Course: MBIO 240
Laboratory Skills

Methods of Microbial Enumeration

Aim: To determine the number of microorganisms present in a sample.

For example, the ability to determine the safety of food, milk, and water depends on Knowing the number of microbes found in such products.

Microbial enumeration is carried out using common methods such as

- ✓ Plate counts (Pour plate; Spread plate)
- ✓ Member filtration
- ✓ Most Probable Number (MPN)

Plate counts:

Microorganisms can be present in thousands or millions in a sample, making it difficult to count their numbers. However, serially diluting the samples makes it easier to determine the count.

Serial Dilution Protocols:

Aim:

- ✓ To dilute the sample to get countable number of colonies.

- ✓ It is a method of diluting a stock solution where concentration decreases by the same quantity in each successive step.

Materials Required: Samples, Test tube, Test-tube racks, Conical flask, Pipettes, Beaker, Distilled water and Autoclave.

Procedure:

- ✓ Assemble the above materials at your workbench.

- ✓ Take 8 test tubes label 10^{-1},10^{-2},10^{-3} up to 10^{-8} and Pour 9ml of distilled water to these test tubes.

- ✓ Above the materials sterilized by autoclaving at 121°C for 15minuts (steam at a pressure about 15 psi).

- ✓ After sterilizing, Take 1gm solid sample or 1ml liquid sample to add 9 ml of distilled water in the first test tube and consider as a stock solution 10^{-1}.

Procedure

- ✓ Using a sterile pipette, transfer 1ml of stock solution to the test tube labeled 10^{-2} and mix well (Mix gently by drawing the solution up and down 3 times).
- ✓ Transfer 1 ml of solution from tube 10^{-2} into tube 10^{-3}. Mix Gently 3times
- ✓ Transfer 1 ml of solution from tube 10^{-3} into tube 10^{-4}. Mix gently 3time. Continue to transfer and mix through tube 10^{-7}.
- ✓ Discard the last 1ml from tube 10^{-7}.
- ✓ Noted : 10^{-8} Used as control (without sample).
- ✓ Examine the tube dilutions using pour plate and spread plate methods.

Calculate dilution.

- ✓The dilution of a sample in a diluent (the liquid used to dilute the sample) can be calculated as:

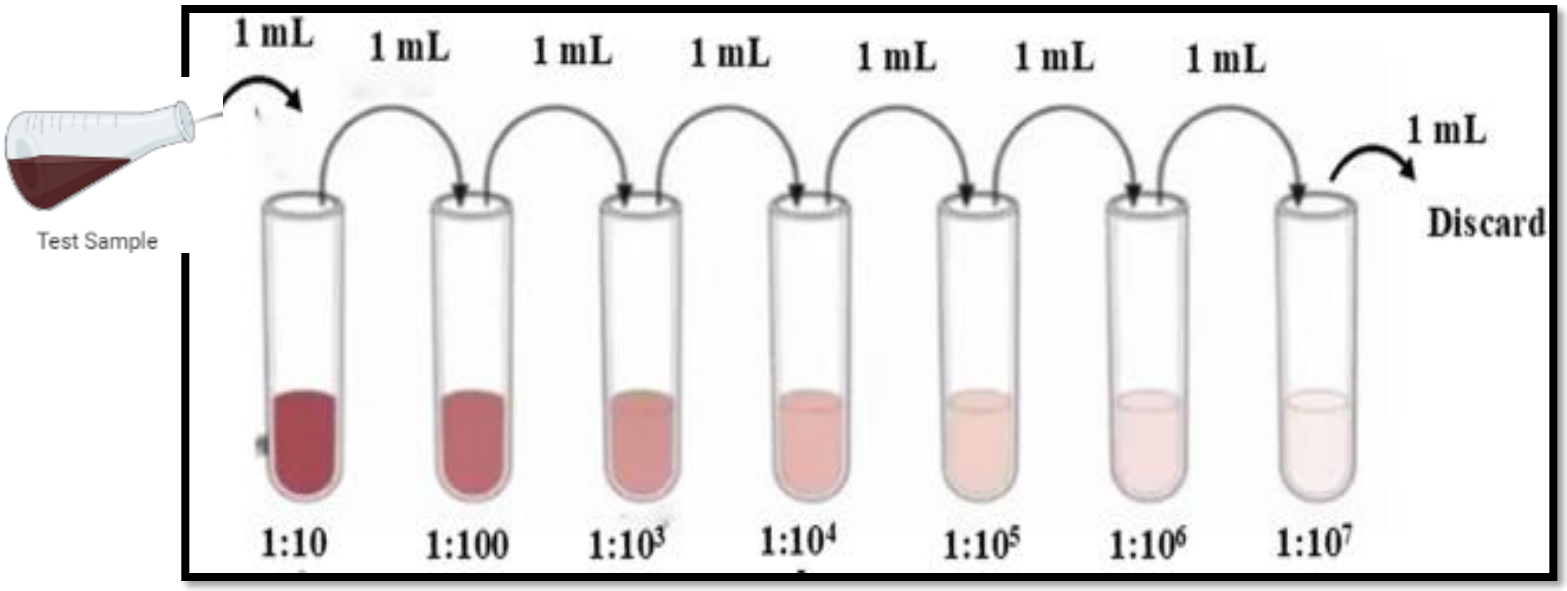
$$\text{Dilution} = \frac{\text{vol. sample}}{\text{vol. sample} + \text{vol. diluent}}$$

