Practical activity (7)

Growing Microbes and Estimating the Number of Microbes

(Teacher's Guide)

I. 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.

II. 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.

III. Teaching notes:

1. Introduce the task by going through the "Background" with students, or have the students read through this part and the "Guiding questions about the design of the experiment" as a pre-class activity.
2. Discuss the design of the experiment with the students, using the "Guiding questions about the design of the experiment".
3. Plan the laboratory work with reference to "Time allocation for the experimental activities" in section IV according to the lesson time of your school.
4. Always remind the students about the safety precautions of each part of the experiment.
5. Go through the "Results" and "Discussion" with the students.

IV. Time allocation for the experimental activities:

Practical work		Duration	
		In class	**Out of class** (To be done by the laboratory technicians)
Lab session 1: Part (1) of the experiment			
1	Streak plate	30 min	
2	Incubation of microbes		3–4 days
Lab session 2: Part (2) of the experiment			
1	Serial dilutions of probiotic drink	20 min	
2	Spread plate	30 min	
3	Pour plate	30 min	
4	Incubation of microbes		3–4 days
Lab session 3: Part (3) of the experiment			
1	Counting of CFU and calculation of bacterial load	30 min	
Total lesson time for the experimental activities		Lab session 1: 30 min. Lab session 2: 1 hr 20 min. Lab session 3: 30 min.	

V. Equipment, materials and preparatory work for the experiments

A. Part (1) of the experiment: Performing streak plate to isolate bacterial colonies

a) Equipment (per group)

- Bunsen burner × 1
- Spark lighter × 1

b) Materials (per group)

- 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

c) Preparatory work

Preparation of PCA plates and aliquot of probiotic drink **(To be done by the teacher / laboratory technicians)**

1. One day before the lab session, dissolve PCA powder in distilled water according to the manufacturer's instructions. Autoclave the solution and pour it into Petri dishes (15–20 ml per dish). Wrap the dishes with parafilm, and store them properly at 4°C until use. Prepare one PCA plate per student.

   Tip: The agar is usually sold in powder form and has to be handled by the laboratory technicians. The agar powder should be dissolved in distilled water upon boiling, according to the manufacturer's instructions. After boiling, cool the agar solution to around 50°C and pour it into Petri dishes (15–20 ml). Let it solidify at room temperature. Invert the plates and store them at 4°C in a zip bag (or the original packaging of the Petri dish and seal with masking tape). Ready-to-use agar plates can be purchased from biotechnology companies.

   Tip: If the agar plates are stored at 4°C, water vapour may condense on the inner surface of the lid of the Petri dishes. Before using, it is recommended to remove the condensed water by placing the Petri dishes inverted with the lid half-open in a clean 37°C incubator for 1 hour. The presence of condensed water may increase the risk of contamination.

2. Commercially available probiotic drinks such as Yakult and Active V can be used in the current experiment. Before the lab session, freshly aliquot the probiotic drink into micro-centrifuge tubes (1 ml per tube). Label the tubes "A", and store them properly at 4°C until use. Prepare one tube per student. Please note that probiotic drinks that contain more than one bacterial species may show colonies of different shapes or colours.

d) Sterilisation and disposal

After the experiment, some equipment and materials to discard (solid or liquid) should be sterilised by steam under pressure (autoclave) at 121°C, 15 psi pressure for at least 30 minutes or immersed in 10% chlorine bleach for at least 2 hours.

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

a) Equipment (per group)

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

b) Materials (per group)

- 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

c) Preparatory work

Preparating and aliquoting of PCA plates, molten PCA solution, PBS and probiotic drink **(to be done by the teacher / laboratory technicians)**

1. One day before the lab session, dissolve PCA powder in distilled water according to the manufacturer's instructions. Autoclave the solution and pour it into Petri dishes (15–20 ml per dish). Wrap the dishes with parafilm, and store them properly at 4°C until use. Prepare nine PCA plates per group.

2. One day before the lab session, dissolve PCA powder in distilled water according to the manufacturer's instructions. Autoclave the solution, and aliquot 120 ml into sterilised bottles. Prepare one bottle per group. Keep the molten PCA solution warm in an incubator or oven until use.

3. Before the lab session, dissolve 10X PBS powder in distilled water according to the manufacturer's instructions. Dilute the 10X solution to 1X with distilled water, and aliquot it into centrifuge tubes (5 ml per tube). Prepare one tube per group.

