Isolation of Bacteria: Viable Titer and Pure Culture
A Three Lab Session Experiment

Bacteria can occur singly and in aggregates in nature. Pure drinking water can have as many as 1,000 bacteria/gram, soil can have more than $10^6$ bacteria/gram. To estimate the number of bacteria in a gram of a sample, the sample must be first mixed thoroughly to break up clumps of bacteria so that a suspension of single cells is achieved. Then the homogenized sample is **serially diluted** so that from some dilutions a reasonable number of cells (20 to 200) are dispensed in aliquots (small sub-samples of the diluted sample) onto sterile agar media in petri plates. The technique we will use is called **spread plating** because after the sample aliquot is put on the agar surface, it will be spread around the total agar surface by a sterile glass rod.

During incubation of the plates each viable cell (not visible to the unaided eye) will give rise to a colony (easily visible). It is always possible that a colony could have arisen from two or more cells that stuck together. The **viable titer** is determined by counting colonies (expressed as Colony Forming Units or CFUs) per volume plated and multiplying by the dilution factor. This method only counts living cells because dead cells do not reproduce to form colonies. This method also only counts culturable cells. Keep in mind that it is estimated only 1% of all bacteria are able to grow under laboratory conditions.

The viable titer is determined **ONLY** from **countable** plates: plates from dilutions that yield at least 20 colonies (so that a statistically significant number has been counted) and less than 200 colonies. When a plate has more than 200 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 grow to form a visible colony.

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

See the Appendix to review dilution problems and how to calculate dilutions, dilution factors and titers. Make sure to recognize the distinction that a one to a million dilution (which can be expressed as $1:1,000,000$ or $1:10^6$ or $10^{-6}$) is different from the dilution factor which is simply $10^6$. Viable titers are usually never expressed as negative logs and must include the units (CFU/ml or CFU/gram).

In this experiment we will use two different media types: Nutrient Agar and M-9 Agar. The number of colonies forming on each type of medium indicates the proportion of different types of bacteria in soil. Later, isolated colonies will be examined for the types of cells and one will be restreaked to obtain a pure culture.

**Nutrient Agar** is a **complex** medium because it contains ingredients which contain unknown amounts or types of nutrients. Complex media are sometimes call “complete” media, which of course they can’t be. Nutrient Agar contains Beef Extract (0.3%), Peptone (0.5%) and Agar (1.5%) in water. Beef extract is the commercially prepared dehydrated form of autolyzed beef and is supplied in the form of a paste. Peptone is casein (milk protein) that has been digested with the enzyme pepsin. Peptone is dehydrated and supplied as a powder. Peptone and Beef Extract contain a mixture of amino acids and peptides, but each preparation is slightly different because they come from different cows and
milkings. Beef Extract also contains water soluble digest products of all other macromolecules (nucleic acids, fats, polysaccharides) as well as vitamins and trace minerals. Although we know and can define Beef Extract in these terms, each batch can not be chemically defined. There are many media ingredients which are complex: yeast extract, tryptone, and others. The advantage of complex media is that they support the growth of a wide range of microbes.

Agar is purified from red algae in which it is an accessory polysaccharide (poly-galacturonic acid) in their cell walls. Agar is added only as a solidification agent. Agar for most purposes has no nutrient value. Agar dissolves >95°C and solidifies at 45°C.

M-9 Agar is a chemically defined medium. All the ingredients are chemically defined: the chemical identity of each ingredient as well as the amount added to the medium is known. M-9 Agar usually contains glucose as the sole carbon and energy source and is used by most bacteria growing on M-9 as the energy source. Ammonium chloride is the nitrogen source and magnesium sulfate is the sulfur and magnesium source. The only bacteria that can grow on this medium will be those that can transport the chemicals into the cells and metabolize it to produce energy and all the metabolic intermediates and monomers for biosynthesis.

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 per Pair
Lab Session One
1. Four 9ml sterile dilution blanks (16x150 mm capped tube) and one 99 ml sterile dilution blank (in a square bottle)
2. Sterile 1,000 µl pipette tips.
3. 5 Nutrient Agar and 5 M-9 Agar plates
4. Sterile 200 µl pipette tips.
5. Balances and weighing boats.
Lab Session Two
1. Gram staining reagents, slides.
2. 1 Nutrient Agar and 1 M-9 Agar plates.
Lab Session Three
1. 1 Nutrient Agar Slant

Procedure
Lab Session One - Viable Titer
1. Mark the sterile dilution blanks in the following manner: the 100 ml dilution blank is $10^{-2}$ and the tubes sequentially are $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$.
2. Weigh one gram of your sample out in a weigh-boat. Add that gram to the $10^{-2}$ dilution blank and shake vigorously for at least 2 full minutes. Make sure the cap is securely tightened during the shaking.
3. Allow 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.

4. Using a fresh, sterile pipette for each succeeding step, transfer 1ml 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. Use the Vortex-mixers. The dilution and plating outline is like the one in Figure 6.11 in the text (page 146, 11th Edition of Brock).

5. Mark one plate for each tube dilution on the bottom with the dilution it will receive. From each dilution beginning with $10^{-2}$ dilution pipette 50 μL of dilution fluid onto each of the corresponding five sterile petri plates. Be sure to use aseptic technique. The lab instructor will demonstrate.

6. Take a glass spreader soaking in alcohol, shake off excess alcohol, and pass the spreader quickly through a Bunsen burner flame. The spreader is sterilized by the alcohol, the flame only removes (rapidly) any excess alcohol.

7. Then using the cool, sterile spreader, spread the aliquot on the plate all over the surface of the agar. Continue to spread all other plates, each with a sterile spreader.

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

**Lab Session Two**

**Viable Titer**

1. Retrieve your plates. Count the number of colonies on each plate beginning with those of the highest dilution. Be sure to count all colonies. When the number of colonies exceeds 200, those plates are considered "Too Numerous To Count" and can be recorded in your results as TNTC.

2. Do the CFU counts make sense? Are there about 10X less between dilutions?

3. Calculate the viable titer for each plating medium. Record your results on the blackboard with those of the rest of the class.

**Population Survey**

1. Find five different colonies that are well isolated from other colonies. Circle each on the back of the plate and assign a number to each. You will need to refer back to each colony later.

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. From each marked colony, perform the Gram Stain using a freshly sterilized loop for each colony. It is important to keep the Gram Stain observation of each colony with the colony description: they are properties of the same organism.

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

**Begin to Obtain a 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 it to 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).

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. The plate should be streaked as:

6. Incubate the plates at 30°C or room temperature until next lab period.

### Lab Session Three

1. Examine the plates streaked for isolation of a pure culture. Are all the colonies identical? If they are, Gram stain a representative colony. Is it the same bacterium 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 plate with the correct colony, and then next period repeat step one above.

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

### Questions

1. State a good reason for cooling the melted agar to 45°C before pouring into the petri plates.

2. Are all the bacteria in your sample counted by this procedure? Is this an overestimate or underestimate of the actual number of viable bacteria? Why?

3. What is a pure culture? How do you prove a culture is pure?

4. Why is it important to obtain pure cultures in research?