PRACTICAL 6 QUANTITATIVE TECHNIQUES

Structure

6.1 INTRODUCTION

In the last two practicals we studied about the process of subculturing and about the methods used to form pure cultures of microorganisms. Now that we are well-versed with the techniques involved in preparing and maintaining cultures, it is also important for us to learn how to evaluate the growth of microbial culture i.e., how to determine microbial number in a given sample. This is done through quantitative techniques, which is the focus of this practical. The direct and indirect methods of microbial estimation are highlighted in this practical.

Objectives

After studying this practical and conducting the activities given herewith, you will be able to:

- discuss the different methods used for microbial estimation,
- enumerate the number of bacteria or other microbes in a given sample,
- differentiate between viable count and total count of microbes,
- evaluate the growth of the given microbial culture, and
- estimate the amount of bacteria by performing spread and pour plate techniques and serial dilution.

6.2 QUANTITATIVE TECHNIQUES: AN INTRODUCTION

Microbial quality of substances like food, milk, air, water, soil etc. can be assessed both qualitatively and quantitatively. Quantitative enumeration of microorganisms in a given sample can be accomplished in a laboratory by employing various methods, either by direct or indirect counts, each having its own advantages and disadvantages. No single method is always best. The most appropriate approach will depend on the experimental situation. These methods can also be used to measure microbial growth, determining growth rate and generation time.

These methods devised for microbial quantitation either measure the cell numbers or determine the cell mass or cell constituents like DNA, protein, O_2 uptake, carbon dioxide production, turbidometric measurement for increase in cell number, membrane filter count and the serial dilution agar plate method etc.

Microbial populations are very large, therefore, most of the methods of counting are based on direct or indirect counting of a very small sample, achieved indirectly by a series of dilutions. Calculations then determine the size of total population.

Let us then learn about these direct and indirect measures of microbial estimation. We begin with the direct method of microbial estimation.

6.3 DIRECT MEASUREMENT OF MICROBES

There are four direct methods of measurement. These are described herewith:

1. *Direct Microscopic Count*

The most obvious way to determine microbial number is through direct counting under a microscope. In this method, a measured volume of the sample is placed within defined area on the microscopic slide and cells are counted under the microscope. In the breed count method for milk, 0.1 ml of the sample, is spread over a marked square centimeter area on a slide. Stain is added and the microbes are counted under a microscope for several different viewing fields. The average number of microbes/field can be calculated and used for calculating the number of microbes per square centimeter area or per 0.1 ml of the sample. Specially designed counting chambers like *Petroff Hausser counting chamber, haemocytometer* and electronic counter like *coulter counter* can also be used for direct measurement.

The advantages and disadvantages of direct microscopic count method are enumerated herewith:

Advantages	Disadvantages
It is easy, inexpensive and relatively quick method.	Small cells are difficult to see under the microscope and may be missed.
It gives information regarding size and morphology of microorganisms.	It gives total count, i.e., both live and dead cells as it is difficult to distinguish between the two.
No incubation time is required.	High concentration of microbes, i.e., about 10 million bacteria/ml is required as very small volume of microbes is sampled. The method is not sensitive to populations of fewer than 1 million.
	Motile cells must be immobilized before counting.
	Phase contrast microscope is needed for unstained cells.

Next let us learn about the plate counts direct method.

2. *Plate Counts*

Plate counts is the most frequently used method to measure microbial numbers. The technique, however, can be used only for those microorganisms, which are able to grow and reproduce on microbial media. Here a diluted sample of microbes is dispersed over suitable nutrient agar medium. There are two ways of performing a plate count – the *spread plate* and *pour plate technique* about which we have already studied in the last practical. Look up these techniques once again. Each microorganism then produces a distinct colony. Original number of viable microorganism in a sample is then calculated from the number of colonies formed and the sample dilution used. The results are reported in form of colony forming units (CFU) per ml of the sample.

