Practical activity (7)

Growing Microbes and Estimating the Number of Microbes

(Student version)

I. Background

The invention of the techniques and media for culturing (growing) bacteria by Robert Koch, a main founder of bacteriology, is one of the most important breakthroughs in the development of modern microbiology. The ability to grow and maintain bacterial culture allows the isolation of specific bacterial strains, and the establishment of pure culture is extremely important to the identification of the causative agents of many infectious diseases, such as tuberculosis and cholera. According to Koch's postulate, the isolation of bacteria from the specimen of a patient and growing that into a pure culture is one of the criteria for confirming a causative agent of an infectious disease.

In addition to isolating single colonies, estimation of bacterial load (counting of the number of bacteria) is a fundamental technique in medical, food and environmental microbiological studies. The spread plate and pour plate techniques for counting bacterial colonies were developed based on the invention of solid culture media. These two counting techniques are often applied to other sophisticated microbiological tests, such as the determination of bacterial load in food and the effectiveness of antimicrobials against different bacteria, which can be directly reflected by the number of colony-forming units formed in the test samples.

In Part (1) of this experiment, you will learn how to isolate bacterial colonies using the streak plate method. In Part (2), you will learn how to determine the number of bacteria in a given probiotic drink, using proper dilutions of the culture and two different plate count methods.

II. Guiding questions about the design of the experiment

Fig. 1 shows the growth pattern of three different bacterial cultures on three agar plates.

1. Which agar plate(s) offer(s) single colonies of a pure bacterial culture?

2. Which agar plate(s) allow(s) a reliable colony count?

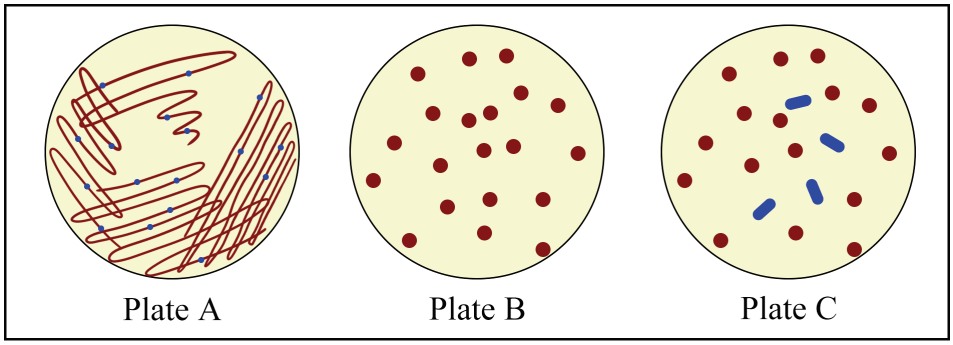


Fig. 1 Schematic diagram showing the growth pattern of bacteria on three agar plates. The lines, dots (large circles) and rod-like shapes on the agar plates are bacterial colonies.

3. If a loopful of a test solution gives rise to numerous single colonies (i.e. too difficult to count) on an agar plate, suggest a way to estimate the number of bacteria present in the test solution.

III. Objectives of the experiment

1. To perform single-colony isolation;
2. To determine the bacterial load of a given sample; and
3. To determine the appropriate dilution factors for colony counting and bacterial load calculation.

