces cerevisiae
5. EFFECT OF pH ON GROWTH OF BACTERIA.

Principle

Growth and survival of microorganisms are greatly influenced by the pH of the environment, and all bacteria and other microorganisms differ as to their requirements. Based on their pH optima, microorganisms may be classified as acidophiles, neutrophiles, or alkalophiles (Figure 17.1). Each 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. These specific pH needs reflect the organisms’ adaptations to their natural environment. For example, enteric bacteria are capable of survival within a broad pH range, which is characteristic of their natural habitat, the digestive system. Bacterial blood parasites, on the other hand, can tolerate only a narrow range; the pH of the circulatory system remains fairly constant at approximately 7.4. Despite this diversity and the fact that certain organisms can grow at extremes of the pH scale, generalities can be made. The specific range for bacteria is between 4 and 9, with the optimum being 6.5 to 7.5. Fungi (molds and yeasts) prefer an acidic environment, with optimum activities at a pH of 4 to 6. Because a neutral or nearly neutral environment is generally advantageous to the growth of microorganisms, the pH of the laboratory medium is frequently adjusted to approximately 7. Metabolic activities of the microorganism will result in the production of wastes, such as acids from carbohydrate degradation and alkali from protein breakdown, and these will cause shifts in pH that can be detrimental to growth. To retard this shift, chemical substances that act as buffers are frequently incorporated when the medium is prepared. A commonly used buffering system involves the addition of equimolar concentrations of K2HP04, a salt of a weak base, and KH2PO4, a salt of a weak acid. In a medium that has become acidic, the K2HP04 absorbs excess H+ to form a weakly acidic salt and a potassium salt with the anion of the strong acid.

\[
\text{K}_2\text{HPO}_4 + \text{HCl} \rightarrow \text{KH}_2\text{PO}_4 + \text{KCl}
\]

Salt of a weak base Strong acid Salt of a weak acid Potassium chloride salt

In a medium that has become alkaline, KH2PO4 releases H+, which combines with the excess OH− to form water, and the remaining anionic portion of the weakly acidic salt combines with the cation of the alkali.
Most media contain amino acids, peptones, and proteins, which can act as natural buffers because of their amphoteric nature. For example, amino acids are zwitterions, molecules in which the amino group and the carboxyl group ionize to form dipolar ions.

**Materials**

**Cultures**
Saline/broth suspensions of 24-hour nutrient broth cultures, adjusted to an absorbance (A) of 0.05 at a wavelength of 600 nm, of *Alcaligenes faecalis*, *Escherichia coli*, and *Saccharomyces cerevisiae*.

**Media**
Per designated student group: 12 Trypticase soy broth (TSB) tubes/plates, three at each of the following pH designations: 3, 6, 7, and 9. The pH has been adjusted with 1N sodium hydroxide or 1N hydrochloric acid.

**Equipment**
Bunsen burner, sterile 1-ml pipettes, mechanical pipetting device, Bausch & Lomb Spectronic 20 spectrophotometer, test tube rack, and glassware marking pencil.

**Procedure** Lab One
1. Using a sterile pipette/inoculation loop, inoculate a series of the appropriately labeled TSB tubes/plates of media, pH values of 3, 6, 7, and 9, with *E. coli* by adding 0.1 ml of the saline culture to each.
2. Repeat Step 1 for the inoculation of *A. faecalis* and *S. cerevisiae*, using a new sterile pipette each time.
3. Incubate the *A. faecalis* and *E. coli* cultures for 24 to 48 hours at 37°C and the *S. cerevisiae* cultures for 48 to 72 hours at 25°C.

**Procedure** Lab Two
1. Using the spectrophotometer as described in Experiment 14, determine the absorbance of all cultures/Record the amount of growth in Trypticase soy plates. Record the readings in the chart provided in the Lab Report.
2. In the second chart provided in the Lab Report, summarize your results as to the overall range and optimum pH of each organism studied.
### Adsorption Readings

<table>
<thead>
<tr>
<th>Microbial Species</th>
<th>pH 3</th>
<th>pH 0</th>
<th>pH 7</th>
<th>pH 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. fuscals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td></td>
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</tbody>
</table>

### pH Summary

<table>
<thead>
<tr>
<th>Microbial Species</th>
<th>pH Range</th>
<th>Optimum pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. fuscals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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6. EFFECT OF TEMPERATURE ON GROWTH OF BACTERIA.