4. Commercially available probiotic drinks such as Yakult and Active V can be used in the current experiment. Before the lab session, freshly aliquot the probiotic drink into micro-centrifuge tubes (1 ml per tube). Label the tubes "A", and store them properly at 4°C until use. Prepare one tube per group.

d) Sterilisation and disposal

After the experiment, some equipment and materials to discard (solid or liquid) should be sterilised by steam under pressure (autoclave) at 121°C, 15 psi pressure for at least 30 minutes or immersed in 10% chlorine bleach for at least 2 hours.

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

a) Equipment (per group)

No equipment needed

b) Materials (per group)

- 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

c) Preparatory work

No specific preparation work required

d) Sterilisation and disposal

After the experiment, some equipment and materials to discard (solid or liquid) should be sterilised by steam under pressure (autoclave) at 121°C, 15 psi pressure for at least 30 minutes or immersed in 10% chlorine bleach for at least 2 hours.

VI. Suggested answers for "guiding questions about the design of the experiment" and Table 1

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

   Plate B

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

   Plates B and C

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 method for estimating the number of bacteria present in the test solution.

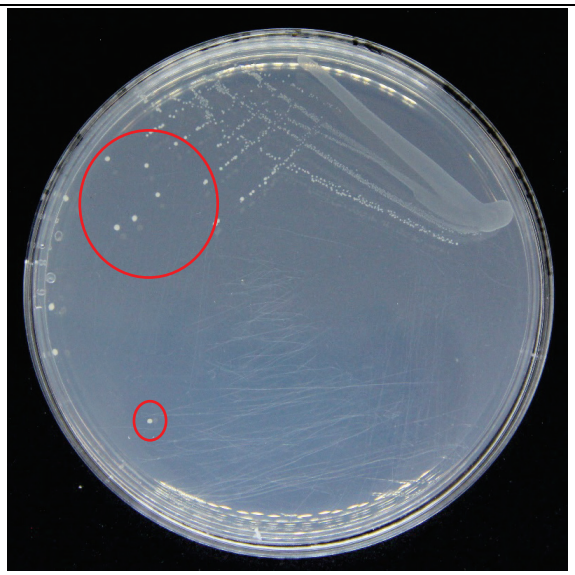
   We can first perform a serial dilution of the test solution and then culture the diluted samples on agar plates using the spread / pour plating techniques. From those culture plates, we can choose the ones with colonies within the countable range 25–250, and use these data for the calculation of the number of bacteria present in the test solution.

Table 1

	Tube A	Tube B	Tube C	Tube D
Dilution fold of sequential 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	*100*	*100 × 100 = 10,000*	*100 × 100 × 100 = 1,000,000*

VII. Results

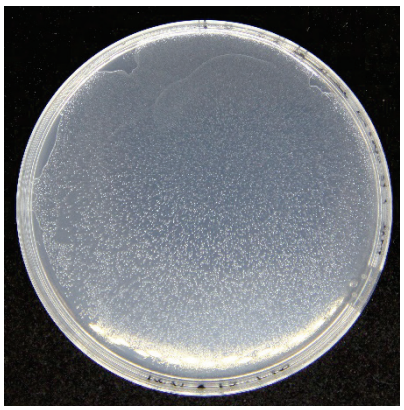
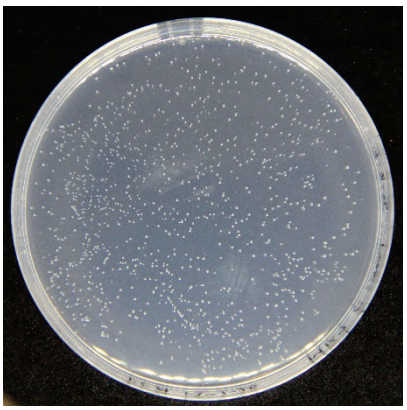
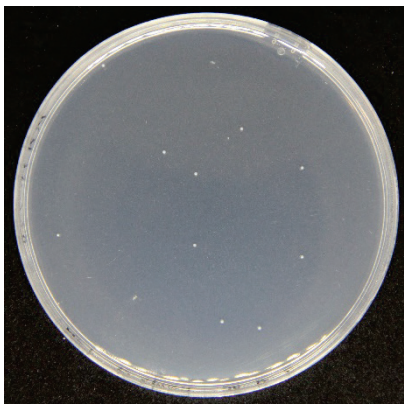
1. Paste the photo or draw a picture of the agar plate obtained in Part (1), and circle the single colonies.