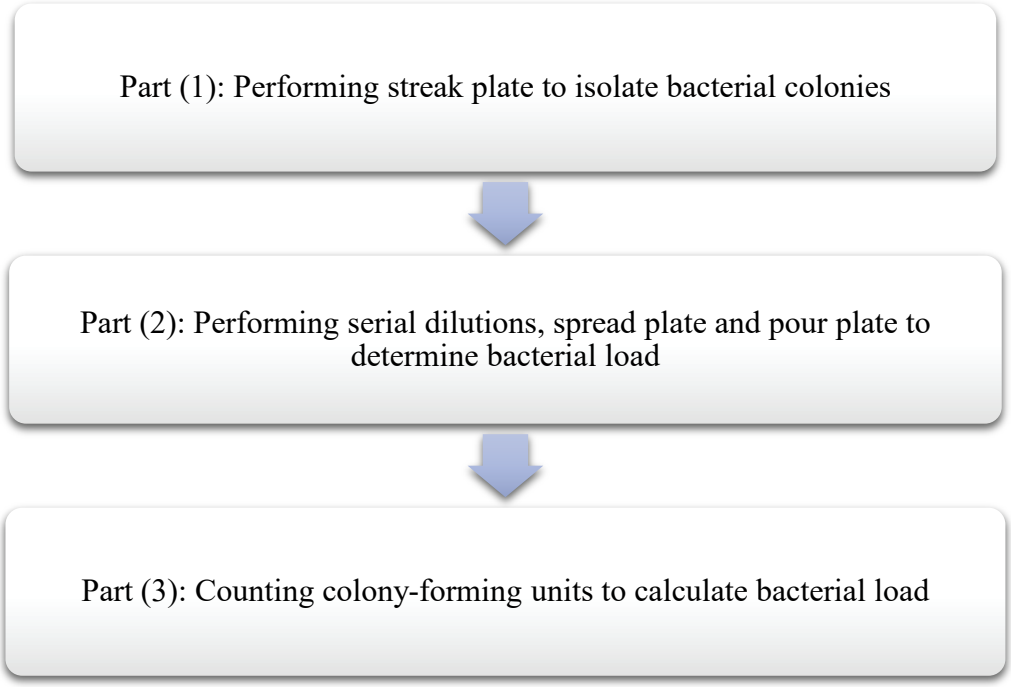
IV. Expected Learning Outcomes

Upon completion of the activities, students should be able to:

1. apply aseptic techniques and safety procedures in inoculating, culturing and disposing of microorganisms;
2. carry out streaking, spreading and pouring techniques in microbiology;
3. outline the techniques of serial dilutions and bacterial load estimation of a sample; and
4. use colony-forming units and appropriate formulae to calculate the bacterial load of a given sample.

V. The experiment

A. Overview



B. Part (1) of the Experiment: Performing streak plate to isolate bacterial colonies

a) Equipment and materials (per group)

Equipment

- Bunsen burner × 1
- Spark lighter × 1

Materials

- Inoculating loop × 1 (per student)
- Plate Count Agar (PCA) plate × 1 (per student)
- Probiotic drink (tube A, 1 ml) × 1 (per student)

- Permanent marker × 1
- 70% ethanol in spray bottle × 1
- Paper towel × 1 box
- Biohazard bag × 1
- Disposal container with 10% chlorine bleach × 1

b) Safety precautions

- Wear a laboratory gown.
- Tie up long hair.
- Wear gloves during the experiment.
- Wash hands to remove all possible contamination before and after the experiment.
- Keep ethanol away from fire.
- Make sure ethanol is fully vaporised before approaching any flame.
- Dispose of or disinfect all materials properly after each experiment.

c) Procedures

1. Disinfect the benchtop and gloved hands with 70% ethanol*.

***Caution: Keep away from fire! DO NOT light the flame until the ethanol has been completely vaporised!**

2. Label the bottom of the agar plate (PCA plate) with "StP" (which stands for "streak plate"), the date of the experiment, your class and class number (Fig. 2).

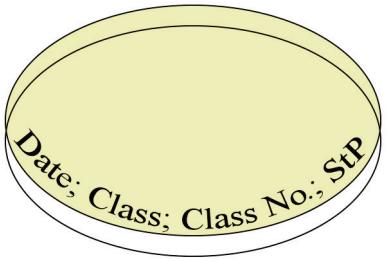


Fig. 2

3. Light a Bunsen burner.

4. Flame the inoculating loop to red hot (Fig. 3) and allow it to cool down#.

#It is essential to flame the metal inoculating loop to red hot to eliminate any previous bacterial residue, so as to prevent contamination of the current sample.

It is also important to cool the loop, as insufficient cooling kills the inoculated bacteria.

If a sterile plastic disposable inoculating loop is provided, flaming is not required.

Use a new inoculating loop when handling different microbial samples.

