Isolation of Microorganisms

Microorganisms occur in natural environment like soil. They are mixed with several other forms of life. Many microbes are pathogenic. They cause a number of diseases with a variety of symptoms, depending on how they interact with the patient. The isolation and growth of suspected microbe in pure culture is essential for the identification and control the infectious agent.

The primary culture from natural source will normally be a mixed culture containing microbes of different kinds. But in laboratory, the various species may be isolated from one another. A culture which contains just one species of microorganism is called a pure culture. The process of obtaining a pure culture by separating one species of microbe from a mixture of other species, is known as isolation of the organisms.

Methods of Isolation

There are special techniques employed to obtain pure cultures of microorganisms. In few cases it is possible to secure pure culture by direct isolation or direct transfer. This can be done only in those situations in which pure culture occurs naturally. Kinds of specimens taken for culturing will depend on the nature and habitat of microbes.

Different pathogens can be isolated from body tissues and fluids such as blood, urine, sputum, pus, faces, spinal fluid, bile, pleural fluids, stomach fluids etc. In the blood stream of a patient suffering with typhoid fever, the bacteria Salmonella typhosa may be present.

A pure culture of this bacterium may be obtained by drawing blood sample using a sterilized hypodermic syringe and treating the blood with anticoagulant such as heparin and potassium oxalate. The presence of the anticoagulant prevents the pathogenic microbe from entrapping in fibrin clot. The sample of the blood may be inoculated into a suitable medium.

Following isolation methods are employed to isolate microbes from mixed cultures

1. Streaking

2. Plating

3. Dilution

4. Enriched procedure, and

5. Single cell technique.

1. Streaking:

This is most widely used method of isolation. The technique consists of pouring a suitable sterile medium into sterile petriplate and allowing the medium to solidify. By means of a sterile loope or straight needle or a sterile bent glass-rod a small amount of growth preferably from a broth culture or bacterial suspension is streaked back and forth across the surface of agar until about one third of the diameter of the plate has been covered.

The needle is then flamed and streaking in done at right angles to and across the first streak. This serves to drag bacteria out in a long line from the initial streak. When this streaking is completed the needle is again flamed and streaking is done at right angles to the second streak and parallel to the first.
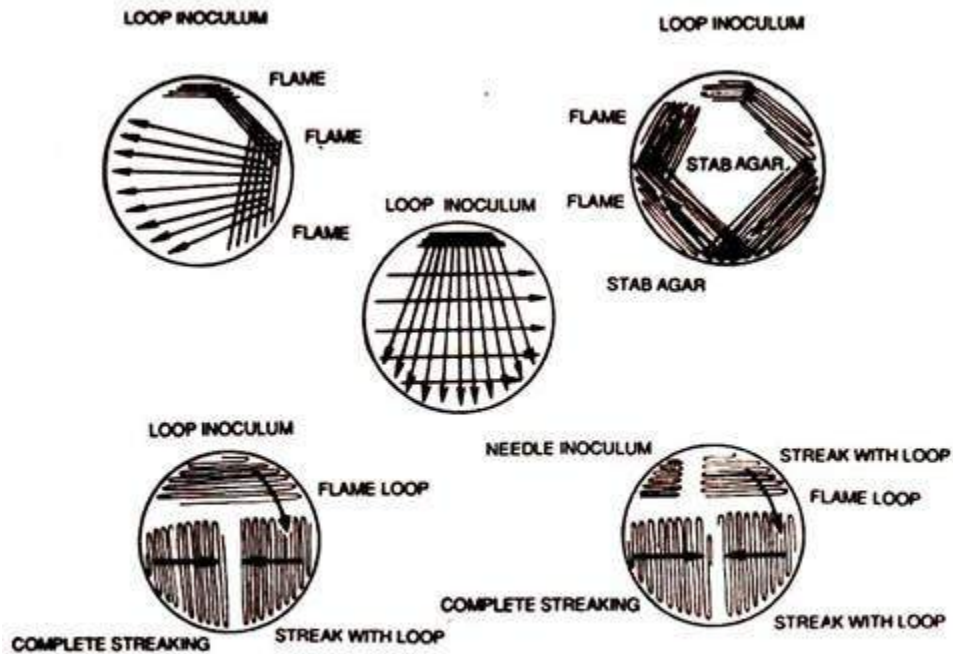
Fig. 2.1 Different patterns used for obtaining pure culture by streaking.