For example, if **1 mL** of a sample was diluted by adding it to **9 mL** of water (diluent), then:

$$\text{Dilution} = \frac{1}{1+9} = 1/10 = 10^{-1}$$

In microbiology, dilutions are usually reported as exponents.

For example: 1/10 = 10-1; 1/100 = 10-2; 1/1000 = 10-3; 1/10000 = 10-4; 1/100000 = 10-5 and so on…



Serial Dilution Steps

Note:

10^{-1} and 10^{-2} = Fungi: Potato Dextrose Agar Medium

10^{-3} and 10^{-4} = Actinomycetes: Starch Casein Nitrite Agar Medium

10^{-4}, 10^{-5}, 10^{-6} = Bacteria: Nutrient Agar medium

Pour plate methods: 1ml sample

Spread Plate Methods: 0.1ml sample

Pour plate and Spread plate technique:

Objectives:

- ✓ To isolate the microorganisms from the liquid specimen or suspension.
- ✓ To calculate viable microbial load by counting colony formation unit (CFU) per ml.
- ✓ To isolate the pure culture of microorganisms from a mixed population.
- ✓ To isolate microorganisms in discrete colonies in order to study colony characters

Materials Required:

- ✓ Serially diluted sample
- ✓ Sterile Petri dishes
- ✓ Nutrient agar (cooled to about 45-50°C)
- ✓ Sterile pipette
- ✓ Bunsen burner
- ✓ Container for aseptic work area.

Procedure:

- ✓ Test sample is firstly serially diluted in appropriate diluent.
- ✓ Then, take 1ml volume of sample from diluted tube is placed in Petri plate.

- Add Melted agar at 44–45°C is mixed with it.
- After homogenous mixing of sample with melted agar, it is kept for solidification.
- Then the petri plates are incubated at appropriate time 24–48hours and 37°C temperature.
- After 24–48 hours, count all the colonies.

- Count the colonies and calculate CFU/mL by using the formula:

$$CFU/mL = \frac{Total\ number\ of\ colonies\ obtained \times dilution\ factor}{volume\ of\ specimen\ used\ (aliquot)}$$

- For optimum count, the number of colonies must be between 30 – 300 CFU/mL.
- Beyond this limit, the whole procedure must be repeated.
- If the number of the colony is less than 30, it is suggested to use the sample of lower dilution.(It is called Too Few To Count – TFTC)
- Whereas, if the total number of colonies exceeds 300, it is suggested to use the sample of higher dilution on successive repeats. .(It is called Too Numerous To Count – TNTC)

Result:

Suppose the plate of the 10^{-4} dilution yielded a count of 123 colonies. Then, the total number of Colony Forming Units in 1 ml of the original sample can be calculated as follows.

