EXPERIMENT 4

ISOLATION OF SOIL BACTERIA: VIABLE TITER and PURE CULTURE

Bacteria in soil occur singly and in aggregates. To estimate the number of bacteria in a gram of soil, the soil must be both diluted and mixed thoroughly so that the aggregates are broken up such that a suspension of single cells is achieved. The cell suspension is then serially diluted so that from some dilutions a reasonable number of cells (30 to 300) are dispensed into Petri plates. The samples in Petri plates are then mixed with sterile, molten (liquid) agar medium which is then allowed to solidify. This method of plating is called pour plating. In a later experiment, we will use spread plating.

Upon incubation each cell will give rise to a colony either in the agar or on the agar surface. It is possible that a colony could have arisen from two or more cells that stuck together. Thus a colony forming unit (CFU) may have originated from one or more cells. The viable titer is determined by counting colonies (CFU's) and multiplying by the dilution factor. This method only counts living cells as dead cells do not reproduce to form colonies**. The viable titer is determined from countable plates: plates from dilutions that yield at least 30 colonies (so that a statistically significant number has been counted) and less than 300 colonies.** When a plate has more than 300 colonies, there is such crowding that fast growing bacteria overwhelm slow growers: the fast growers either remove nutrients or produce inhibitory end products before slow growers can form a visible colony.

Viable titer = (CFU/volume plated) x Dilution factor

Appendix V reviews dilution problems and how to calculate dilutions, dilution factors and titers.

Later, isolated colonies will be examined for the types of cells and one will be restreaked to obtain a pure culture. A pure culture is defined as the progeny from one cell. Actually we will be making an axenic culture from a clone (colony). Assuming that one cell could have given rise to the colony, we call these pure cultures even though we have no technical proof of that. Proof of pure culture involves showing that all the colonies on the restreak are identical and Gram staining these to demonstrate all the cells in the resulting colonies are identical and the same as those on the original plate.

MATERIALS

1. 4 9 ml sterile dilution blanks (16x150 mm capped tube)
   1 100 ml sterile dilution blank (square bottle)
2. 6 sterile 1 ml pipettes.

3. Molten (45^{o}C) Nutrient Agar prepared in Experiment 3.
4. 10 sterile Petri plates.
5. Balances and weighing boats.
6. Nutrient Agar plate (Day Two)
7. Gram staining reagents, slides.

PROCEDURE

First Lab Period

1. Mark the sterile dilution blanks in the following manner: the 100 ml dilution blank is 10^{-2} and the
9 ml tubes sequentially are 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}.

2. Go outside and with a weigh boat obtain some soil.

3. Weigh one gram of soil out in another weigh boat. Add that gram to the 10^{-2} dilution blank and shake vigorously for at least 1 full minute. Make sure the cap is securely tightened during the shaking.

4. All the 10^{-2} dilution to sit for a short period. Then aseptically transfer 1 ml from this dilution to the 10^{-3} tube. The instructor will demonstrate how to make an aseptic transfer. Mix thoroughly.

5. Using a fresh, sterile pipette for each succeeding step, transfer 1 ml from the 10^{-3} dilution to the 10^{-4} dilution blank, then from the 10^{-4} to the 10^{-5}, then from the 10^{-5} to the 10^{-6}. Each time the sample transferred must be thoroughly mixed with the dilution fluid before being transferred to the next tube. The dilution and plating outline is given on the next page:

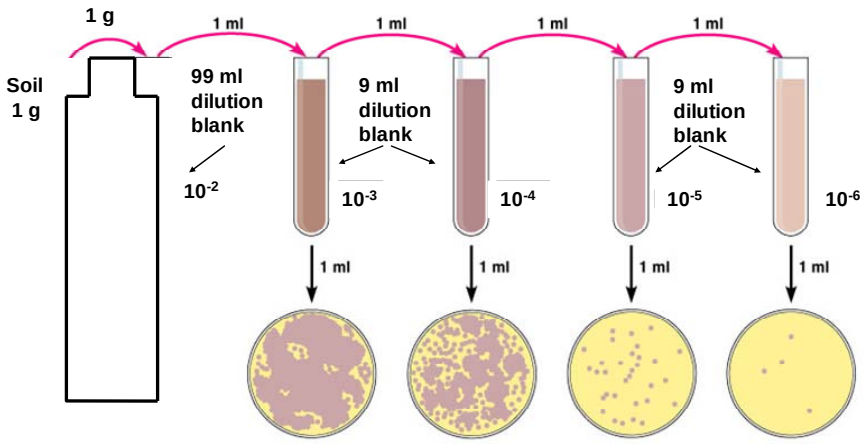


Figure 1. Plating procedure

6. Mark two plates for each tube dilution on the bottom with the dilution it will receive. From each dilution tube (but not the 10^{-2}) place 1 ml of dilution fluid into each of two sterile Petri plates. Be sure to use aseptic technique.

