**Module: A3259C Aseptic Techniques**

## Lesson 1: Basic Techniques in Microbiology Practical I

### Introduction

Spread plate and pour plate methods are two commonly employed methods for determining viable cell count in a given sample. They differ from direct count method and turbidity method in their ability to distinguish between viable and non-viable cells. They are particularly useful in calculating the number of organisms in food, water, and environmental samples.

When employing these methods, it is always important to use the appropriate positive and negative controls to accurately analyze the results of the experiments. This helps in understanding if the sample has been contaminated due to insufficient sterilization or inappropriate incubation and storage conditions. These results can help in eliminating false positive results from our inference.

### Question 1

What would serve as your positive and negative control in this experiment?
In this experiment, positive control would be a sample of diluted *Escherichia coli* organisms, and negative control would be a 0.9% saline solution.


Positive and negative controls are required in all experiments.

**Positive control**


- Diluted culture - same volume as sample being plated
- Ensure that all reagents are working

**Negative control**


- Same volume as sample being plated
- Ensure that saline used for dilution is not contaminated

### Question 2

What is the purpose of using agar in the media?
Agar is a polysaccharide medium that helps make the nutrient broth solid so that bacteria can easily be counted and observed on the plates. It serves as a medium in which components such as blood can easily be mixed for studying bacterial growth. Also, once agar solidifies, it only melts at 85 ˚C, making it conducive for the growth of thermophilic bacteria.

### Methodology

#### Dilutions

- Three centrifuge tubes were labelled with appropriate dilution factors – 10X, 100X, and 1000X.
- 9 ml of 0.9% saline was added in each tube.
- 1 ml of the given sample was added to the tube labelled 10X and mixed well, giving us the first dilution.
- 1 ml from the tube labelled 10X was added to the tube labelled 100X and mixed well, giving us the second dilution.
- 1 ml from the tube labelled 100X was added to the tube labelled 1000X and mixed well, giving us the third dilution.

#### Spread plate technique

- 6 LB Agar plates were labelled as Neat, 10X, 100X, 1000X, Positive control and Negative control respectively.

- 0.1 ml of the undiluted sample was added to the plate labelled Neat, and spread evenly using a sterile L-shaped spreader.
- 0.1 ml of samples from dilutions 10X, 100X, and 1000X were added to the respectively labelled plates and spread evenly using a sterile L-shaped spreader.
- Similarly, 0.1 ml of positive control and negative control each was spread on the respectively labelled plates.

**Pour plate technique**

- 0.1 ml of sample from the tube labelled 100X was added to molten agar and mixed well.
- After it had sufficiently cooled down, this agar was poured into a petri dish.
- After the agar had solidified, all plates were sealed with parafilm, inverted, and incubated at 37°C for 24-48 hours.

**Question 3**

Why is it important to allow the nutrient agar to cool to 45$^{o}$C before pouring it onto the plate with the water sample?

Many organisms in water get destroyed at higher temperatures. If the temperature of nutrient agar is more than 45 ˚C when mixed with the water sample, it might kill the microorganisms present resulting in no growth on the agar plate.

**Question 4**

What is the purpose of parafilming the plates? Is it always necessary to parafilm all agar plates?

Parafilming agar plates ensure that the plates are not contaminated with microorganisms in the air, and to prevent loss of moisture from the plate. If the inoculation of the plate is done in a Laminated Air Flow (LAF) chamber using standard aseptic practices, then parafilming is not necessary as all precautions have been taken to avoid contamination.

**Question 5**

Should the plates be incubated upright or inverted?

Agar plates are incubated in an inverted position to avoid condensation drops from falling on to the agar and contaminating the culture.

**Results and Discussions**

Following aseptic techniques, the given sample was serially diluted and plated using the spread plate and pour plate techniques. Appropriate positive and negative control plates were also set up. After incubation, the plates were examined for bacterial growth.

Positive control – The plate was filled with numerous colonies that were uncountable with naked eye. This shows that the appropriate control was used for the experiment and this growth should be expected on the experimental plates as well.

Negative control – The plate had a single colony that is indicative of contamination. This contaminant could have been present in the agar medium or in the negative control sample. It may also have been introduced during plating indicating use of incorrect technique.

Experimental plates (Spread Plate Technique) – Plates with all four dilutions showed a large number of colonies that could not be counted with naked eye. This indicates that the given sample has a huge number of microorganisms. In order to count the number accurately, the sample needs to be diluted further to isolate single colonies on the plate.

Experimental plate (Pour Plate Technique) – The plate had a countable number of colonies and these were enumerated to give a figure of 292 colonies on the plate. Multiplying with the dilution factor, the final number of microorganisms in the sample was found to be 292 x $10^4$ organisms. Thus, using the pour plate techniques it was possible to find out the number of microorganisms in the given sample.

**Question 6**

Describe what can be observed on the agar plates (positive control) after incubation.
The positive control of the agar plate shows the presence of several small round colonies with smooth edges, thus indicating bacterial growth.

**Question 7**

Would a microscope be required for the observation of colonies?
No, the colonies can be seen with the naked eye as a colony does not represent a single organism but is a collection of millions of organisms.