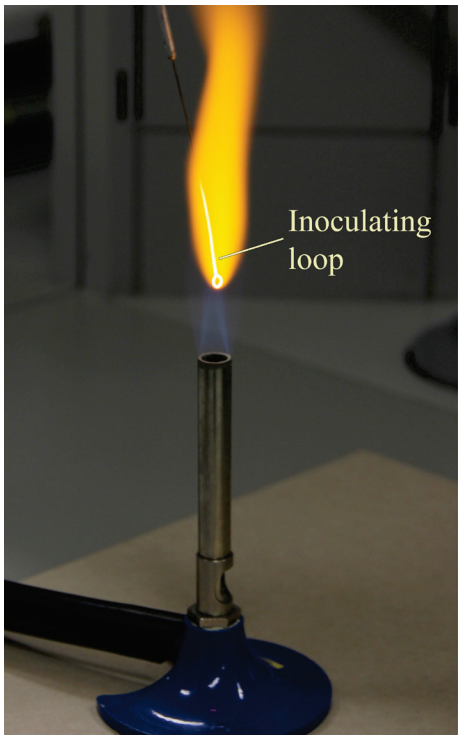


Fig. 3

5. Aseptically* obtain a loopful of the probiotic drink from tube A (Fig. 4).

*Note: Please refer to Steps 3–4 of Appendix 1 for details of the aseptic technique.

Fig. 4

6. Raise the lid of the agar plate to about 45° (Fig. 5)^ and put the inoculating loop onto the agar.

^Note: This is to minimise the exposure of agar to the atmosphere.

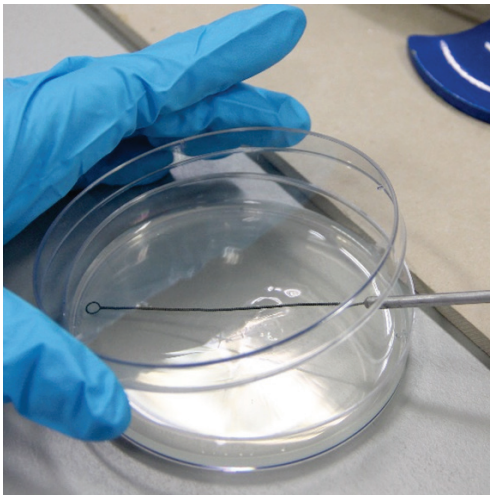


Fig. 5

7. Streak the first quadrant (part ⓐ) of the agar plate in a zigzag pattern, as shown in Fig. 6a.

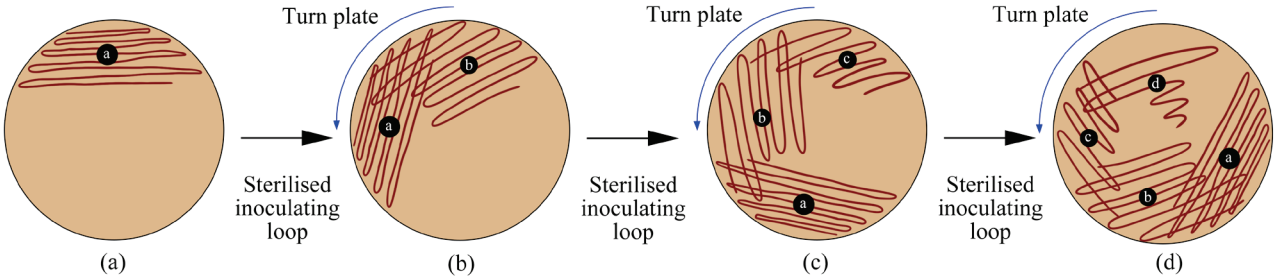


Fig. 6 Streak plate technique for isolation of bacterial colonies. The brown arrow indicates the direction trait of streaking.

8. Replace the lid.

9. Rotate the plate 90º.

10. Flame the loop and allow it to cool.

11. Overlap the previous quadrant 2–3 times and streak into the next quadrant (part ⓑ) in a zig-zag pattern, as shown in Fig. 6b.

12. Repeat Steps 8–11 to finish the third (part ⓒ) and fourth (part ⓓ) quadrants, as shown in Fig. 6c and Fig. 6d respectively*.

> *Note: The principle of isolating single colonies by streaking is to collect a fraction of bacteria from one quadrant and spread them to the next quadrant; thus, the density of bacteria can be gradually reduced and it allows further separation of bacteria into single colonies in the subsequent quadrants. The flaming between each turn is to prevent carryover of an excessive number of bacteria from the previous quadrant.
>
> In practice, a professional laboratory technician / microbiologist performs streaking without flaming by using different sides of the loop for spreading the bacteria in each quadrant. However, it is recommended that students, as beginners flame the loop at each turn.

13. Replace the lid, flame-sterilise the inoculation loop and allow it to cool.

14. Shut off the Bunsen burner.

15. Invert the plate and incubate it at room temperature# or in an incubator set at 25–30°C for 3–4 days.

> #Note: Room temperature may vary, depending on the ambient conditions of the laboratory environment. Hence, the incubation time may also vary.