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
	TNTC	*TNTC*	*TNTC*	*TNTC*	*TNTC*	*TNTC*	*9*	*10*	*11*
Mean	--			--			*10*		
CFU/ml	--			--			*10 × 1,000,000 / 0.1 = 1×10^8*		

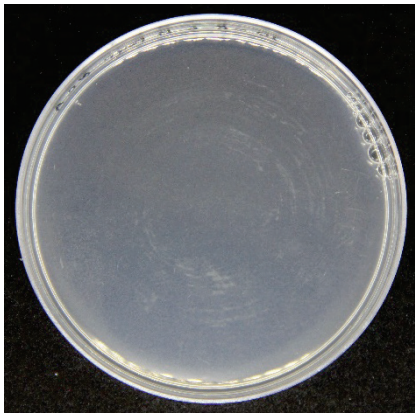
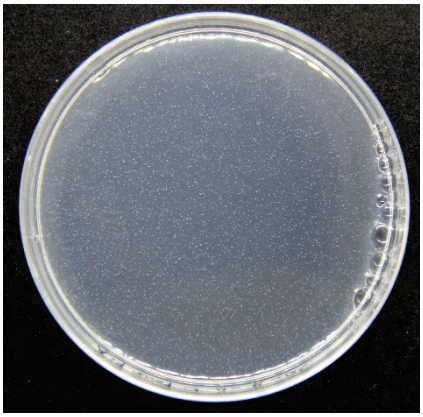
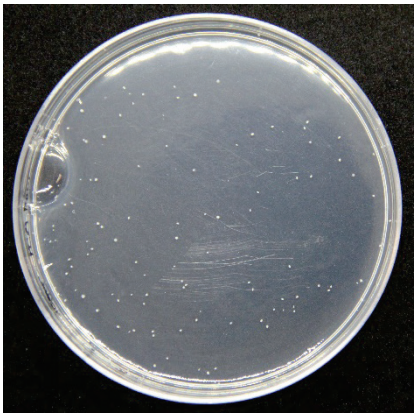
Results of spread plates (for reference only)

1:100	1:10,000	1:1,000,000
		

3. Data 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
	TNTC	*TNTC*	*TNTC*	*TNTC*	*TNTC*	*TNTC*	*102*	*98*	*100*
Mean	--			--			*100*		
CFU/ml	--			--			*100 × 1,000,000 = 1×10^8*		

Results of pour plates (for reference only)

1:100	1:10,000	1:1,000,000
		

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

1×10^{10} CFU (i.e. 10 billion)

(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

Number of bacteria per ml: 10 × 1,000,000 / 0.1 = 1×10^{8} CFU/ml

Volume of the probiotic drink: 100 ml

The total number of bacteria loaded in the bottle of the probiotic drink

= number of bacteria per ml × volume

= 1×10^{8} CFU/ml × 100 ml

= 1×10^{10} CFU

ii. By means of pour plate count

Number of bacteria per ml: 100 × 1,000,000 = 1×10^{8} CFU/ml

Volume of the probiotic drink: 100 ml

The total number of bacteria loaded in the bottle of the probiotic drink

= number of bacteria per ml × volume

= 1×10^{8} CFU/ml × 100 ml

= 1×10^{10} CFU

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

The number of bacteria found is similar to what the manufacturer claims.

VIII. Discussion

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

   The principle of isolating single colonies on an agar plate by streaking is to collect a fraction of bacteria from one quadrant and spread them to the next quadrants. Thus, the density of bacteria can be gradually reduced across the quadrants, which allows further separation of bacteria into single colonies.

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

   The dilution with CFU between 25 and 250, i.e. the one with the probiotic drink diluted in the 1:1,000,000 dilution.

3. Why are three plates needed for CFU counting?

   To minimise the effect of pipetting variation.

   [Note for teachers: Standard deviation can only be determined with at least three data points.
   The standard deviation is a measure of the variation of the data obtained, thus reflecting how precisely the experiment was done and how reliable the data were (the data should be discarded if SD is larger than the mean.]

IX. References

da Silva N, Taniwaki MH, Junqueira VCA, Silveira NFA, do Nascimenta MS, Gomes RAR. Basic plate count techniques for enumeration of microorganisms. *Microbiological Examination Methods of Food and Water: A Laboratory Manual*, 2nd Edition. Leiden, the Netherlands: CRC Press / Balkema; 2018, p. 25

da Silva N, Taniwaki MH, Junqueira VCA, Silveira NFA, do Nascimenta MS, Gomes RAR. Aerobic plate count. *Microbiological Examination Methods of Food and Water: A Laboratory Manual*, 2nd Edition. Leiden, the Netherlands: CRC Press / Balkema; 2018, p. 65.