2. Plating:

It includes diluting of a mixture of microorganisms until only a few hundred bacteria are left in each millilitre of the suspension. A very small amount of the dilution is then placed in a sterile petriplateby means of a sterile loop or pipette. The melted agar medium is cooled to about 45°C and is poured into plate. The microorganism and agar are well mixed. When the agar is solidified the individual bacterium will be held in place and will grow to a visible colony.
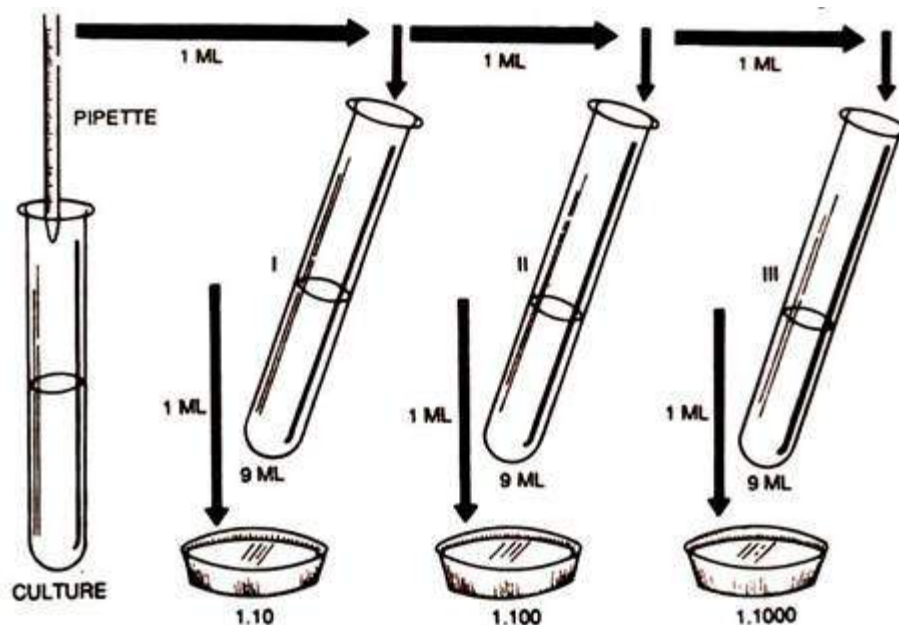
Fig. 2.2 Dilution method of isolation.

3. Dilution:

This method is used for the microorganisms which cannot be easily isolated by streaking or plating method. Sometimes when several organisms are present in a mixture, with one organism predominating, the predominating form may be isolated by this method. For example, when raw milk is allowed to sour at room temperature it will, at the time of curding, have a mixture of microorganisms with high percentage of Streptococcus lactis.

If 1 ml of the sour milk is taken into a tube containing 9 ml. of sterile milk (in which no organisms are present) then 1 ml. of this mixture is transferred with a sterile pipette into a second tube of sterile milk and the procedure is repeated i.e. from second to third tube, third to fourth tube until a series of about 10 tubes are inoculated. By this serial dilution, the chances are that a pure culture of S. lactis will be obtained.

4. Enrichment Procedure:

This procedure involves the use of media and conditions of cultivation which favour the growth of the desired species. For example, when a man suffers with

typhoid, the intestinal discharge posses small number of Salmonella typhosa when compared with E. coli and other forms.

It is almost impossible to isolate the typhoid organisms because they represent only a fraction of a per cent of the total microorganisms present. The media are therefore derived, which allow the rapid growth of the desired organisms, at the same time inhibiting the growth of other microorganisms.

5. Single Technique:

This is one of the most ideal and difficult method of securing pure culture. In this method a suspension of the pure culture is placed on the under-side of a sterile cover-glass mounted over a moist chamber on the stage of the microscope.