7. Take a flask of Nutrient Agar (that you made in Experiment 3) from the 45^{o}C water bath and aseptically pour molten agar into each Petri plate for that set. Pour in 15 ml, the exact amount is not important: you need to get enough to cover the bottom of the plate and mix with the 1 ml inoculum in the plate. Rapidly but carefully pour all plates of that set. Then gently swirl each plate on the bench so that the inoculum gets thoroughly mixed with the agar. It is important to do this thoroughly, but not too vigorously so as to get the molten agar on the Petri plate top.

9. Allow all the plates to stand without moving so that the agar may solidify and set completely.

10. Invert the plates and stack into pipette canisters and place in the incubator or at room temperature until next period (2 days).

Second Lab Period

Viable Titer

1. Remove the plates from the incubator. Count the number of colonies on each plate beginning with those of the highest dilution. Be sure to count all colonies, those in the agar can be tiny while those on the surface of the agar are usually much larger. When the number of colonies exceeds 300, those of lower dilutions are "Too Numerous To Count" and can be recorded in your lab notebook as TNTC.
2. Calculate the viable titer. Record your results on the blackboard with those of the rest of the class.

Population Survey

1. Find three different surface colonies that are well isolated from other colonies. Circle each on the back of the plate and assign a number to each.

2. Describe each colony morphology. Refer to the Appendix to describe the size, shape, margin, elevation, consistency, color, transparency and other terms to give an accurate description of the colonies.

3. For each colony, perform the Gram Stain to observe the cellular morphology and Gram reaction of the bacteria which make up each colony. It is important to keep the Gram Stain observation of each colony along with the colony description: they are properties of the same organism. Check Appendix I for Cell and Colony Morphology.

4. Are all the colonies composed of only one cell type? What types of cells predominate in your population survey?

Pure Culture

1. Choose one colony that appears to be composed of only one cell type.

2. Flame an inoculating loop, when cool gently touch the surface of the colony you will restreak onto a Nutrient Agar plate.

3. Streak these cells as a primary streak on the surface of a Nutrient Agar Plate. This streak should not occupy more than 1/4 of the plate, see the diagram below.

4. Flame the loop, allow it to cool and streak across the primary streak once or twice and then continue streaking the secondary streak without going back into the primary streak.

5. Flame the loop, allow it to cool and streak across the secondary streak once or twice and then continue streaking the tertiary streak without going back into the secondary or primary streak (Figure 2 and 3).