16. Wrap the edge of the agar plate with parafilm and store it at 4ºC until lab session 3.

C. Part (2) of the experiment: Performing serial dilutions, spread plate and pour plate to determine bacterial load

a) Equipment and materials (per group)

Equipment

- Bunsen burner × 1
- Spark lighter × 1
- Micropipettes (P5000, P1000, P200 and P20) and sterile tips

Materials

- 2.0 ml micro-centrifuge tube × 3
- Glass spreader × 1
- PCA plate × 9
- Molten PCA solution (120 ml) × 1
- Sterile Petri dish × 9
- Probiotic drink (tube A, 1 ml) × 1
- Phosphate buffered saline (PBS, 5 ml) × 1
- 250 ml beaker with 70% ethanol × 1
- 20 ml measuring cylinder × 1

- Permanent marker × 1
- 70% ethanol in spray bottle × 1
- Paper towel × 1 box
- Biohazard bag × 1
- Disposal container with 10% chlorine bleach × 1

b) Safety precautions

- Wear a laboratory gown.
- Tie up long hair.
- Wear gloves during the experiment.
- Wash hands to remove all possible contamination before and after the experiment.
- Keep ethanol away from fire.
- Make sure ethanol is fully vaporised before approaching any flame.
- Dispose of or disinfect all materials properly after each experiment.

c) Procedures

Dilution of bacterial sample

1. Disinfect the benchtop and gloved hands with 70% ethanol*.

***Caution: Keep away from fire! DO NOT light the flame until the ethanol has been completely vaporised!**

2. Label three 2.0 ml micro-centrifuge tubes "B", "C" and "D".

3. Light a Bunsen burner.

4. Use the P1000 micropipette, pipette 1485 µl# PBS for each of the 2.0 ml micro-centrifuge tubes (Fig. 7).

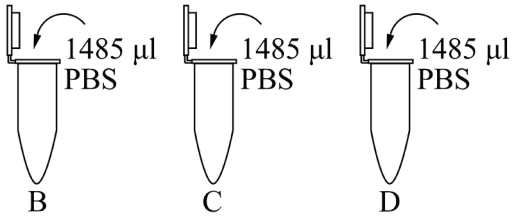


Fig. 7

#Note: The maximum volume of P1000 is 1000 µl or 1 ml.
To transfer a volume of 1485 µl, 2 draws of 742.5 µl are needed.

5. Use the P20 micropipette, transfer 15 µl solution from tube A to tube B. Hence, the solution in tube B is a 100-fold dilution of the solution in tube A (Fig. 8).

6. Close the tube cap. Mix the contents of tube by turning it upside down three times.

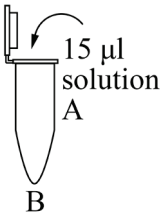


Fig. 8

7. Using the P20 micropipette, transfer 15 µl solution of tube B to tube C. Hence, the solution in tube C is a 100-fold dilution of the solution in tube B (Fig. 9).

8. Close the tube cap. Mix the content of the tube by turning it upside down three times.

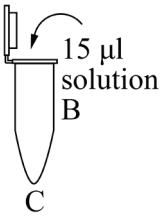


Fig. 9

9. Using the P20 micropipette, transfer 15 µl solution of tube C to tube D. Hence, the solution in tube D is a 100-fold dilution of the solution in tube C (Fig. 10).

10. Close the tube cap. Mix the content of the tube by turning it upside down three times.

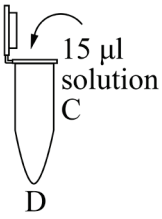


Fig. 10

Please complete the table below.

Table 1

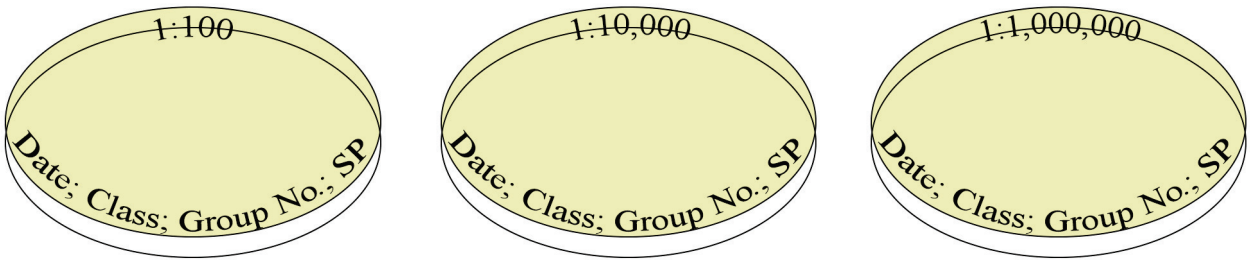
	Tube A	Tube B	Tube C	Tube D
Dilution fold of the serial dilution*	0	100-fold dilution of tube A	100-fold dilution of tube B	100-fold dilution of tube C
Dilution fold of the probiotic drink	0			