While looking through the microscope, a single cell is removed with the help of sterile micropipette and transferred to a small drop of sterile medium on a sterile cover-glass and is mounted on a sterile hanging drop slide, which is then incubated at suitable temperature. If the single cell germinates in this drop, few cells are transferred into a tube containing sterile culture medium which is placed in the incubator to obtain pure culture originated from single cell.
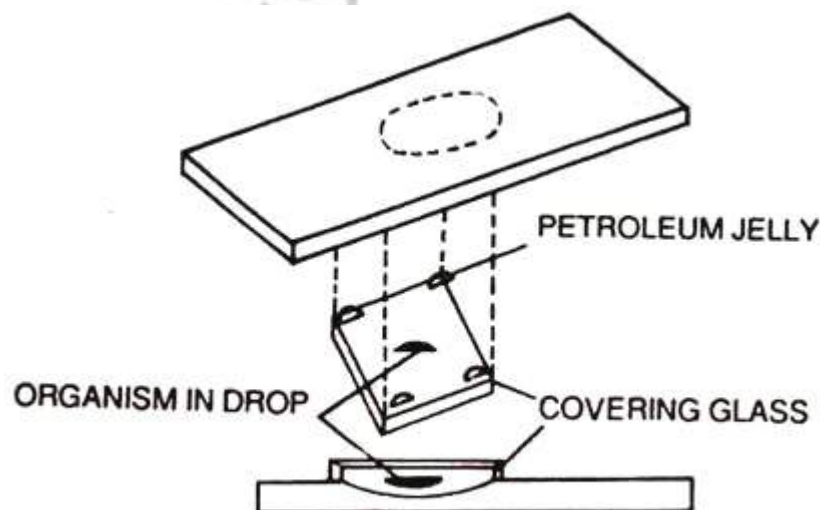
PETROLEUM JELLY

ORGANISM IN DROP

COVERING GLASS

Fig. 2.3   Hanging drop slide preparation.

Preparation of Media:

There are three main steps in the preparation of media:

(a) Preparation as solutions of chemicals and adjusting the pH.

(b) Dispensing the media, and

(c) Sterilization.

A broth is prepared by dissolving the appropriate amount of the components in distilled water and pH is adjusted by the addition of either dilute NaOH or Hcl. The broth is dispensed into clean rimless 'Pyrex' test tubes which are plugged with non-absorbant cotton wool plugs. The test tubes are placed in wire baskets which are covered with grease proof paper.

The media are sterilized by autoclaving at a temperature of 121 °C and a pressure of 151 b/in2 for 15 minutes. But medium containing heat- sensitive substances are sterilized either by filtering the solution at room temperature, using bacteria-proof filter or by a process called Tyndallization.

In this method, the liquids are steamed for one hour a day on three consecutive days and the liquids are incubated at 25-30°C. During the first steaming, all the heat sensitive vegetative cells are killed, leaving only the spores. During the first incubation period, most of the spores germinate in to vegetative cells. These vegetative cells are killed by the second steam period.

In the second incubation period, the rest of the spores germinate into vegetative cells which are killed by the third steaming period. In this way, the liquids are sterilized without temperature rising above 100°C.

Enumeration of Microorganisms

Introduction (Plate Count)

The laboratory microbiologist often has to determine the number of bacteria in a given sample, as well as having to compare the amount of bacterial growth under various conditions. Enumeration of microorganisms is especially important in dairy microbiology, food microbiology, and water microbiology. Since the enumeration of microorganisms involves the use of extremely small dilutions and extremely large numbers of cells, scientific notation is routinely used in calculations.

The number of bacteria in a given sample is usually too great to be counted directly. However, if the sample is serially diluted and then plated out on an agar surface, single isolated bacteria can form visible isolated colonies.
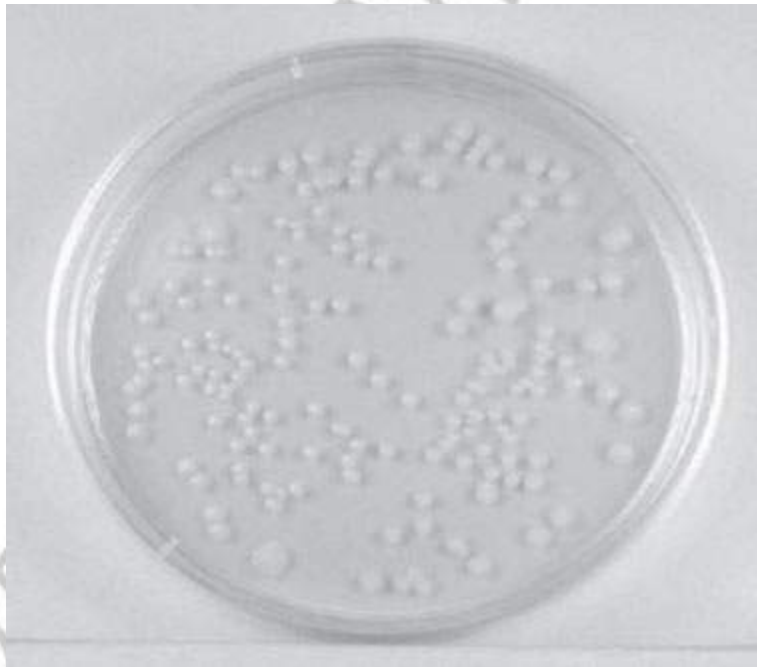
Figure Single isolated colonies obtained during the plate count.