Colony forming unit/ml can be expressed as:

$$CFU/ml = \frac{\text{No. of Colonies formed} \times \text{dilution factor}}{\text{Volume sampled}}$$

Dilution factor is the reciprocal of the dilution used. As number of microorganisms in original sample is not known, the original sample is diluted several times in sterile water blanks to ensure that separate colonies are developed and some colony counts will be in the specified range. The process is called *serial dilution*. The procedure is shown in Figure 6.1. Usually a ten-fold serial dilution is used in the process where 1 ml of the sample is transferred to a 9 ml of the diluent to get 1:10 dilution. 1 ml of this is transferred to another tube of 9 ml of diluent to get 1:100 dilution and so on till the required dilutions are obtained.

Figure 6.1: Serial dilution process

It is important that the dilution used should produce a limited number of colonies on plate. When many cells are present, overcrowding of cells may not allow many colonies to develop and may also result in many colonies to fuse. This leads to inaccuracies in count. Similarly, too less colonies are not statistically significant. According to Food and Drug Administration, only those plates having 25-250 colonies should be considered for calculation; but most microbiologists use plates with 30-300 colonies to get the microbial numbers in the sample. Plates getting colonies less than 30 are marked too less to count (TLTC) and getting more than 300 are marked as too numerous to count (TNTC) and are discarded.

The advantages and disadvantages of using the plate counts method are enumerated next.

Advantages	Disadvantages
It is simple and sensitive method.	It takes time, usually 24 hours or more is required to obtain visible colonies.
It provides viable count as only live cells will grow and produce the colony.	Microbial count obtained depends upon the culture media used for plate counts.
Even very few cells in a sample can be counted.	It is not a very useful method for actinomycetes and moulds because in plate counts, it is the asexual spores, which are counted.
It allows isolation of discrete colonies which can be sub-cultured to achieve pure culture.	More glassware is needed.
Even a particular cell type in a mixed population can be counted by using specific culture media and growth conditions.	Errors in result may arise due to errors in dilution or plating.
	Results are expressed as counts forming unit per ml (CFU/ml) rather than as number of microbes. It is so because many microbes are present in chains and clusters and it is difficult to be absolutely certain that each colony has arise from a single cell. A colony might be produced from a short segment of a chain or from a bacterial clump.

There are many factors, which may lead to errors in count.

(i) Counts may be low if the agar medium used in the technique does not support the microbial growth.

(ii) The hot agar used in pour plate may kill the cells, leading to low microbial count.

(iii) Low count may result if clump of cells is not broken up and microorganisms are not dispersed well.

(iv) Colony development also depends on incubation conditions and incubation time. Short incubation time may result in development of lesser colonies because different cells on plate grow at different time.

(v) It is difficult to count very small colonies and may give inaccurate count. For more accurate counts, a special colony counter can be used.

Having studied about the plate counts method, we move on to the next technique, which is membrane filtration technique.

3. *Membrane Filtration Technique*

Another method used for determining the bacterial number is the *filtration technique*. The margain figure illustrates the membrane filter assembly. This technique can be used to know the microbial count in liquid samples containing relatively very small quantity of microbes. Both *viable* and *total counts* can be obtained. A known volume of the sample is passed through a special membrane filter having pores small enough to trap bacteria and other microbes.

For *viable count*, the membrane filter is placed over the agar medium surface or over a pad soaked in liquid medium and incubated for 24 hours. Each cell trapped form a separate colony. The numbers of colonies, then formed, indicate the number of microbes present in the volume of sample, which is drawn through a membrane filter. The colonies are counted

by hand or by using quebec colony counter about which you have already studied in Practical 2.

Total count can be obtained microscopically by using fluorescent dyes such as acridine orange or DNA stain DAPI. The counts obtained microscopically are usually higher than those from culture technique because the former method cannot differentiate between live and dead cells.

Membrane filtration technique is applied frequently for detection and enumeration of coliforms.

The advantages of membrane filtration technique are enumerated next.