*Note: Serial dilution means the sequential (or stepwise) dilutions of the test solution sample, in which the proportion between the test sample and the solvent at each step stays the same and thus generates the resulting concentrations with a geometric progression.

Perform spread plate

11. Collect nine PCA plates. Label the bottom of each plate with the date of the experiment, your class, group number and "SP" (which stands for "spread plate").

12. Label the bottom of three of the plates "1:100", another three plates "1:10,000" and another three plates "1:1,000,000" (Fig. 11).



3 plates 3 plates 3 plates

Fig. 11

13. After labeling the plates, turn them over so the lids face up.

14. Finger-flick tube B (Fig. 12).

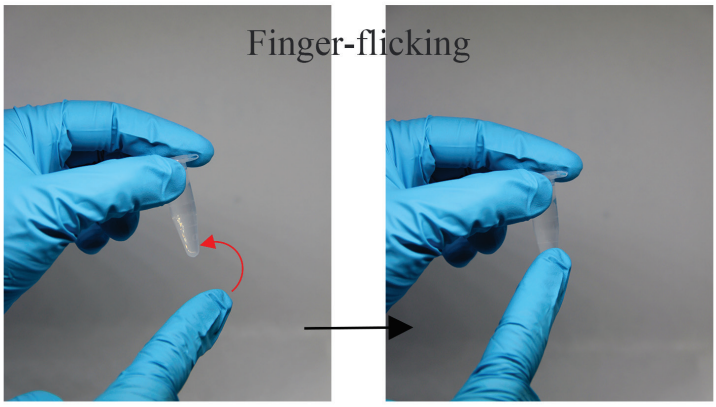


Fig. 12

15. Using the P200 micropipette, aseptically* pipette 0.1 ml solution of tube B to one of the PCA plates marked "1:100" (Fig. 13).

*Note: Please refer to Steps 3–4 of Appendix 1 for details of the aseptic technique.

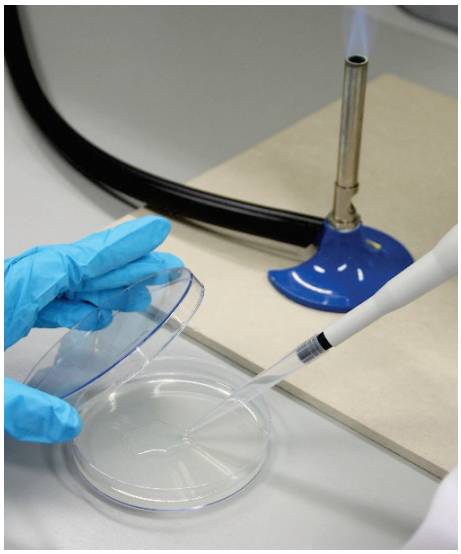


Fig. 13

16. Dip a glass spreader into the beaker of 70% ethanol. Pass it through the burner flame gently* and allow it to cool (Fig. 14).

***Caution: Do not put a hot spreader into the ethanol, as the ethanol may catch fire. In case the ethanol catches fire, cover the beaker with a fireproof board (larger than the beaker) to cut off the oxygen supply, thus extinguishing the fire.**

Fig. 14

17. Uniformly spread the transferred sample over the entire agar plate with the flame-sterilised spreader while continually rotating the plate (Fig. 15).