The number of colonies can be used as a measure of the number of viable (living) cells in that known dilution. owever, keep in mind that if the organism normally forms multiple cell arrangements, such as chains, the colony-forming unit ay

consist of a chain of bacteria rather than a single bacterium. In addition, some of the bacteria may be clumped ogether. Therefore, when doing the plate count technique, we generally say we are determining the number of colony-forming units (CFUs) in that known dilution. By extrapolation, this number can in turn be used to calculate the number of CFUs in the original sample.

Normally, the bacterial sample is diluted by factors of 10 and plated on agar. After incubation, the number of colonies on a dilution plate showing between 30 and 300 colonies is determined. A plate having 30–300 colonies is chosen, because this range is considered statistically significant. If there are less than 30 colonies on the plate, small errors in dilution technique or the presence of a few contaminants will have a drastic effect on the final count. Likewise, if there are more than 300 colonies on the plate, there will be poor isolation and colonies will have grown together.

Generally, one wants to determine the number of CFUs per milliliter (mL) of sample. To find this, the number of colonies (on a plate having 30–300 colonies) is multiplied by the number of times the original mL of bacteria were diluted (the dilution factor of the plate counted). For example, if a plate containing a 1/1,000,000 dilution of the original mL of sample shows 150 colonies, then 150 represents 1/1,000,000 the number of CFUs present in the original mL. Therefore, the number of CFUs per mL in the original sample is found by multiplying 150 × 1,000,000, as shown in the formula below:

Number of CFUs per ml of sample = number of colonies (30–300 plate) × the dilution factor of the plate counted
In the case of the example above, 150 × 1,000,000 = 150,000,000 CFUs per mL.

For a more accurate count, it is advisable to plate each dilution in duplicate or triplicate and then find an average count.

Direct Microscopic Method (Total Cell Count)
In the direct microscopic count, a counting chamber consisting of a ruled slide and a coverslip is employed. It is constructed in such a manner that a known volume is delimited by the coverslip, slide, and ruled lines. The number of bacteria in a small

known volume is directly counted microscopically and the number of bacteria in the larger original sample is determined by extrapolation.
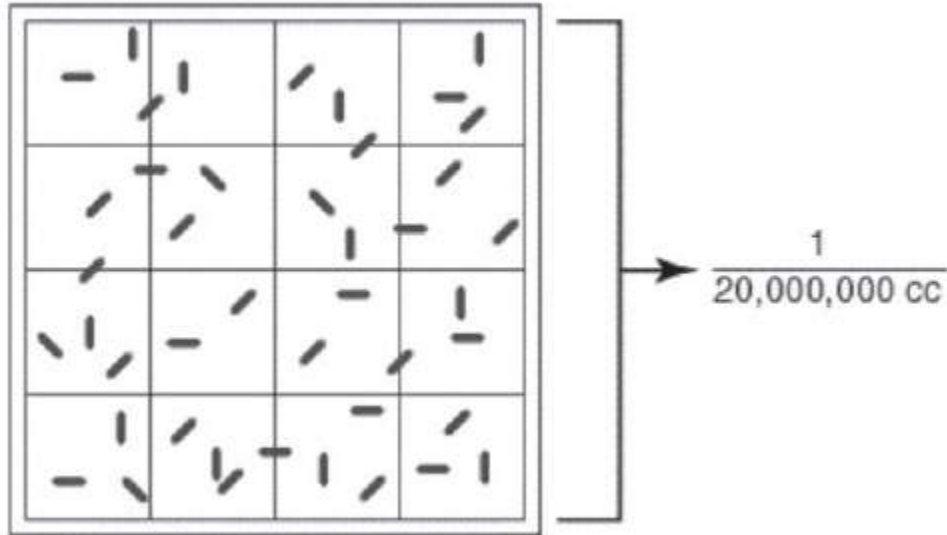