6. Incubate the plates at 30^{o}C or room temperature until next lab period.

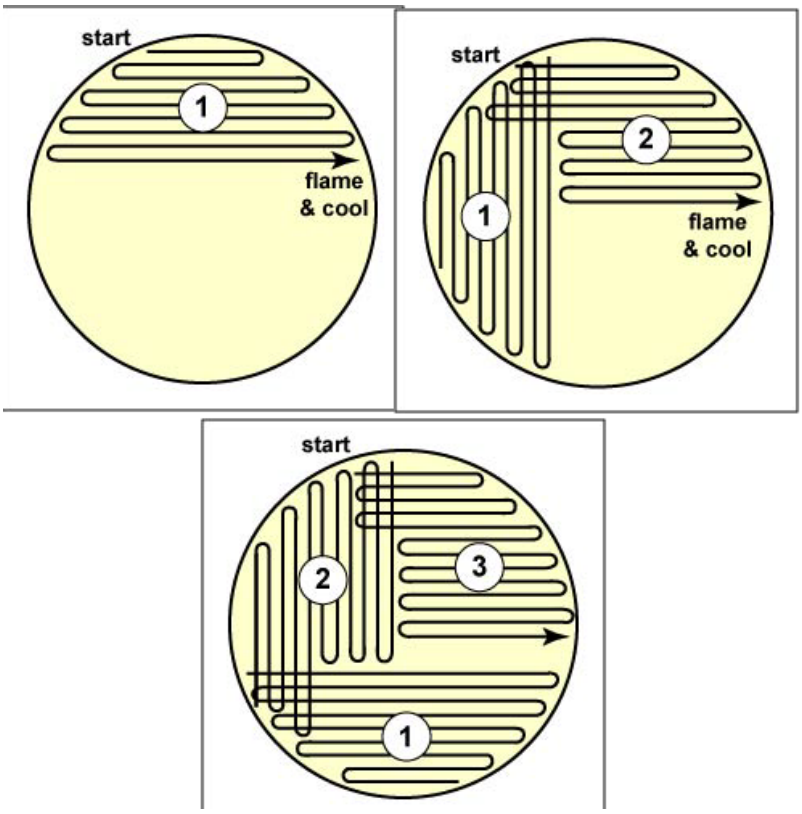


Figure 2. Procedure for streaking a plate for isolation

Figure 3. Bacteria streaked on agar plate

Third Lab Period

1. Examine the Nutrient Agar plate restreaked for a pure culture. Are all the colonies identical? If they are, Gram stain a representative colony. Is it composed of the same bacteria found in the original colony?

2. If your plate contains more than one colony type, Gram stain each and determine which has the identical cell morphology and Gram reaction as the original colony. At this point, you still do not have a pure culture. You will need to restreak another Nutrient Agar plate with the correct colony, and then next period repeat step one above.

3. When you have achieved a pure culture, then streak the same colony that you Gram stained onto a Nutrient Agar slant. Incubate for 1 day and place in the refrigerator. This is your isolate from soil and you will be using this isolate in future experiments.

QUESTIONS

1. Why must you cool the melted agar to 45^{o}C before pouring into the Petri plates.

2. Are all the bacteria in soil counted by this procedure? Is it possible that this medium could not support the growth of certain bacterial types? Thus, is the Nutrient Agar count of the bacteria in soil an overestimate or underestimate of the actual number of viable bacteria in soil?

3. How would you design an experiment to test the answers to Question 2?

4. Suppose someone gave you a slant containing bacteria and said: "here's a pure culture". How do you prove that this is really a pure culture?

5. Why is it important to obtain pure cultures?

HINTS

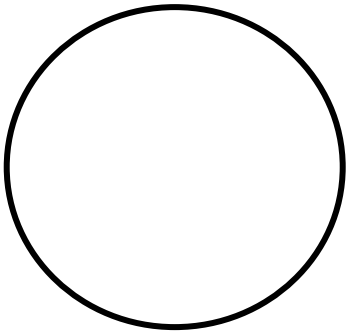
1. Each time the loop is flamed, allow it to cool on the agar at least 10 to 15 seconds before streaking the culture. Wait until the loop stops “hissing.”
2. Use a loopful of culture from the source tube only when applying the first streak in quadrant 1 of the petri plate. Do not return to the source tube for more culture when streaking quadrants 2 to 4.
3. Be sure to flame and cool loops between all inoculations to avoid incidental cross-contamination of cultures.
4. An inoculated plate is always incubated in an inverted position to prevent condensation from falling onto the surface of the plate and interfering with discrete colony formation.

Daily Lab Report: Soil Titer and Pure culture - 1 Name:

RESULTS

GRAM STAINS and COLONY MORPHOLOGY

Isolate 1



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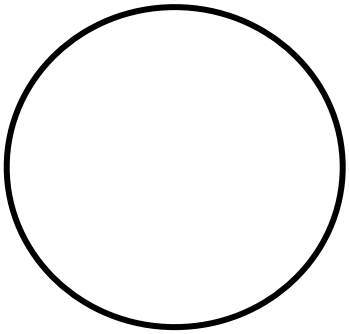
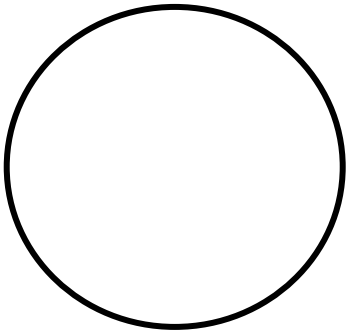
Colony morphology:

Isolate 2



Colony morphology:

Isolate 3



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Colony morphology:

Calculation of viable titer (30-300 rule):

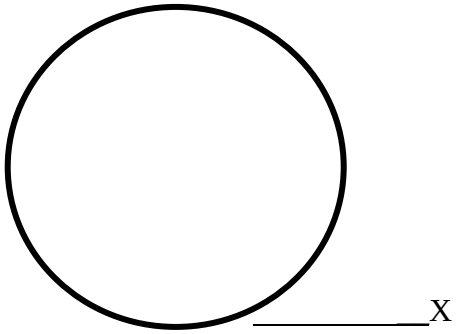
Viable titer = (CFU/volume plated) x Dilution factor

Daily Lab Report: Pure culture - 2 Name:

Pure culture restreak

GRAM STAIN and COLONY MORPHOLOGY

Pure culture isolate



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Colony Morphology: