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Culturing and Enumerating Bacteria from Soil Samples

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Overview

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Surface soils are a heterogeneous mixture of inorganic and organic particles that combine together to form secondary aggregates. Within and between the aggregates are voids or pores that visually contain both air and water. These conditions create an ideal ecosystem for bacteria, so all soils contain vast populations of bacteria, usually over 1 million per gram of soil.

Bacteria are the simplest of microorganisms, known as prokaryotes. Within this prokaryotic group, there are the filamentous microbes known as actinomycetes. Actinomycetes are actually bacteria, but they are frequently considered to be a unique group within the classification of bacteria because of their filamentous structure, which consists of multiple cells strung together to form hyphae. This experiment uses glycerol case media that select for actinomycete colonies, during dilution and plating. Typically, actinomycetes are approximately 10% of the total bacterial population. Bacteria and actinomycetes are found in every environment on Earth, but the abundance and diversity of these microbes in soil is unparalleled. These microbes are also essential for human life and affect what people eat, drink, breathe, or touch. In addition, there are bacterial species that can infect people and cause disease, and there are bacteria that can produce natural products capable of healing people. Actinomycetes are particularly important for producing antibiotics, such as streptomycin. Bacteria are critical for nutrient cycling, plant growth, and degradation of organic contaminants.

Bacteria are highly diverse in terms of the number of species that can be found in soil, in part because they are physiologically and metabolically diverse. Bacteria can be heterotrophic, meaning they utilize organic compounds, such as glucose, for food and energy, or autotrophic, meaning they utilize inorganic compounds, such as elemental sulfur, for food and energy. They can also be aerobic, utilizing oxygen for respiration, or anaerobic, utilizing combined forms of oxygen, such as nitrate or sulfate, to respire. Some bacteria can use oxygen or combined forms of oxygen and are known as facultative anaerobes.

Principles

One way to enumerate the number of bacteria present in a soil sample is to utilize dilution and plating methodology. This methodology utilizes agar as a medium for bacterial growth, a process termed, “culturable technology.” Because of the vast numbers of bacteria found within soils, a small sample of soil is serially diluted in water, prior to being plated on agar within a Petri plate. Typically, a small amount of soil contained within 0.1 to 1 mL of the diluted soil suspension is “spread” over the surface of the agar plate. The plates contain agar, which is molten when hot, but solid when cool. In addition to the agar, nutrients, such as peptone yeast or a product commercially available as R_2A, are added to the medium to allow for the growth of heterotrophic bacteria.

Dilution and plating is an inexpensive and relatively simple technology for the enumeration of soil bacteria. However, there are several drawbacks to the technique. Some common errors and assumptions associated with dilution and plating assays are as follows: it is assumed that every single soil bacterium gives rise to a colony, but in reality a colony may arise from a clump of cells, resulting in an underestimation of true culturable count. During serial dilution of the soil, soil particles can settle out (fall to the bottom), so the true aliquot of soil is not passed on into the next dilution. Many soil microbes are viable but non-culturable. Slow growing bacteria may not result in visible colonies within a reasonable time frame (1-2 weeks).

Also, anaerobic bacteria do not grow under aerobic conditions, and bacteria that do grow are selected for by the nutrients added to the medium. Thus R_2A selects for heterotrophic bacteria, while elemental sulfur selects for autotrophic sulfur oxidizers. Overall, it is estimated that only 0.1 to 1% of all soil bacteria can be cultured. Therefore, dilution and plating of soil bacteria only accounts for culturable bacteria and underestimates the true viable soil population by one to two orders of magnitude. An example of heterotrophic bacterial colonies that resulted from soil dilution and plating is shown in **Figure 1**. Note that approximately 1 million bacterial cells are needed for a colony to be visible to the naked eye.

This experiment demonstrates the dilution and spread plating methodology used to enumerate the number of bacteria within a soil sample. Specifically, two media are used: one designed for all bacteria, and the other that selects for actinomycetes. Once the bacterial colonies have grown on the agar plates, isolate the pure cultures of selected colonies by using a streak plate technique. Such pure cultures can then be further analyzed and characterized for specific traits and functions.

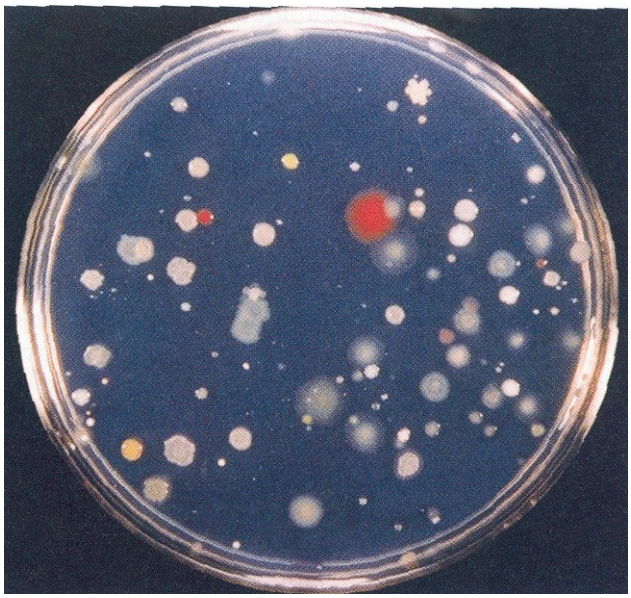

Figure 1. Heterotrophic colonies on an R_2A agar plate. A number of discrete colonies with diverse morphology arise after dilution and plating from soil. Permission for use granted by Academic Press.