Advantages:

(i) Both total and viable counts can be obtained.

(ii) Specific microorganisms can be selected by using special media.

Finally, let us learn about the last direct method used for microbial estimation i.e., most probable number technique.

4. *Most Probable Number (MPN)*

MPN is a statistical estimating technique which is based on the fact that more the number of bacteria in a sample, more is the dilution needed to reduce the number to a point where no bacteria is left to grow in the tubes in a dilution series.

What are the advantages of MPN? The advantages include:

(i) The method is useful when the microbes cannot be grown on solid media.

(ii) It is also useful when liquid differential media is used for microbial identification.

With MPN, we end our discussion on the direct estimation methods. Next, we shall review the indirect methods of microbial estimation.

6.4 INDIRECT METHODS OF MICROBIAL ESTIMATION

Indirect methods, i.e., methods other than those counting the microbial cells can also be used for quantitation purpose. Three commonly used methods are –

(1) Dry weight estimation or measurement of cell mass,

(2) Turbidity, and

(3) Metabolic activity.

Let us get to know about these methods.

1. *Measurement of Cell Mass*

You may recall reading earlier that filamentous bacteria and moulds cannot be counted satisfactorily by employing plate count method. One of the approaches used commonly for estimating these organisms in a substance and their growth is to determine the dry weight. The cells growing in the liquid media are filtered or centrifuged, washed to remove extraneous material, dried in oven and then weighed. Change in dry weight can be used in following growth.

The advantages and disadvantages of measurement of cell mass are enumerated herewith:

Advantage	Disadvantages
It is a useful technique for estimating fungal and actinomycetes growth.	It is time consuming and not very sensitive.
	Bacterial mass is very small, so a large volume of bacterial sample is needed to collect a sufficient measurable quantity.

Next, we move on to the turbidity measurement method.

2. *Turbidity Measurement*

Presence of sufficient number of microbes makes the liquid medium turbid. Turbidity also increases with the multiplication of microbes. Estimation of turbidity can be used both for the quantitation of microbes and monitoring microbial growth. The technique depends on the fact that cells scatter the light striking them and the amount of scattering is directly proportional to the biomass of cells present and indirectly to the cell number.

The scattering of light can be measured by *spectrophotometer* or *photometer*. In the instrument, a beam of light is passed through a cell suspension and the amount of unscattered light is detected by a light sensitive detector. Photometer uses a simple filter to generate incident light whereas in spectrophotometer, prism is used instead of a filter. Commonly used wavelengths for bacteria are 540 nm, 600 nm and 660 nm. More the number of microbes in the suspension, less is the light reaching to the detector and read as percent transmission or absorbance (OD, optical density, a logarithmic expression).

The advantages and disadvantages of using turbidity measurement are enumerated next.

Advantages	Disadvantage
It is an easy, rapid and sensitive technique.	High amount of microbial cells about million to 100 million cells per milliliter is needed for estimation.
Turbidity measurement can be done without destroying the sample.	

Finally, a word about the metabolic activity method.

3. *Metabolic Activity and Cell Constituents*

Metabolic activity like amount of acid produced or CO_2 produced is directly proportional to the number of bacteria present, so can be used to measure bacterial number.

In a similar manner, the amount of certain substances, like protein or nitrogen content, chlorophyll content etc. is constant in each cell and, therefore, can be used for detecting microbial content.

With a brief discussion on metabolic activity and cell constituents, we end our study about the quantitative techniques used for microbial estimation. We hope you now have a fairly good insight into how to estimate the amount of bacteria in a given sample. So then let us check your knowledge on this topic. Answer the questions given in section Review Questions.

6.5 REVIEW QUESTIONS

1. Name the direct methods of enumeration of bacterial cells in the sample.

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2. Explain serial dilution. Why is it required for quantifying the microbes in a sample?

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3. What are the advantages and disadvantages of direct microscopic count and plate count?

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4. Mention the two methods used for plate count. What are their merits and demerits?