**Question 8**

**Positive control plate**

| Dilution Factor | Number of CFU per plate | |
|---|---|---|
| | Plate of group 1 | Plate of group 2 |
| *1000x / $10^3$* | *TNTC (too numerous to count)* | *TNTC (too numerous to count)* |

a) State the purpose of positive control plates.

**Positive control plates are used to compare and confirm that the growth on the experimental plates represent the organisms in the sample and not the presence of contaminants.**

b) State the **conclusion** of your results above.

The plate was filled with numerous colonies that were uncountable with naked eye. This shows that the appropriate control was used for the experiment and this growth should be expected on the experimental plates as well.

**Question 9**

**Negative control plate**

| Dilution Factor | Number of CFU per plate | |
|---|---|---|
| | Plate of group 1 | Plate of group 2 |
| *0x / Saline* | *1 CFU* | *NIL* |

a)State the purpose of negative control plates.
Negative control plates are used to ensure that there is no contamination in the plated samples and that correct technique has been applied while plating.

b) State the **conclusion** of your results above.

The plate had a single colony that is indicative of contamination. This contaminant could have been present in the agar medium or in the negative control sample. It may also have been introduced during plating indicating use of incorrect technique.

**Question 10**

**Spread plates**

a) Determine the bacterial concentration (CFU/ml) in the samples.

| Dilution Factor | Number of CFU per plate | | | Bacterial concentration (CFU/ml) |
|---|---|---|---|---|
| | Group A | Group B | Average | |
| **Neat** | *TNTC (too numerous to count)* | *TNTC (too numerous to count)* | *TNTC (too numerous to count)* | *TNTC (too numerous to count).* |
| **$10^1$** | *TNTC (too numerous to count)* | *TNTC (too numerous to count)* | *TNTC (too numerous to count)* | *TNTC (too numerous to count).* |
| **$10^2$** | *TNTC (too numerous to count)* | *TNTC (too numerous to count)* | *TNTC (too numerous to count)* | *TNTC (too numerous to count).* |
| **$10^3$** | *TNTC (too numerous to count)* | *TNTC (too numerous to count)* | *TNTC (too numerous to count)* | *TNTC (too numerous to count).* |

c) State the **conclusion** of your results above.

Plates with all four dilutions showed a large number of colonies that could not be counted with naked eye. This indicates that the given sample has a huge number of microorganisms. In order to count the number accurately, the sample needs to be diluted further to isolate single colonies on the plate.

d) Referring to the results recorded in a), comment on your **serial dilution technique**.

The number of colony forming units (CFUs) in each dilution was huge and uncountable with naked eye. This indicates that the sample needs to be diluted further to isolate individual colonies on the plate.

**Question 11**

**Pour plates**

a) Determine the bacterial concentration (CFU/ml) in the samples.

| Dilution Factor | Number of CFU per plate | | | Bacterial concentration (CFU/ml) |
|---|---|---|---|---|
| | Group A | Group B | Average | |
| *102* | *296* | *288* | *296 – 288 = 584*<br>*584/2 = 292* | *292 x $10^3$ x 1m/0.1m*<br>*= 292 x $10^4$* |

| | | | | |
|---|---|---|---|---|
| | | | | |

b) State the **conclusion** of your results above.
The colonies were small, round, with smooth edges and they could be seen inside the agar medium. This was because the sample was mixed with the liquid media before it was poured onto the plate. The colonies were individually counted and multiplied with the dilution factor to get the final bacterial concentration in the given sample.
The plate had a countable number of colonies and these were enumerated to give a figure of 292 colonies on the plate. Multiplying with the dilution factor, the final number of microorganisms in the sample was found to be 292 x $10^4$ organisms. Thus, using the pour plate techniques it was possible to find out the number of microorganisms in the given sample.

c) Comment on the **differences between spread and pour plate techniques**:

- The major difference between the two techniques is the way in which the inoculum is mixed with the agar medium. In spread plate technique, the sample is spread on the surface of the solidified agar and in pour plate technique, the sample is mixed into the liquid agar before pouring in the plates.
- Pour plate technique allows the growth of aerobes, anaerobes, as well as facultative anaerobes whereas spread plate technique allows the growth of specific colony forming units.
- In pour plate, colonies are seen in the agar medium, and in spread plate, colonies are observed on the surface of the agar medium.

## Conclusion

Based on the above results, it is evident that the given sample has a large number of microorganisms. In plates that were inoculated using the spread plate technique, all four dilutions were unable to give isolated colonies that could be counted with the naked eye. This shows that the sample is heavily concentrated and needs to be diluted further to isolate single colonies on the plate. The plate that was inoculated using the pour plate technique showed growth of single colonies that could be counted. Hence, using pour plate technique, the number of microorganisms in the given sample could be enumerated.

## References

Sanders, Erin. (2012). Aseptic Laboratory Techniques: Plating Methods. Journal of visualized experiments : JoVE. e3064. 10.3791/3064.

Taylor, Raymond & Allen, Martin & Geldreich, Edwin. (1983). Standard plate count: A comparison of pour plate and spread plate methods. Journal American Water Works Association - J AMER WATER WORK ASSN. 75. 35-37. 10.1002/j.1551-8833.1983.tb05055.x.

Varghese, Naveena & Joy, P.P. (2014). Microbiology Laboratory Manual.