**Dilution plating to determine microbial density in liquid culture STUDENT**

**Introduction**

By diluting a culture of bacteria, spreading it on an agar plate and incubating that plate it is possible to count individual bacterial colonies. Each colony arises from a single bacterial cell so you will then be able to calculate an estimate of the number of viable bacterial cells in the original culture. In this activity you will use this technique on two cultures of *Bacillus subtilis*.

**Aim**

To find the density of viable bacterial cells in two cultures of *Bacillus subtilis* by dilution plating.

**Intended class time**

* 60 minutes on the first day and 30 minutes a day later.

**Chemicals**

|  |  |
| --- | --- |
| 70% ethanol | HSE warning symbol Highly flammableHarmful if swallowed |
| *Bacillus subtilis* | Although *Bacillus subtilis* is not considered pathogenic the possibility of mutation or contamination means all cultures should be treated as potentially pathogenic. Spills must be disinfected. Used cultures must be disposed of by sterilisation.  |
| Antibacterial (1% Virkon®) | No known health hazard but avoid cotact with skin and eyes |
| Sterile LB Broth | No known hazard |

**Equipment**

* Bunsen burner
* Antibacterial spray (Virkon®)
* Antibacterial waste pot (Virkon®)
* Bactericidal hand-wash
* Paper towels
* 10 sterile nutrient agar plates
* 8 sterile Bijou bottles
* Sterile LB broth
* 5 ml 24 h culture of *Bacillus subtilis*
* 5 ml 48 h culture of *Bacillus subtilis*
* Tray
* Two sterile 10 cm3 pipettes
* Ten sterile 1 cm3 pipettes
* 100 µl pipette and sterile tips
* Glass spreader
* Marker pen
* 70% ethanol provided in a glass Petri dish with a lid
* Tape

**Health and Safety**

* Ethanol is highly flammable and you have a naked flame on your bench. Great care is therefore needed to minimise the risk of fire. Have only a small volume of ethanol in your wide dish, keep it covered when not in use and well away from the Bunsen burner.
* Wear a lab coat and goggles when working with microorganisms.
* Cover any skin cuts or abrasions.
* All spills must be immediately disinfected using 1% Virkon® solution left in place for 10 minutes.

**Procedure**

**Part 1 – diluting the cultures and spreading the plates**

*Note: At all times in this investigation, a roaring Bunsen flame should be burning on the bench. This is needed for sterile working. However, it is a fire hazard. Keep your dish of ethanol well away from the Bunsen burner and keep the dish covered when not in use.*

1. Wipe your bench with antibacterial spray and a paper towel. Pour 1% Virkon® solution into your tray to cover the bottom with a thin layer, leave in place for 10 minutes and then wipe away.
2. Take five sterile agar plates and label them: **24 h 100, 24 h 10-1, 24 h 10-2, 24 h 10-3** and **24 h 10-4**
3. Take the other five sterile agar plates and label them: **48 h 100, 48 h 10-1, 48 h 10-2, 48 h 10-3** and **48 h 10-4**
4. Take four sterile Bijou bottles and label them: **24 h 10-1, 24 h 10-2, 24 h 10-3** and **24 h 10-4**
5. Take the other four sterile Bijou bottles and label them: **48 h 10-1, 48 h 10-2, 48 h 10-3** and **48 h 10-4**
6. You will now make a serial dilution so that you have samples from each culture diluted 10, 100, 1000 and 10,000 times. Decide on the volume of sterile LB broth you will put into each of your sterile Bijou bottles and the volume of culture that you will need to add to each.

**Volume of LB broth:…………………… Volume of *Bacillus subtilis* culture:………………………………**
7. Wash your hands thoroughly using anti-bacterial hand wash and dry with a paper towel.
8. Working near the flame use a sterile pipette to add the volume of sterile LB broth you have decided on to each of the eight Bijou bottles. Flame the top of the culture bottle and Bijou bottles when opening and closing.
9. Now take the 24 h culture and use a sterile pipette to add the correct volume to the Bijou bottle labelled **24 h 10-1**. Flame the tops of the bottles when opening and closing. Mix well.
10. Next use the 24 h 10-1 dilution you have just made to make your 24 h 10-2 dilution as in step 9, and so on for 24 h 10-3 and 24 h 10-4.
11. Now repeat steps 9 and 10 with the 48 h culture.
12. Put a sterile tip on to your pipette and then, working near the flame, carefully open the 24 h culture and use the pipette to remove 100 µl of culture, flaming the top of the culture bottle when opening and closing.
13. Lift the lid of the **24 h 100** agar plate very slightly and pipette the 100 µl of culture into the centre of the plate, close the lid. Discard the pipette tip into the antibacterial waste pot.
14. Next, take the glass spreader, dip it in ethanol and flame it in a blue Bunsen flame, hold it close to the flame until it is cool, then use it to spread the culture evenly across the surface of the agar plate.
15. Now repeat steps 12 to 14 with the other four 24 h diluted cultures, the 48 h culture and the four 48 h diluted cultures. You will now have all ten agar plates spread with the correct dilution of the correct culture.
16. Use 2-4 small pieces of sticky tape to tape the lid of each plate securely onto the base but do not seal it completely.
17. Disinfect your tray with 1% Virkon®, and wash your hands thoroughly with bactericidal hand-wash.
18. Incubate the plates overnight at 20-25°C.

**Part 2 – observing the colonies and calculating original densities**

1. After one day, look at the plates but do not open them.
2. To record your observations, draw and annotate each of your ten spread plates.
3. In each set of five plates, identify one where you can see individual colonies.
4. Count the number of colonies on the plate you have chosen from each set and record this number and the dilution factor for that plate.
5. Use your data to calculate an estimate of the density of viable bacteria in each of the two original cultures and record your results using appropriate units.

**Extension questions**

1. What other methods could be used to find the density of bacteria in a culture? Why might some of these methods, even when carried out carefully and accurately, give results different from the dilution plating method?
2. When observing your spread plates, how can you be confident that all the colonies you see are *Bacillus subtilis*?
3. One of the cultures you were provided with was 24 h older than the other. Use your results to calculate the percentage increase in cell number that has taken place in the 24 h that separates the two cultures.
4. Assuming that the bacteria have been dividing at a constant rate throughout those 24 h, how long does it take each cell to divide?

**To submit**

For this piece of work to count towards Practical Activity Group 7 of the GCE Biology Practical Endorsement, you need to have annotated drawings of your observations, colony counts and calculations. You also need to have considered the above questions as the answers to these questions will aid you in preparation for your written examinations.