Number of colonies : 123

Dilution factor: 10^{-4}

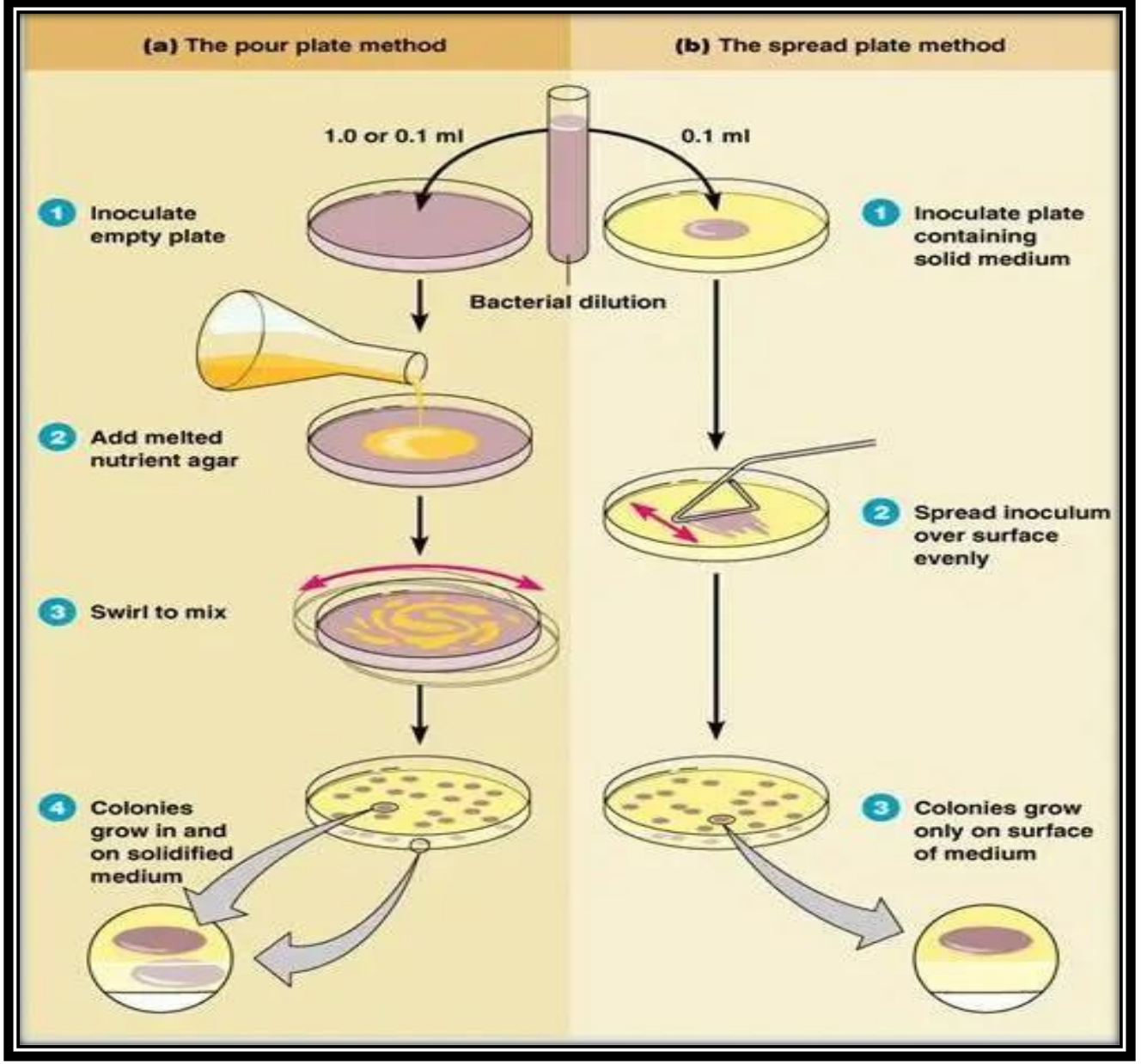
Volume plated : 1

$$\text{CFU} = \frac{123 \times 10^{-4}}{1} = 1{,}230{,}000 \text{ CFU/ml}$$

CFU = 1,230,000 cells/mL in original sample

Spread plate technique:

- In this method, appropriately 0.1ml diluted sample is placed on the surface of solidified agar.
- Then the drop of sample is spread over agar surface using bent glass rod.
- Plate is incubated for sufficient time and temperature, then number of colonies are counted.
- Calculation of number or organisms is done similarly as in pour plate technique.