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5. Distinguish between dilution and dilution factor.

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6. What is the limit of colonies number in agar plate count method? What is the significance of this limit?

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7. What is meant by TNTC and TLTC?

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8. Which method is good for filamentous bacteria and moulds and why?

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9. What is the principle of turbidometric method?

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10. Name the method used for estimating microbial counts in liquid material.

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11. Why is a viable count more sensitive than a microscopic count?

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12. Describe how you would dilute a bacterial culture by 10^{-5}.

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Well, are you having trouble answering some of the questions given herewith? Do not panic. We suggest you read the content given in this practical once again and then try answering the questions.

Well done. Now carry out the Exercises 1 and 2 given next, which will help you internalize what you have learnt so far. So then, let us get started. After completing the exercises, do not forget to submit the review question answers along with the exercises for evaluation.

EXERCISE 1

ESTIMATION OF THE AMOUNT OF BACTERIA BY PERFORMING POUR PLATE TECHNIQUE

Date : .....

Aim : To estimate the amount of bacteria in a given sample by performing pour plate technique.

Requirements

Culture : 24 hours *E. coli* culture in nutrient broth.

Media : 10 tubes of 9 ml normal saline in each tube, molten nutrient agar,

Equipments and Glassware : Sterile petri plates, sterile test tubes, 1 ml sterile pipettes, incubator, bunsen burner, water bath, quebec colony counter, Glass marker.

Theory/Principle:

Viable plate count is routinely employed for bacterial/microbial quantitation in a given sample. As discussed earlier, the method involves the spreading of microbial cells on agar surface so that each cell can multiply and produce a distinct colony. Plates having colonies ranging between 30 and 300 are considered for calculation as these are most valid statistically.

A plate count is done either by pour plate or spread plate method. Write the theory/ principle behind the plate count method in the space provided herewith. You may look up the details of pour plate method given in section 5.3 in Practical 5. Now briefly give the theory here and also highlight advantages and disadvantages of pour plate method

The colonies are counted by hand or by using Quebec colony counter. This procedure is repeated for all dilutions to be plated. Dilutions should be plated in duplicate for greater accuracy.

Give the formula for calculating bacterial counts in the original sample in the space provided.

CFU/ml =

Procedure:

Now carry out the exercise following the steps enumerated herewith.

1. Prepare ten fold dilutions of *E. coli* culture in diluent till 10^{-7} dilution is obtained. For making dilution, label 7 tubes of sterile water blanks as 10^{-1} through 10^{-7}. Look at the Figure 6.1 for details. Mix the *E. coli* culture by rolling the tube between the palms for uniform distribution of cells. Aseptically transfer 1 ml of culture with a sterile pipette to a tube marked 10^{-1}. This is 1:10 dilution (10 times diluted culture). Discard the pipette in a beaker of disinfectant.

2. Thoroughly mix the 1:10 dilution and with a fresh sterile pipette, transfer 1 ml of 10^{-5} dilution to a tube marked 10^{-2} to achieve 1:100 or 100 times dilution.

3. Repeat the step 2 till get 10^{-7} dilution, i.e., 1 ml of 10^{-2} is transferred to a tube labeled 10^{-3} (1000 times dilution) and 1 ml from 10^{-3} to a tube marked 10^{-4} (10,000 times dilution) and so on. Each time a fresh sterile pipette has to be taken.

4. Liquefy the nutrient agar medium by boiling and maintain in a liquid state at a temperature of 45^{o}C by keeping in a water bath.

5. Label the sterile petri plates as 10^{-5}, 10^{-6} and 10^{-7} in duplicate. Add 1 ml of each 10^{-5}, 10^{-6} and 10^{-7} to the labeled petri plates.

6. Aseptically pour 20-25 ml of molten nutrient agar at temperature of 45^{o}C to each petri plate having dilution. Gently rotate the plate to ensure uniform distribution of cells in the medium.