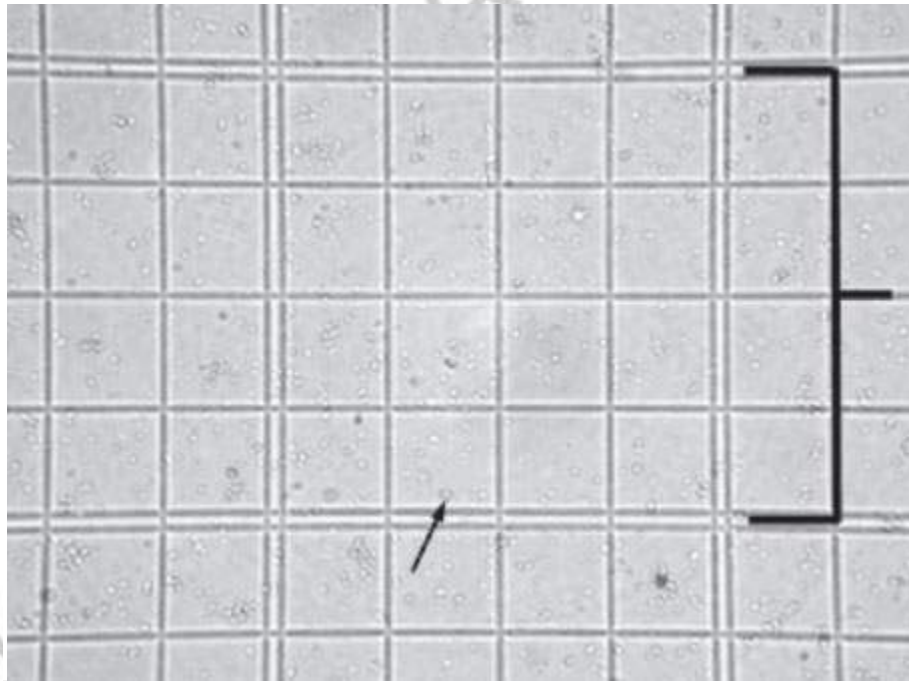**Figure** Large double-lined square of a Petroff-Hausser counter.

Figure Petroff-Hausser Counter as seen through a microscope.

The double-lined "square" holding 1/20,000,000 cc is shown by the bracket. The arrow shows a bacterium. The square holds a volume of 1/20,000,000 of a cubic centimeter. Using a microscope, the bacteria in the square are counted. For

example, has squares 1/20 of a millimeter (mm) by 1/20 of a mm and is 1/50 of a mm deep. The volume of 1 square, therefore, is 1/20,000 of a cubic mm or 1/20,000,000 of a cubic centimeter (cc). The normal procedure is to count the number of bacteria in 5 large double-lined squares and divide by 5 to get the average number of acteria per large square. This number is then multiplied by 20,000,000, since the square holds a volume of 1/20,000,000 cc, to find the total number of organisms per cc in the original sample.

If the bacteria are diluted (such as by mixing with dye) before being placed in the counting chamber, then this dilution must also be considered in the final calculations.

The formula used for the direct microscopic count is:

number of bacteria per cc = the average number of bacteria per large double-lined square × the dilution factor of the large square (20,000,000) × the dilution factor of any dilutions made prior to placing the sample in the counting chamber, e.g., mixing the bacteria with dye.