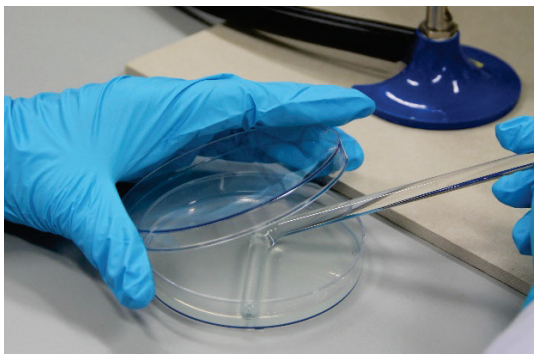


Fig. 15

18. Repeat Steps 14–17 for the two remaining plates marked “1:100” .

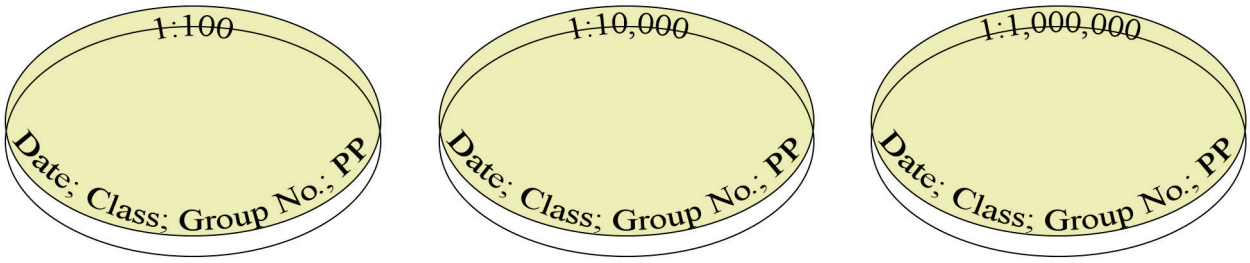
19. Repeat Steps 14–17 using the solution in tube C for the 3 plates marked “1:10,000”.

20. Repeat Steps 14–17 using the solution in tube D for the 3 plates marked “1:1,000,000”.

Perform pour plate

21. Collect nine sterile empty Petri dishes. Label the bottom of each dish with the date of experiment, your class, group number and “PP” (which stands for “pour plate”).

22. Label the bottom of three of the Petri dishes “1:100”, (Fig. 16), another three dishes “1:10,000” and another three dishes “1:1,000,000”.



3 dishes 3 dishes 3 dishes

Fig. 16

23. After labeling the dishes, turn them over so the lids face up.

24. Finger-flick tube B. Use the P1000 micropipette to aseptically* transfer 1 ml solution of tube B to one of the empty Petri dishes marked "1:100" (Fig. 17).

*Note: Please refer to Steps 3 – 4 of Appendix 1 for details of the aseptic technique.

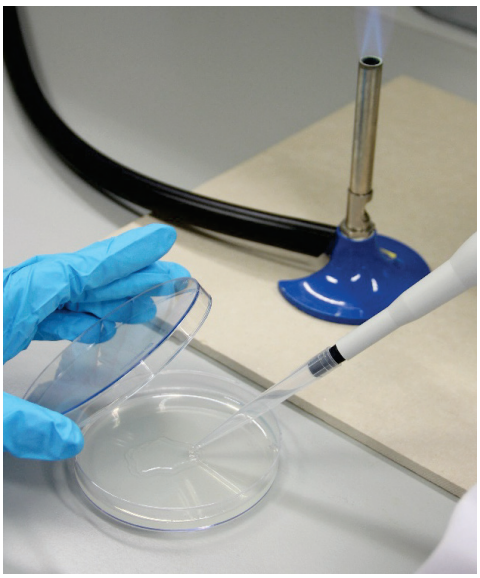


Fig. 17

25. Using the 20 ml measuring cylinder, transfer 11 ml molten PCA solution to the dish. Swirl the dish gently to mix the solution,# and allow it to solidify (Fig. 18).

#Note: A gentle swirl is highly recommended in order to avoid any bubble formation in the agar.

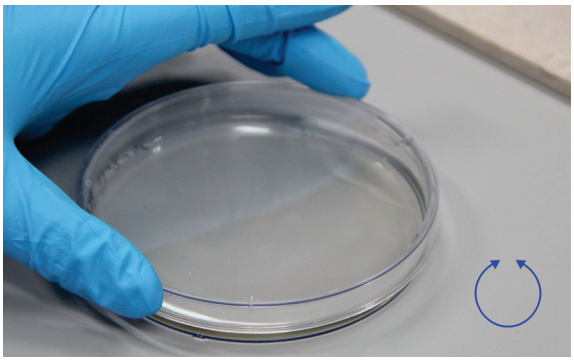


Fig. 18

26. Repeat Steps 24–25 for the two remaining "1:100" dishes.

27. Finger-flick tube C. Aseptically transfer 1 ml solution of tube C to one of the empty Petri dishes marked "1:10,000".