7. After solidification of agar, incubate the plates at 37^{o}C for 24 hours in an inverted position. Following incubation, cell counts are made both for surface and subsurface colonies. For manual counting, each colony is counted and marked with a marker on the bottom of the plate. Calculations are made to get the microbial number in original sample, using the formula given earlier in section 6.3.

Precautions:

1. Dilution should be made carefully.

2. Use fresh sterile pipette for making each dilution.

3. Aseptic condition should be made.

4. Proper mixing of nutrient agar with the bacterial culture should be made for even distribution of cells.

5. Plates containing colonies between 30 and 300 should be used for microbial counting.

6. Count all surface and subsurface colonies.

Observations and Results:

Now record your observations in the format given herewith. Calculate the CFU/ml. First write the formulae in the space provided herewith, then calculate CFU/ml in the given sample.

Formula for CFU/ml =

Observations				Result
Dilution plated	Volume of the dilution plated	Number of colonies	Bacterial counts/ml of samle	Average counts/ml of sample

Inference / Conclusion:

CFU/ml in the given sample is

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Submit the exercise for evaluation.

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Counsellor Signature

QUANTITATIVE DETERMINATION OF VIABLE MICROBES BY SPREAD PLATE METHOD

EXERCISE 2

Date.....

Aim : To determine quantitatively the amount of viable microbes in a given sample by spread plate method.

Requirements

Culture : 24 hours *E. coli* culture in nutrient broth.

Media : Nutrient agar plates, 7 tubes of 9 ml diluent in each tube.

Equipments and Glassware : Incubator, spreader, 1 ml pipettes, bunsen burner, test tube racks.

Theory/Principle:

Spread plate is commonly used to know the microbial content of any substance. It is of advantage when one deals with heat sensitive microbes which might be killed during pour plate technique. For studying colony characteristics, also the spread plate method is frequently used.

Briefly write down the theory/principle of the Spread Plate method in the space provided herewith. Look up section 5.3 in Practical 5 for details about spread plate method.

Procedure:

Now carry out the exercise following the steps included herewith:

1. Label the diluent tubes from 10^{-1} through 10^{-7} and prepare dilutions as you did in previous exercise (Exercise 1).
2. Label the nutrient agar plates as 10^{-5}, 10^{-6} and 10^{-7} in duplicate.
3. Pour 0.1 ml of each dilution from 10^{-5} through 10^{-7} over the agar surface of labeled plates in the centre.
4. Spread the inoculum over the entire surface by means of a sterile bent glass rod (spreader) till it is absorbed completely. Spreader can be made sterile by dipping in alcohol followed by passing through a flame of Bunsen burner or by autoclaving.
5. Keep the plates for incubation at 37^{o}C for 24 hours.
6. Following incubation, observe and made the colony count. Calculate colony forming units/ml of the original sample by multiplying the number of colonies counted by the dilution factor and divided by the volume used. Use only dilution plates yielding colonies between 30 and 300 for calculations. Plates with more than 300 colonies are designated as too numerous to count (TNTC) and with fewer than 30 colonies are designated as too few to count (TFTC).

Precautions:

1. Dilution should be made carefully.
2. Use fresh sterile pipette for making each dilution.
3. Aseptic conditions should be made.
4. Plates containing colonies between 30 and 300 should be used for microbial counting.
5. Count all colonies.

Observations and Results:

Under dilution plated, note the dilution factor i.e., 10^{-1} or 10^{-2} etc. Under volume of dilution plated, note the volume, which is inoculated in the plate. For instance, here we used 1 ml of diluted sample so the volume is 1 ml. Observe and count the colonies as appearing on the plate. Calculate the bacterial counts/ml using the formula given above. Now record the observations in the format given herewith.

Observations				Result
Dilution Plated	**Volume of dilution Plated**	**No. of colonies**	**Bacterial counts/ml of sample**	**Average count/ml of sample**

Inference/Conclusion:

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Now submit the exercise for evaluation.

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Counsellor Signature