Principle

Microbial growth is directly dependent on how temperature affects cellular enzymes. With increasing temperatures, enzyme activity increases until the three-dimensional configuration of these molecules is lost because of denaturation of their protein structure. On the other hand, as the temperature is lowered toward the freezing point, enzyme inactivation occurs and cellular metabolism gradually diminishes. At 0°C, biochemical reactions cease in most cells. Bacteria, as a group of living organisms, are capable of growth within an overall temperature range of minus 5°C to 80°C. Each species, however, requires a narrower range that is determined by the heat sensitivity of its enzyme systems. Specific temperature ranges consist of the following cardinal (significant) 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 cell enzymes are destroyed and the organism dies.

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

All bacteria can be classified into one of three major groups, depending on their temperature requirements:

1. Psychrophiles: Bacterial species that will grow within a temperature range of −5°C to 20°C. The distinguishing characteristic of all psychrophiles is that they will grow between 0° and 5°C.

2. Mesophiles: Bacterial species that will grow within a temperature range of 20°C to 45°C. The distinguishing characteristics of all mesophiles are their ability to grow at human body temperature (37°C) and their inability to grow at temperatures above 45°C. Included among the mesophiles are two distinct groups:
   a. Those whose optimum growth temperature is in the range of 20°C to 30°C are plant saprophytes.
   b. Those whose optimum growth temperature is in the range of 35°C to 40°C are organisms that prefer to grow in the bodies of warm-blooded hosts.

3. Thermophiles: Bacterial species that will grow at 35°C and above. Two groups of thermophiles exist:
a. **Facultative thermophiles:** Organisms that will grow at 37°C, with an optimum growth temperature of 45°C to 60°C.

b. **Obligate thermophiles:** Organisms that will grow only at temperatures above 50°C, with optimum growth temperatures above 60°C.

**Materials**

**Cultures**

24- to 48-hour nutrient broth cultures of *Escherichia coli*,

**Media**

Per designated student group: four Trypticase soy agar plates.

**Equipment**

Bunsen burner, inoculating loop, refrigerator set at 4°C, two incubators set at 37°C and 60°C, sterile Pasteur pipette, test tube rack, and glassware marking pencil.

**Procedure** Lab One

1. Score the underside of all plates into four quadrants with a glassware marker. Label each section with the name of the test organism to be inoculated. When labeling the cover of each plate, include the temperature of incubation (4°C, 20°C, 37°C, or 60°C).

2. Aseptically inoculate each of the plates with *E. coli* by means of a single line of inoculation in its appropriately labeled section.

3. Incubate all plates in an inverted position at each of the four experimental temperatures (4°C, 20°C, 37°C, or 60°C) for 24 to 48 hours.

**Procedure** Lab Two

a. Observe all the cultures for the presence of growth. Record your observations: (1+) for scant growth; (2+) for moderate growth; (3+) for abundant growth; and (−) for the absence of growth. Evaluate the amount of growth. Record and classify the cultures as psychrophiles, mesophiles, facultative thermophiles, or obligate thermophiles.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td></td>
</tr>
<tr>
<td>20°C</td>
<td></td>
</tr>
<tr>
<td>37°C</td>
<td></td>
</tr>
<tr>
<td>60°C</td>
<td></td>
</tr>
</tbody>
</table>

**Observations and Results:**

[Table and content]
7. DETERMINATION OF THERMAL DEATH POINT

PRINCIPLE: Thermal death

Thermal death is the death of a population of microorganisms due to exposure to an elevated temperature. Thermal death also involves the destruction of the membranes surrounding microorganisms such as bacteria. The high temperatures can cause the phospholipid constituents of the membrane to dissolve and thus destroy the membrane structure. Finally, the high heat will also cause the destruction of the nucleic acid of the target microorganism. In the case of double-stranded DNA, the heat will result in the disassociation of the two DNA strands.