Overview of Pour plate and spread plate method

Activity:

Calculate CFU

Suppose after plating 0.1ml of the sample of 10^{-3} dilution tube and you got 52 colonies on agar plate, then calculate CFU/ml present in original sample.

Member filtration:

Objective of Membrane Filter (MF) Technique:

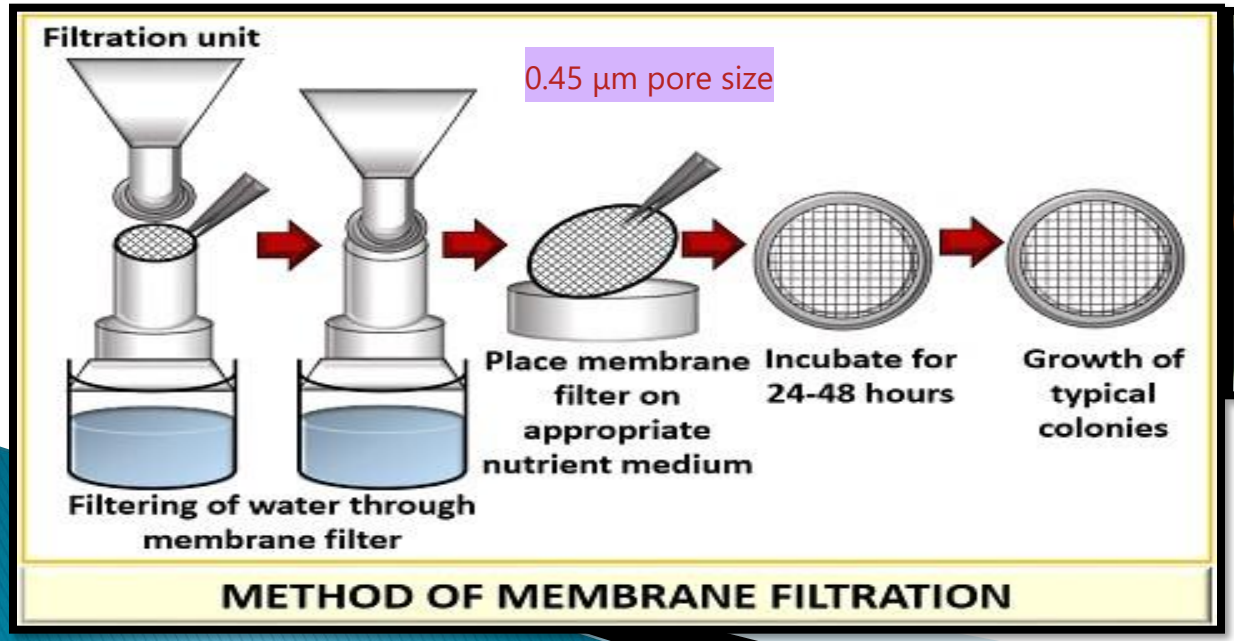
- To determine the quality of water samples using the membrane filter method.

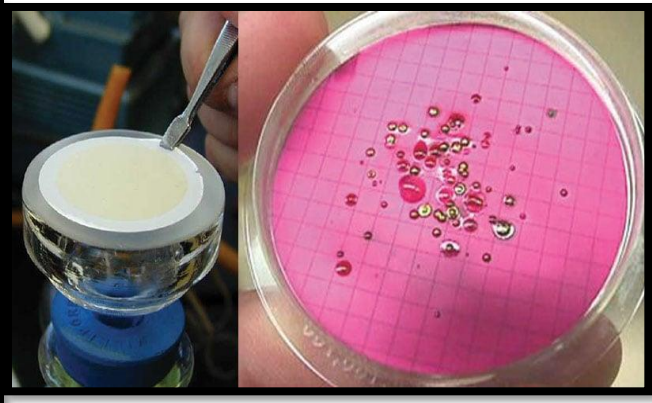
Materials and Equipment:

- ✓ Sterile membrane filter (typically 0.45 µm pore size for water testing)
- ✓ Filtration unit (funnel and base)
- ✓ Vacuum source
- ✓ Sterile petri dishes with absorbent pads and a selective culture medium (e.g. Endo agar for coliforms)
- ✓ Sterile forceps
- ✓ Water sample
- ✓ Sterile water
- ✓ Incubator

Step-by-step Procedures:

- ✓ Collect the sample and make any necessary dilutions.
- ✓ Select the appropriate nutrient or culture medium.
- ✓ Flame the forceps, and remove the membrane from the sterile package.
- ✓ Place the membrane filter into the funnel assembly.
- ✓ Flame the pouring lip of the sample container and pour the sample into the funnel.
- ✓ Turn on the vacuum and allow the sample to draw completely through the filter.
- ✓ Flame the forceps and remove the membrane filter from the funnel.
- ✓ Place the membrane filter into the prepared Petri dish.
- ✓ Incubate at the proper temperature and for the appropriate time period.
- ✓ Count the colonies under 10 - 15 X magnification.
- ✓ Confirm the colonies and report the results.





Growth of Colonies on the membrane filter surface.

selective culture medium (e.g. Endo agar for coliforms)

Most Probable Number (MPN) Test:

Aim:

- To enumerate the number of coliform bacteria present in the water sample.
- To identify the coliform bacteria present in the water sample.

MPN is most commonly applied for quality testing of water i.e. *to ensure whether the water is safe or not in terms of bacteria present in it.*

Principle:

- Water to be tested is diluted serially and inoculated in lactose broth, coliforms if present in water utilizes the lactose present in the medium to produce acid and gas.
- The presence of acid is indicated by the colour change of the medium and the presence of gas is detected as gas bubbles collected in the inverted Durham tube present in the medium.
- The number of total coliforms is determined by counting the number of tubes giving positive reaction and comparing the pattern of positive results *(the number of tubes showing growth at each dilution)* with standard statistical tables.

MPN test is performed in three steps:

- Presumptive test
- Confirmed test
- Completed test

Presumptive test:

- ✓ The presumptive test is a screening test to sample water for the presence of coliform organisms.
- ✓ If the presumptive test is negative, no further testing is performed, and the water source is considered microbiologically safe.
- ✓ If, however, any tube in the series shows acid and gas, the water is considered unsafe and the confirmed test is performed on the tube displaying a positive reaction.