Turbidity

When we mix the bacteria growing in a liquid medium, the culture appears turbid. This is because a bacterial culture acts as a colloidal suspension that blocks and reflects light passing through the culture. Within limits, the light absorbed by the bacterial suspension will be directly proportional to the concentration of cells in the culture. By measuring the amount of light absorbed by a bacterial suspension, one can estimate and compare the number of bacteria present.
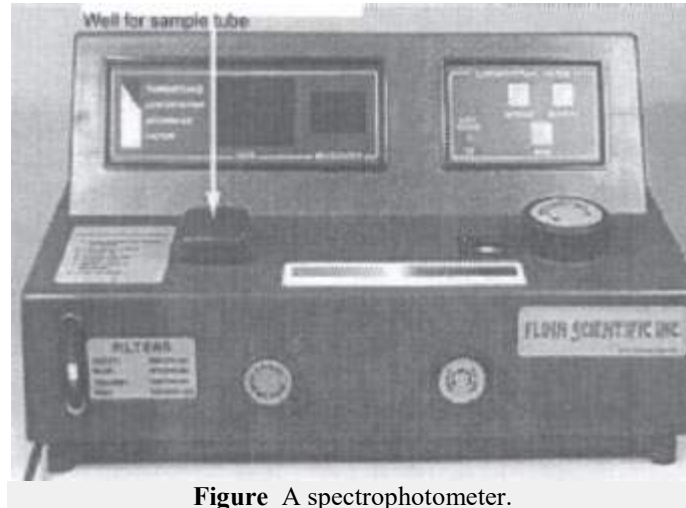
Figure A spectrophotometer.

The instrument used to measure turbidity is a spectrophotometer. It consists of a light source, a filter that allows only a single wavelength of light to pass through, the sample tube containing the bacterial suspension, and a photocell that compares the amount of light coming through the tube with the total light entering the tube.

The ability of the culture to block the light can be expressed as either percentage of light transmitted through the tube or amount of light absorbed in the tube.
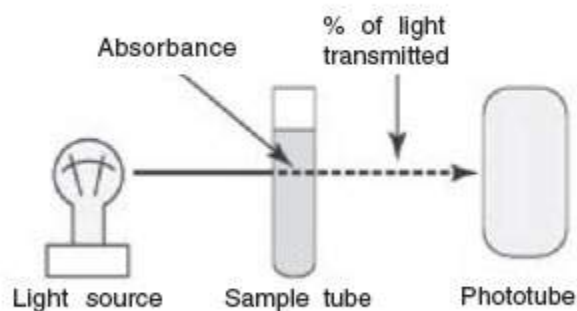

Figure A spectrophotometer.

The percentage of light transmitted is inversely proportional to the bacterial concentration. (The greater the percent transmittance, the lower the number of bacteria.) The absorbance (or optical density) is directly proportional to the cell

concentration. (The greater the absorbance, the greater the number of bacteria.) Turbidimetric measurement is often correlated with some other method of cell count, such as the direct microscopic method or the plate count. In this way, turbidity can be used as an indirect measurement of the cell count. For example:

1. Several dilutions can be made of a bacterial stock.
2. A Petroff-Hausser counter can then be used to perform a direct microscopic count on each dilution.
3. Then, a spectrophotometer can be used to measure the absorbance of each dilution tube.
4. A standard curve comparing absorbance to the number of bacteria can be made by plotting absorbance versus the number of bacteria per cc.
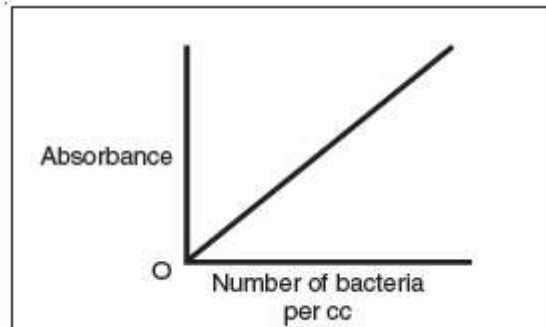

Figure A standard curve plotting the number of bacteria per cc versus absorbance.

1.