Procedure

1. Preparation of Soil Dilutions

1. To begin the procedure, weigh out 10 g of soil sample and add to 95 mL of deionized water. Shake the suspension well, and label as “A”.
2. Before the soil settles, remove 1 mL of the suspension with a sterile pipette and transfer it to a 9-mL deionized water blank. Vortex thoroughly, and label as “B”.
3. Repeat this dilution step three times, each time with 1 mL of the previous suspension and a 9-mL deionized water blank. Label these sequentially as tubes C, D, and E. This results in serial dilutions of 10^{-1} through 10^{-5} grams of soil per mL

2. Making Spread Plates for Bacterial Culture

1. To grow bacterial colonies, take three pre-prepared peptone-yeast agar plates and label them as C, D, and E. Vortex samples C, D, and E, and pipette 0.1 mL onto each plate. This increases the dilution value further, by a factor of ten (C = 10^{-3}, D = 10^{-4}, E = 10^{-5}).
2. Next, dip a glass spreader into ethanol. Place the spreader in a flame for a few seconds to ignite and burn off the ethanol. This will sterilize the spreader.
3. Hold the spreader above the first plate until the flame is extinguished. Open the plate quickly, holding the lid close by. Touch the spreader to the agar away from the inoculum (Inoculum = cells used to begin a culture) to cool, and then spread the drop of inoculum around the surface of the agar until traces of free liquid disappear. Replace the plate lid.
4. Re-flame the spreader and repeat the process with the next plate, working quickly so as not to contaminate the agar with airborne organisms
5. Incubate the bacteria plates at room temperature for 1 week. Make sure the plates are inverted during the incubation to prevent drops of moisture from condensation from falling onto the agar surface.

3. Making Spread Plates for Actinomycetes

1. To grow actinomycetes, take three pre-prepared glycerol-casein plates and label them as B, C, and D. Using the techniques shown previously, spread plate 0.1 mL from the suspensions B, C, and D. The lower dilutions are used because actinomycetes are typically present as $1/10^{th}$ of the bacterial population (B = 10^{-2}, C = 10^{-3}, D = 10^{-4}).
2. Incubate the actinomycete plates (inverted) at room temperature for 2 weeks.

4. Bacterial and Actinomycete Counts

1. After incubation, examine all of the bacteria plates carefully, and note differences in colony size and shape. When grown on agar, bacteria produce slimy colonies ranging from colorless to bright orange, yellow, or pink. In contrast, actinomycete colonies are chalky, firm, leathery, and will break under pressure, where other bacterial colonies will smear. This allows colonies to be distinguished by touch with a sterile loop.
2. Count and record the number of bacterial colonies, including any actinomycetes. Only count plates with 30-200 colonies per plate.

5. Isolation of Pure Cultures

1. Select individual bacterial colonies from any of the plates. More colonies can be selected if there is particular interest in the soil. Use a high dilution plate, as it tends to have pure colonies that are separated well. Choose only colonies that are well-separated from neighboring colonies and look morphologically distinct from each other.
2. Sterilize the loop by dipping it in alcohol and flaming it. Quickly open the Petri dish of interest, and touch the loop to a bare spot in the agar to cool it. Then, remove a small amount of a colony of interest onto the loop.
3. Taking a fresh peptone-yeast plate, make a streak a few centimeters long on one side. Sterilize and cool again, then make a streak that crosses the initial streak only on the first pass. Repeat this process twice more in the same manner. This streaking “dilution” results in cells on the loop being separated from one another. Place the plate in a dark area to incubate at room temperature for two weeks.

Results

A 10-g sample of soil with a moisture content of 20% on a dry weight basis is analyzed for viable culturable bacteria via dilution and plating techniques. The dilutions were made as shown in **Table 1**. 1 mL of solution E is pour-plated onto an appropriate medium and results in 200 bacterial colonies.

$$\text{Number of CFU per g moist soil} = \frac{1}{\text{dilution factor}} \times \text{number of colonies}$$

$$= \frac{1}{10^5} \times 200 \text{ CFU/g moist soil}$$

$$= 2.00 \times 10^7 \text{ CFU/g moist soil}$$

But, for 10 g of moist soil,

$$\text{Moisture content} = \frac{\text{moist weight} - \text{dry weight (D)}}{\text{dry weight (D)}}$$

Therefore,

$$0.20 = \frac{10 - D}{D} \text{ and}$$

$$D = 8.33 \text{ g}$$

$$\text{Number of CFU per g dry soil} = 2.00 \times 10^7 \times \frac{1}{8.33} = 2.4 \times 10^7$$

Step		Dilution
10 g soil (weight/volume)	95 mL saline (solution A)	10^{-1}
1 mL solution A (volume/volume)	9 mL saline (solution B)	10^{-2}
1 mL solution B (volume/volume)	9 mL saline (solution C)	10^{-3}
1 mL solution C (volume/volume)	9 mL saline (solution D)	10^{-4}
1 mL solution D (volume/volume)	9 mL saline (solution E)	10^{-5}

Table 1: Dilution and plating of the samples.

Applications and Summary

There are two fundamental applications of dilution and plating of soil bacteria. The first application is the enumeration of culturable bacteria within a particular soil. The quantification of the number of soil bacteria gives an indication of soil health. For example, if there are 10^6 to 10^8 culturable bacteria present per gram of soil, this would be considered a healthy number. A number less than 10^6 per gram indicates poorer soil health, which may be due to a lack of nutrients as found in low organic matter soils; abiotic stress imposed by extreme soil pH values (pH < 5 or > 8); or toxicity imposed by organic or inorganic anthropogenic contaminants.

The second major application is the visualization and isolation of pure cultures of bacteria. The pure cultures can subsequently be characterized and evaluated for specific characteristics that may be useful in either medical or environmental applications. Examples include: antibiotic production; biodegradation of toxic organics; or specific rhizobia useful for nitrogen fixation by leguminous crops, such as peas or beans.