Thermal death can be related to time. A term known as the thermal death time is defined as the time required to kill a population of the target microorganism in a water-based solution at a given temperature. The thermal death time of microorganisms can vary, depending on the thermal tolerance of the microbes. For example, thermophilic bacteria such as Thermophilus aquaticus that can tolerate high temperatures will have a thermal death time that is longer than the more heat-sensitive bacterium Escherichia coli.

Another aspect or measure of thermal death is termed the thermal death point. This is defined as the lowest temperature that will completely kill a population of a target microorganism within 10 minutes. This aspect of thermal death is useful in purifying water via boiling. Whereas Escherichia coli populations will be readily killed within 10 minutes at 212°F (100°C), spores of bacteria such as Bacillus subtilis and Clostridium perfringens will have a higher thermal death point, because a higher temperature is required to kill spores within 10 minutes.

Required Materials:

2 Nutrient agar plates, 1 Empty glass test tube, 1 Large beaker Heavy duty tongs for lifting beaker

hot plate, Thermometer, Test tube holder, Inoculating loops.

Procedure:

1. Label the agar plates and the test tubes appropriately.
2. Using a sterile inoculating loop, transfer one loopful of the *E. coli* broth culture to the control sector of the *E. coli* agar plate.

3. Using another sterile inoculating loop, transfer one loopful of the *B. subtilis* broth culture to the control sector of the *B. subtilis* agar plate.

4. 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.

5. Monitor the temperature, and when the thermometer reads the assigned temperature (either 63°C (± 1°C) or 72°C (± 1°C)), etc., use the tongs to remove the beaker from the hot plate and set it on the bench top.

6. Place the broth culture test tubes (*E. coli* and *B. subtilis*) into the water bath and begin timing. Maintain the culture broths at assigned temperature for 10 minutes. After 10 minutes, remove one loopful of the broth and subculture to the appropriate sector on the appropriate agar plate. Be sure to monitor the thermometer to insure that the water bath remains at the specified temperature for the entire 15 minute incubation period.

7. When complete, incubate the two agar plates at 35°C for 18-24 hours. Discard the broth culture tubes into the biohazard container.

8. Following incubation, record the results in the following table. For each sector on the agar plate, indicate + (use the growth control as a guide), to indicate growth and (-) to indicate no growth.

**Thermal Death point**

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 minutes</td>
<td>63°C 72°C 80°C 100°C</td>
</tr>
</tbody>
</table>

*Escherichia coli*

*Bacillus subtilis*
8. ISOLATION OF COLIPHAGES FROM RAW SEWAGE (DEMONTRATION)

Principle
Isolates of bacterial viruses (bacteriophages) can be obtained from a variety of natural sources, including soil, intestinal contents, raw sewage, and some insects such as cockroaches and flies. Their isolation from these environments is not an easy task because the phage particles are usually present in low concentrations. Therefore, isolation requires a series of steps:

1. Collection of the phage-containing sample at its source.
2. Addition of an enriched susceptible host cell culture to the sample to increase the number of phage particles for subsequent isolation.
3. Following incubation, centrifugation of the enriched sample for the removal of gross particles.
4. Filtration of the supernatant liquid through a bacteria-retaining membrane filter.
5. Inoculation of the bacteria-free filtrate onto a lawn of susceptible host cells grown on a soft agar plate medium.
6. Incubation and observation of the culture for the presence of phage particles, which is indicated by plaque formation in the bacterial lawn.

Materials

Cultures
Lab One: 5-ml 24-hour broth cultures of E. coli B and 45-ml samples of fresh sewage collected in screw-capped bottles. Lab Two: 10-ml 24-hour broth cultures of E. coli B.

Media
Per designated student group: Lab One: One 5-ml tube of bacteriophage nutrient broth, 10 times normal concentration. Lab Two: Five tryptone agar plates and five 3-ml tubes of tryptone soft agar.