2. Once the standard curve is completed, any dilution tube of that organism can be placed in a spectrophotometer and its absorbance read. Once the absorbance is determined, the standard curve can be used to determine the corresponding number of bacteria per cc.
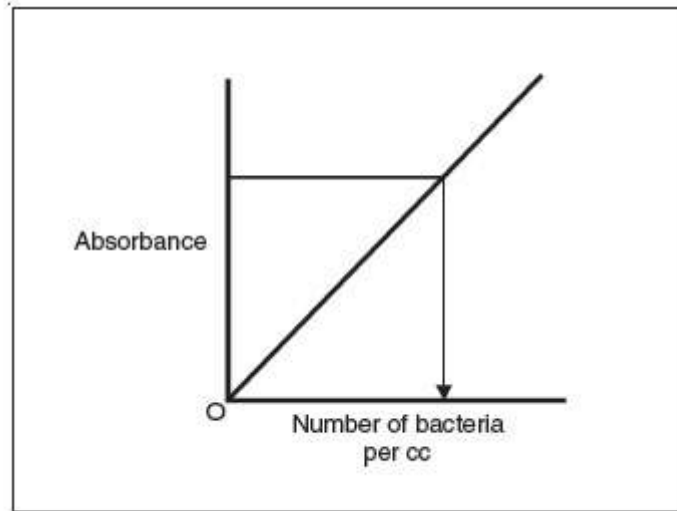
Figure Using a standard curve to determine the number of bacteria per cc in a sample by measuring the sample's absorbance.

1.

Materials: 6 tubes each containing 9.0 mL of sterile saline, 3 plates of trypticase soy agar, 2 sterile 1.0-mL pipettes, pipette filler, turntable, bent glass rod, dish of alcohol.

Organism: Trypticase soy broth culture of Escherichia coli.

2.

Procedure

Plate Count

1. Take 6 dilution tubes, each containing 9.0 mL of sterile saline. Aseptically dilute 1.0 mL of a sample of E. coli, as shown in and described as follows:
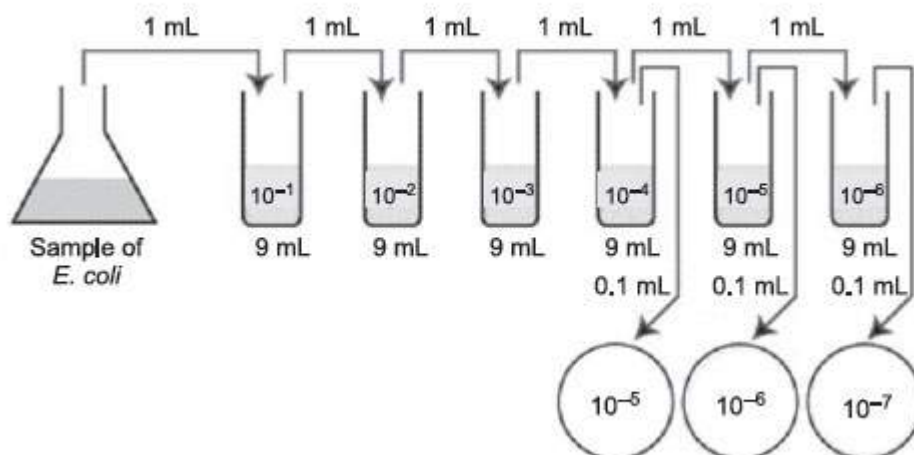
Figure Plate count dilution procedure.

1.

2. Remove a sterile 1.0-mL pipette from the bag. Do not touch the portion of the pipette tips that will go into the tubes and do not lay the pipette down. From the tip of the pipette to the "0" line is 1 mL; each numbered division (0.1, 0.2, etc.) represents 0.1 mL.

3. Insert the cotton-tipped end of the pipette into a blue 2-mL pipette filler.

4. Flame the sample flask, insert the pipette to the bottom of the flask, and withdraw 1.0 mL (up to the "0" line of the sample) by turning the filler knob towards you. Draw the sample up slowly so that it isn't accidentally drawn into the filler itself. Reflame and cap the sample.

5. Flame the first dilution tube and dispense the 1.0 mL of sample into the tube by turning the filler knob away from you. Draw the liquid up and down in the pipette several times to rinse the pipette and help mix. Reflame and cap the tube.

6. Mix the tube thoroughly by either holding the tube in one hand and vigorously tapping the bottom with the other hand or by using a vortex mixer. This is to assure an even distribution of the bacteria throughout the liquid.

7. Using the same procedure, aseptically withdraw 1.0 mL from the first dilution tube and dispense into the second dilution tube. Continue doing

this from tube to tube as shown in until the dilution is completed. Discard the pipette in the biowaste disposal containers at the front of the room and under the hood. These pipetting and mixing techniques will be demonstrated by your instructor.