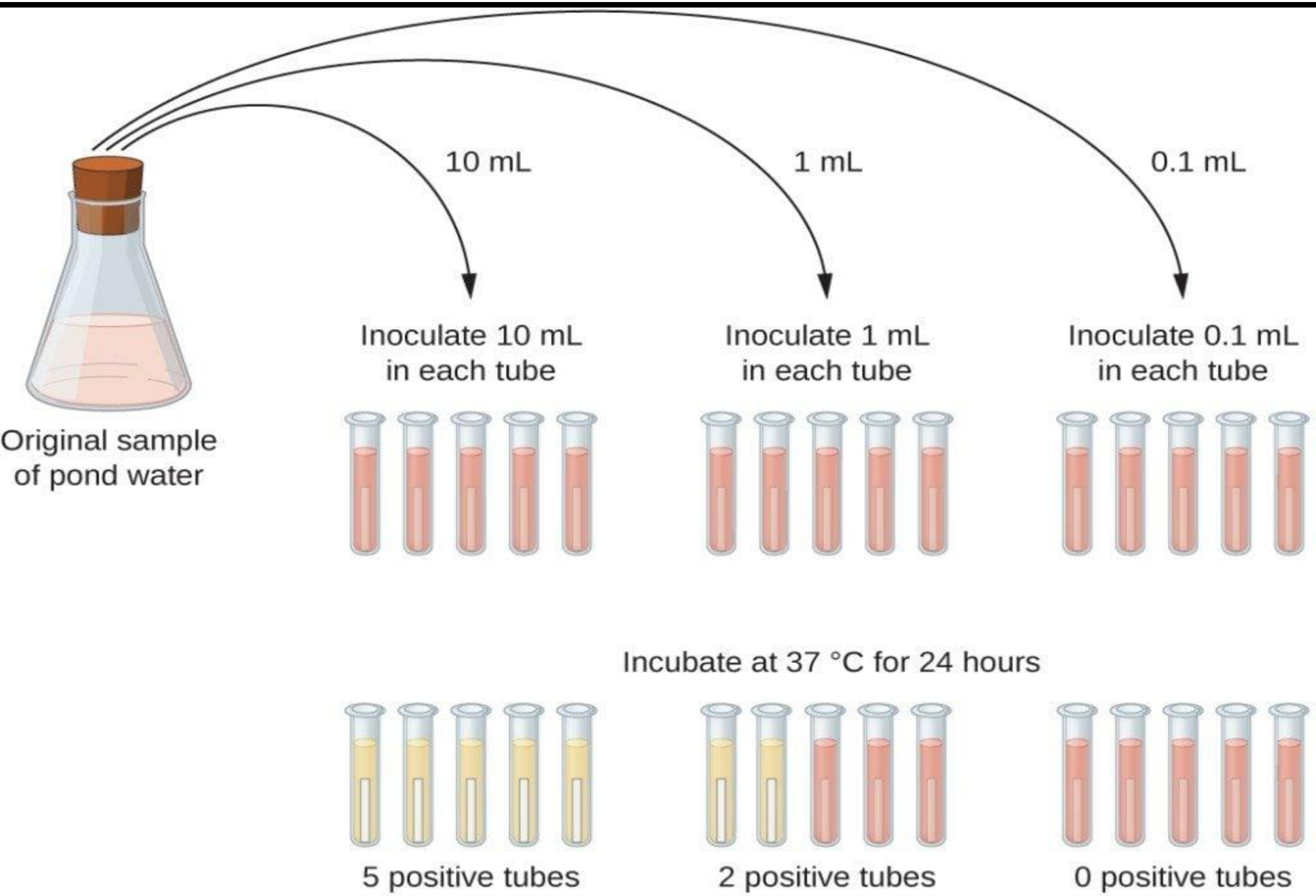
Requirements:

- ✓ Medium: Lactose broth or MacConkey broth or Lauryl tryptose (lactose) broth.
- ✓ Glassware's: Test tubes of various capacities and Durham tube
- ✓ Others: Sterile pipettes.

Procedures: (Step-by-step)

- Prepare Lactose broth or MacConkey broth of single and double strength in test tubes with Durham's tube and autoclave it.
- Take three sets of test tubes containing five tubes in each set; one set with 10 ml of double strength (DS) other two containing 10 ml of single strength (SS) .
- Using sterile pipettes, transfer 10 ml of water to each of the DS broth tubes. Transfer 1 ml of water sample to each of 5 tubes of one set of SS broth and transfer 0.1 ml water to five tubes of remaining last set of SS broth tubes.
- Incubate the tubes at 37°C for 24 hours.
- After incubation, observe the gas production in Durham's tube and the colour change of the media.
- Record the number of positive results from each set and compare with the standard chart to give presumptive coliform count per 100 ml water sample.

Presumptive test



Result:

For example, a water sample tested shows a result of 3–2–1

3 × 10 mL positive

2 × 1 mL positive

1 × 0.1 mL positive

So, MPN value of 17, i.e. the water sample contains an estimated 17 coliforms per 100 ml

MPN values per 100 ml of sample and 95% confidence limits for various combinations of positive and negative results (when five 10-ml, five 1-ml and five 0.1 ml test portions are used)

No. of tubes giving a positive reaction :			MPN (per 100 ml)	95% confidence limits	
5 of 10 ml	5 of 1 ml	5 of 0.1 ml		Lower	Upper
0	0	0	<2	<1	7
0	1	0	2	<1	7
0	2	0	4	<1	11
1	0	0	2	<1	7
1	0	1	4	<1	11
1	1	0	4	<1	11
1	1	1	6	<1	15
2	0	0	5	<1	13
2	0	1	7	1	17
2	1	0	7	1	17
2	1	1	9	2	21
2	2	0	9	2	21
2	3	0	12	3	28
3	0	0	8	1	19
3	0	1	11	2	25
3	1	0	11	2	25
3	1	1	14	4	34
3	2	0	14	4	34
3	2	1	17	5	46

Standard Statistical Tables

Activity:

Calculate MPN Value

What is the MPN value if a water sample test gives a positive result of 2-3-0 (2× 10 mL positive, 3 × 1 mL positive, 0 × 0.1 mL positive)?