28. Add 11 ml molten PCA solution to the dish. Swirl the dish gently to mix the solution, and allow it to solidify.

29. Repeat Steps 27–28 for the two remaining "1:10,000" dishes for tube C.

30. Finger-flick tube D. Aseptically transfer 1 ml solution of tube D to one of the empty Petri dishes marked "1:1,000,000".

31. Add 11 ml molten PCA solution to the dish. Swirl the dish gently to mix the solution, and allow it to solidify.

32. Repeat Steps 30–31 for the two remaining "1:1,000,000" dishes for tube D.

33. Turn off the Bunsen burner.

34. Discard all used tubes and tips in the designated disposal container with 10% chlorine bleach (no autoclave processing required) or in a biohazard bag for autoclave processing afterwards.

Incubation of bacterial growth

35. When the agar solution gets solidified, invert all the 18 PCA plates and incubate them at room temperature* or in an incubator set at 25 − 30°C for 3–4 days.

> *Note: Room temperature may vary, depending on the ambient conditions of the laboratory environment. Hence, the incubation time may also vary.

D. Part (3) of the Experiment: Counting colony forming units to calculate bacterial load

a) Equipment and Materials (per group)

Equipment

No equipment needed

Materials

- PCA plate from Part (2) × 18

- Permanent marker × 1
- 70% ethanol in spray bottle × 1
- Paper towel × 1 box
- Biohazard bag × 1
- Disposal container with 10% chlorine bleach × 1

b) Safety Precautions

- Wear a laboratory gown.
- Tie up long hair.
- Wear gloves during the experiment.
- Wash hands to remove all possible contamination before and after the experiment.
- Keep ethanol away from fire.
- Make sure ethanol is fully vaporised before approaching any flame.
- Dispose of or disinfect all materials properly after each experiment.

c) Procedures

1. Count the colony-forming units (CFU)* on all PCA plates after the culture incubation. Mark TNTC (Too Numerous To Count) if the number of colonies on the plate is higher than 250 CFU.

> *Note: Only plates with a colony number between 25 and 250 can be used for counting.
>
> Use the term "colony-forming unit" (CFU) instead of the term "colony" to reflect the fact that a single colony observed on an agar plate may not be formed by a single bacterium.
>
> In fact, such a colony can be formed by several bacteria (more than one bacterium) growing in proximity. If we present our data using the term "colony", then the actual number of bacteria in the test sample may be underestimated.

2. Calculate the number of CFU per ml for each dilution of the sample, using the following equation:

$$\boldsymbol{CFU/ml} = \left[\left(\frac{\boldsymbol{CFU\ of\ Plate\ 1 + CFU\ of\ Plate\ 2 + CFU\ of\ Plate\ 3}}{\boldsymbol{3}}\right)\right] \boldsymbol{x\ Dilution\ Fold}$$

3. After observation and analysis, dispose of all PCA plates in the designated disposal container with 10% chlorine bleach (no autoclave processing required) or in a biohazard bag for autoclave processing afterwards.

VI. Results

1. Paste the photo or draw a picture of the agar plate obtained in Part (1), and circle the single colonies.

2. Data table for Part (2): Spread plate count

Dilution fold	1:100			1:10,000			1:1,000,000		
CFU	Plate 1	Plate 2	Plate 3	Plate 1	Plate 2	Plate 3	Plate 1	Plate 2	Plate 3
Mean									
CFU/ml									

3. Date table for Part (2): Pour plate count

Dilution fold	1:100			1:10,000			1:1,000,000		
CFU	Plate 1	Plate 2	Plate 3	Plate 1	Plate 2	Plate 3	Plate 1	Plate 2	Plate 3
Mean									
CFU/ml									

4. (a) How many bacteria does the manufacturer claim that the probiotic drink has?

_____

(b) According to your experimental data, estimate the total number of bacteria found in the probiotic drink used in the experiment.

i. By means of spread plate count

_____

_____

_____

_____

_____

_____

ii. By means of pour plate count

(c) Is the number of bacteria found similar to what the manufacturer claims in the product description?

VII. Discussion

1. How does streak plating help in the isolation of single colonies?

2. Which dilution of the probiotic drink is most suitable for counting the bacterial load?

3. Why are three plates needed for CFU counting?