8. Using a new 1.0-mL pipette, aseptically transfer 0.1 mL from each of the last 3 dilution tubes onto the surface of the corresponding plates of trypticase soy agar as shown in figure. Note that since only 0.1 mL of the bacterial dilution (rather than the desired 1.0 mL) is placed on the plate, the bacterial dilution on the plate is 1/10 the dilution of the tube from which it came. Using a turntable and sterile bent glass rod, immediately spread the solution over the surface of the plates as follows:
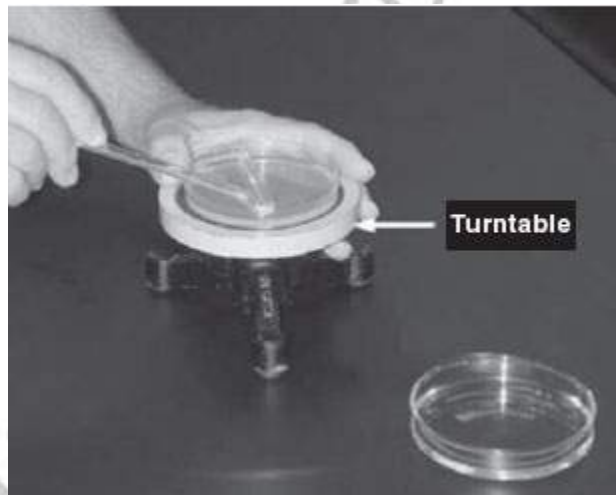

Figure Using a bent glass rod and a turntable to spread a bacterial sample.

9.

10. Place the plate containing the 0.1 mL of dilution on a turntable.
11. Sterilize the glass rod by dipping the bent portion in a dish of alcohol and igniting the alcohol with the flame from your burner. Let the flame burn out.

12. Place the bent portion of the glass rod on the agar surface and spin the turntable for about 30 seconds to distribute the 0.1 mL of dilution evenly over the entire agar surface.
13. Replace the lid and resterilize the glass rod with alcohol and flaming.
14. Repeat for each plate.
15. Discard the pipette in the biowaste disposal containers at the front of the room and under the hood.
16. Incubate the 3 agar plates upside down at 37°C until the next lab period. Place the used dilution tubes in the disposal baskets in the hood.

Direct Microscopic Method

1. Pipette 1.0 mL of the sample of E. coli into a tube containing 1.0 mL of the dye methylene blue. This produces a ½ dilution of the sample.
2. Using a Pasteur pipette, fill the chamber of a Petroff-Hausser counting chamber with this ½ dilution. Turntable
3. Place a coverslip over the chamber and focus on the squares using 400X (40X objective).
4. Count the number of bacteria in 5 large double-lined squares. For those organisms on the lines, count those on the left and upper lines, but not those on the right and lower lines. Divide this total number by 5 to find the average number of bacteria per large square.
5. Calculate the number of bacteria per cc as follows:

Number of bacteria per cc = the average number of bacteria per large square × the dilution factor of the large square (20,000,000) × the dilution factor of any dilutions made prior to placing the sample in the counting chamber, such as mixing it with dye (2 in this case).

1.

Turbidity

Your instructor will set up a spectrophotometer demonstration illustrating that as

the number of bacteria in a broth culture increases, the absorbance increases (or the percent of light transmitted decreases).

Results

Plate Count

Choose a plate that appears to have between 30 and 300 colonies.

1. Sample 1/100,000 dilution plate
2. Sample 1/1,000,000 dilution plate
3. Sample 1/10,000,000 dilution plate.

Count the exact number of colonies on that plate using the colony counter (as demonstrated by your instructor).

Calculate the number of CFUs per mL of original sample as follows:

$$\text{Number of CFUs per mL of sample} = \text{Number of colonies (30–300 plate)} \times \text{the dilution factor of the plate counted}$$

_____ = Number of colonies

_____ = Dilution factor of plate counted

_____ = Number of CFUs per mL.

Record your results on the blackboard.

Direct Microscopic Method

Observe the demonstration of the Petroff-Hausser counting chamber.

Turbidity

Observe your instructor's demonstration of the spectrophotometer.