Equipment
Lab One: Sterile 250-ml Erlenmeyer flask and stopper. Lab Two: Sterile membrane filter apparatus, sterile 125-ml Erlenmeyer flask and stopper, 125-ml flask, 1000-ml beaker, centrifuge, Bunsen burner, forceps, 1-ml sterile disposable pipettes, sterile Pasteur pipette, mechanical pipetting device, test tube rack, and glassware marking pencil.

Procedure Lab One

Enrichment of Sewage Sample
1. Aseptically add 5 ml of bacteriophage nutrient broth, 5 ml of the E. coli B broth culture, and 45 ml of the raw sewage sample to an appropriately labeled sterile 250-ml Erlenmeyer flask.
2. Incubate the culture for 24 hours at 37°C.

**Procedure** Lab Two

**Filtration and Seeding**

1. Following incubation, pour the phage-infected culture into a 100-ml centrifuge bottle or several centrifuge tubes and centrifuge at 2500 rpm for 20 minutes.
2. Remove the centrifuge bottle or tubes, being careful not to stir up the sediment, and carefully decant the supernatant into a 125-ml beaker.
3. Pour the supernatant solution through a sterile membrane filter apparatus to collect the bacteria-free, phage-containing filtrate in the vacuum flask below. Refer to Experiment 50 for the procedure in assembling the filter membrane apparatus.
4. Melt the soft tryptone agar by placing the five tubes in a boiling waterbath and cool to 45°C.
5. Label the five tryptone agar plates and the five tryptone agar tubes 1, 2, 3, 4, and 5, respectively.
6. Using a sterile 1-ml pipette, aseptically add 0.1 ml of the *E. coli* B culture to all the molten soft-agar tubes.
7. Using a sterile Pasteur pipette, aseptically add 1, 2, 3, 4, and 5 drops of the filtrate to the respectively labeled molten soft-agar tubes. Mix and pour each tube of soft agar into its appropriately labeled agar plate.
8. Allow agar to harden.
9. Incubate all the plates in an inverted position for 24 hours at 37°C.

**Procedure** Lab Three

1. Examine all the culture plates for plaque formation, which is indicative of the presence of coliphages in the culture.
2. Indicate the presence (+) or absence (-) of plaques in each of the cultures in the chart in the Lab Report.

**Observations and Results**

<table>
<thead>
<tr>
<th>DROPS OF PHAGE FILTRATE</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLAQUE FORMATION (+) OR (-)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Phage Plaques*
Figure 40.1 Procedure for isolation of coliphages from raw sewage
9. STUDY OF CYANOBACTERIA

Nostoc:

Classification
Kingdom: Bacteria
Division: Cyanobacteria
Class: Cyanophyceae
Order: Nostocales
Family: Nostocaceae
Genus: Nostoc

1. Thalli are present in the form of colony.

2. Ball like colony is enveloped by a gelatinous sheath

3. Balls are greenish to bluish-green in colour.

4. Each colony contains thousands of straight or twisted filaments or trichomes.
5. Each trichome is surrounded by its individual sheath and called the filament.

6. A trichome is contorted and consists of many cells arranged in a beaded manner.

7. Each cell is somewhat cylindrical or spherical in shape. In filaments there are present some large, spherical or cylindrical, colourless empty cells called hetero cysts.

9. Heterocysts are generally intercalary but in the young condition, they may be terminal.

10. Two polar nodules are present in each heterocyst.

11. Some cells of the filament become enlarged and filled with the food material. These thick-walled cells are called akinetes. Akinetes are generally present in chain.