MPN values per 100 ml of sample and 95% confidence limits for various combinations of positive and negative results (when five 10-ml, five 1-ml and five 0.1 ml test portions are used)

No. of tubes giving a positive reaction :			MPN (per 100 ml)	95% confidence limits	
5 of 10 ml	5 of 1 ml	5 of 0.1 ml		Lower	Upper
0	0	0	<2	<1	7
0	1	0	2	<1	7
0	2	0	4	<1	11
1	0	0	2	<1	7
1	0	1	4	<1	11
1	1	0	4	<1	11
1	1	1	6	<1	15
2	0	0	5	<1	13
2	0	1	7	1	17
2	1	0	7	1	17
2	1	1	9	2	21
2	2	0	9	2	21
2	3	0	12	3	28
3	0	0	8	1	19
3	0	1	11	2	25
3	1	0	11	2	25
3	1	1	14	4	34
3	2	0	14	4	34

Standard Statistical Tables

Confirmed Test:

- Some microorganisms other than coliforms also produce acid and gas from lactose fermentation. In order to confirm the presence of coliform, a confirmatory test is done.

- From each of the fermentation tubes with positive results transfer one loopful of medium to 3 mL Brilliant green bile broth tube Brilliant green bile broth tube, and also, agar slant.

- Incubate the inoculated Brilliant green bile broth tube at 37°C and inspect gas formation after 24 ± 2 hours. If no gas production is seen, further incubate up to a maximum of 48 ±3 hours to check gas production.

- The agar slants should be incubated at 37°C for 24± 2 hours and Gram-Stained preparations made from the slants should be examined microscopically.

Result:

The formation of gas in Brilliant green bile broth tube and the demonstration of Gram-negative, non-spore-forming bacilli in the corresponding agar indicates the presence of a member of the coliform group in the sample examined.

The absence of gas formation in Brilliant green bile broth tube or the failure to demonstrate Gram-negative, non-spore-forming bacilli in the corresponding agar slant constitutes a negative test *(absence of coliforms in the tested sample)*.

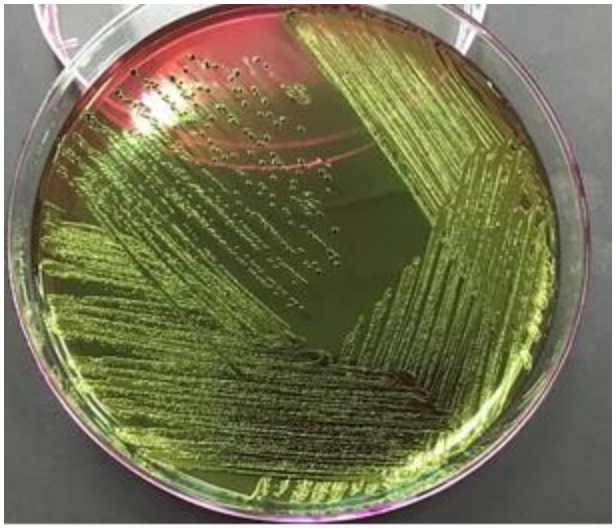
Completed Test

- Since some of the positive results from the confirmatory test may be false, it is desirable to do completed tests. For this inoculum from each positive tube of the confirmatory test is streaked on a plate of EMB or Endo agar.

- In this process, a loopful of a sample from each positive BGLB tube is streaked onto selective medium like Eosin Methylene Blue agar or Endo's medium.
- Each plate is incubated at 37°C and another at 44.5± 0.2°C for 24 hours.

Result:

- Coliforms produce colonies with a greenish metallic sheen which differentiates it from non-coliform colonies (show no sheen).
- The presence of typical colonies on high temperature (44.5 ±0.2) indicates the presence of thermotolerant ***E.coli.***



Coliform (*E. coli*) on EMB agar


many
Thanks!