& & & & & & & &
Scytonema

Phylum: **Cyanobacteria**

Class: **Cyanophyceae**

Order: **Nostocales** (Hormogonales) Family: **Scytonemataceae**

Genus: **Scytonema**

1. The plant body is a filament and is surrounded by mucilage sheath, like in other blue green algae.
2. The sheath is lamellated and of golden yellow or brown color. The filaments possess either intercalary or terminal heterocysts.
3. The genus is characterized by its unique type of branching. This is known as "false branching" or "Gemminate branching". The filament breaks up near a heterocyst and the broken end protrudes out of the sheath as a branch. If both the free ends protrude out as branches there will be two branches and it is known as "gemminate branching". If only one develops into a branch, it is known as "false branching". Later the branches develop their own mucilage sheath.
4. Cells are cylindrical or rectangular in shape. Heterocysts are found in between the vegetative cells. The terminal cell is usually hemispherical.
5. There is no sexual reproduction:

6. Asexual reproduction is brought about by the formation of hormogonia.
Stigonema:

**Class**: Cyanobacteria (Cyanobacteria)

**Order**: Stigonematales

**Family**: Stigonemataceae

**Genus**: Stigonema

1. Thallus possess a filamentous and hetertrichous habit. The filaments are variuosly curved and irregularly branched.

2. The branches may arise from all sides of the main axis and may branch and rebranch repeatedly.

   The branches are true branch and lateral which retain the continuity with the main filament.

3. The cells are characteristically spherical but in young axis and branches they are flattened due to mutual pressure. In old filaments the lie at a distance from one another and these cells may be surrounded by their individual sheath. In mature filaments, adjacent sister cells are connected with each other by pit connections.

4. Heterocysts are present both in the main filament as well as in the branches. They may be terminal or intercalary but lateral in position.

5. Reproduction by hormogonia.

&&&&&&&&&&&&&&&
Oscillatoria

Kingdom: Bacteria  
Phylum: Cyanobacteria  
Class: Cyanophyceae  
Order: Oscillatoriales  
Family: Oscillatoriaceae  
Genus: Oscillatoria

1. Its body is composed of single row of cells. These cells form trichomes.
2. Its trichomes are unbranched filaments. They are covered by very thin mucilaginous sheath.
3. All cells of a trichome are similar in shape except apical cells. The apical cells are convex at the tip.
4. All other cells are broader and cylindrical. In some species, the apical cells may end in subacute point. In some cases, it may have cap or calyptra at the tip.
5. Reproduction by hormogonia.
ENTAMOEBA:

Kingdom :Protista

Subkingdom:Protozoa

Phylum :Sarcomastigophora

Subphylum:Sarcodina

Class :Lobosea

Order :Amoebida

Family: Endamoebidae

Genus :Entamoeba

Species: histolytica

1. The trophozoites range in size from 18 to 40 μm (mean 25 μm) and do not have a definite shape.

2. Their cytoplasm has an outer, clear ectoplasm and inner, granular endoplasm.

3. The trophozoites of *F. histolytica* engulf red blood cells, bacteria, yeast and other debris.

4. The trophozoites contain a single, spherical, 3 to 5 μm nucleus. The nucleus has a delicate nuclear membrane.

5. On the internal surface of the nuclear membrane there are minute granules known as chromatin dots.

6. In the center of the nucleus is a single dense karyosome or nucleolus.

7. The trophozoites of *E. histolytica* live in the mucosal folds of the large intestine and divide by binary fission.

&&&&&&&&
BALANTIDIUM COLI

KINGDOM Chromalveolata
PHYLUM Ciliophora
CLASS Litostomatea
ORDER Vestibuliferida
FAMILY Balantiididae
GENUS Balantidium

1. Shape and size: Ovoid with tapering anterior end; 50–100 um long, 40–70 um wide

2. Motility: Rotary, boring; may be rapid

3. Nuclei: 1 large kidney-bean-shaped macronucleus may be visible in unstained preparation; 1 small round micronucleus adjacent to macronucleus, difficult to see

4. Cytoplasm: May be vacuolated; may contain ingested bacteria and debris; anterior cytostome

5. Cilia: Body surface covered with longitudinal rows of cilia; longer near cytostome

PLASMODIUM GAMETOCYTE

Gametocytes: After a variable number of schizogony cycles, gametogony is initiated. In gametogony, instead of merozoites, a gametocyte is formed inside an RBC. There are two types of gametocytes- female gametocyte and male gametocyte. They are the exit forms of malarial parasite. As these forms do not develop further, syngamy- sexual process – can not take place in human beings further development of gametocytes and syngamy takes place in mosquitoes.