

Bacterial Growth

Objectives

1. Measure growth of a bacterial culture by spectrophotometry
2. Quantify viable cells in a bacterial culture by standard plate count
3. Observe differentially stained bacteria using light microscopy
4. Determine the effectiveness of some common chemical disinfectants and antibiotics

Background

A. Growth measurements

When attempting to study the basic processes of life, biologists often turn to “simpler” organisms to make their observations and develop working theories. Observations are then made on more complex biological systems to determine if the information obtained from the simpler organisms can be extrapolated to the higher forms. For this lab we shall use prokaryotic organisms, bacteria, to examine the growth process.

Bacteria are a diverse group of small, single-celled organisms in the kingdoms Eubacteria and Archaeobacteria. Found in virtually every extreme of all habitats, they have existed on earth longer and are more widely distributed than any other group of organisms. Bacteria have their genetic material organized in a circular DNA molecule that is not surrounded by a nuclear membrane. Reproduction is by binary fission with the formation of two equal size progeny. During active bacterial growth the size of the population continuously doubles, one cell becomes 2, 2 become 4, etc. in a geometric progression. When bacteria are inoculated into a fresh medium, the resulting culture exhibits a characteristic growth curve of four distinct phases (Fig. 1). During the lag phase the cells prepare for synthesis of DNA and enzymes needed for cell division. There is no increase in cell number. This is followed by the log phase where the culture reaches its maximum rate of growth for specific conditions. The time required for the population to double is known as the **generation time**. The generation time varies between organisms and under different environmental conditions. The graphical determination of doubling time can be made by extrapolation (Fig. 2). As the bacteria multiply, nutrients are exhausted and inhibitory metabolic end products accumulate. These conditions give rise to the stationary phase which represents no net increase in numbers (growth rate equals death rate). Eventually there will be a decline in cell number-the death phase.

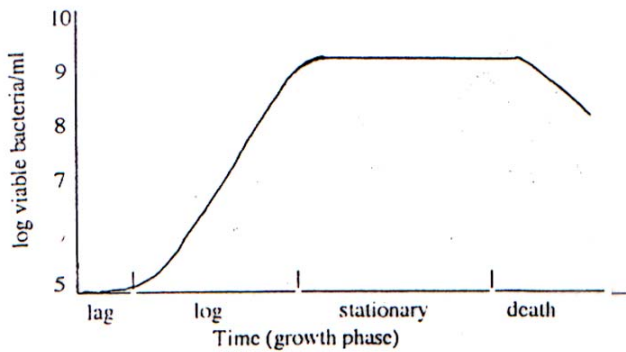


Figure 1. Bacterial Growth Curve

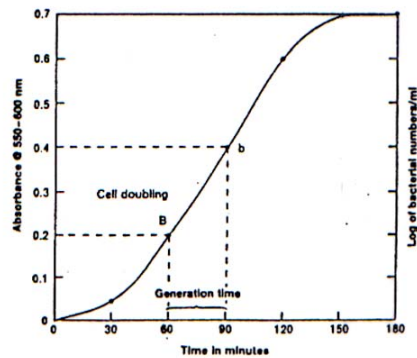


Figure 2. Determination of Generation Time

In this lab you will estimate numbers of bacteria by the two most widely used methods: viable plate count and spectrophotometric analysis. In liquid culture, the medium appears more and more cloudy as the bacteria increase in number by division. A tube of bacteria will tend to reflect light so that less light is transmitted through the tube. A spectrophotometer can measure the amount of light passing through the tube, or conversely the amount of light absorbed. These measurements of turbidity or optical density (OD) are not direct measurements of bacterial numbers, but an indirect measurement of cell biomass that includes both living and dead cells.

As the bacterial cell population increases, the amount of transmitted light decreases, increasing the absorbance reading on the spectrophotometer. (Fig. 3,4). If one takes readings of the same culture over time, the absorbance readings will increase as the cell number increases.



Figure 3. Flask on left contains sterile medium; flask on right medium inoculated with *E. coli* the day before. Note the turbidity in second flask due to bacterial cells.

This can then be graphed to show the growth curve for the particular conditions being tested. There are some limitations with this method, though. A growth curve that includes the lag, log, and stationary phase will take several hours to complete and the relationship between cell number and absorbance will begin to deviate from linearity at high cell densities. Generally an absorbance reading or O.D. of 0.8 is about as high as one should try to measure. To give you an idea of how the turbidity measurements

correspond to actual numbers, more than a million cells /ml need be present in order to get even a trace of a measurement on the spectrophotometer.

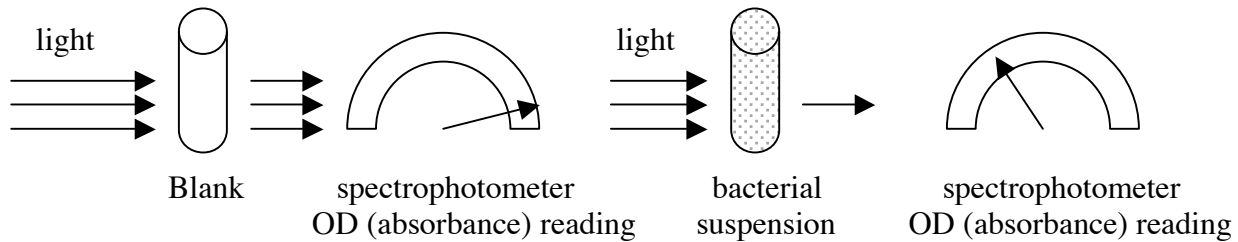


Figure 4. Spectrophotometric determination of cell densities

To quantify viable cells a plate count is done. A sample of bacteria is diluted in a sterile medium until the numbers are very low. This diluted sample of bacteria is then transferred onto an agar plate and spread out evenly so that each cell is separate from the others. Each viable cell will continue to divide into a discrete **colony** of millions of bacterial cells which can now be seen with the naked eye. These colonies can then be counted. Keeping in mind that each colony arose from a single cell that was plated onto the agar, the number of colonies can be used to determine the number of bacterial cells present in the original culture.

For this portion of the lab you will use *Escherichia coli*, a bacterium which is found by the hundreds of grams in the human lower digestive track. Of all microbes, *E. coli* is probably the most utilized by biologists and biochemists. It has fairly simple growth requirements and a rapid growth rate making it possible to observe its growth curve in one laboratory period.

B. Gram Stain

Most bacteria are characterized by having not only a cell membrane but also a cell wall which lies outside of the cell membrane. This cell wall is composed mostly of peptidoglycan and helps to maintain osmotic pressure and the cell's characteristic shape. Some taxonomic groups of bacteria also have an outer membrane that is attached to the peptidoglycan by small lipoprotein molecules (Fig. 5). This difference in outermost cell structure is the basis for classification of bacteria by a differential staining technique known as the Gram stain. **Gram-positive** cells (those without an outer membrane) stain purple in the procedure, **gram-negative** cells (which have the outer membrane) stain red or pink. The usual first step in any bacterial identification is the determination of whether or not it is a G+ or G- bacterium. A sample of the bacteria in question is first stained with the primary cationic dye crystal violet. Since most bacteria carry a net negative charge at pH 7 they pick up the dye. At this point morphological features such

will diffuse into the agar medium. The concentration decreases the further away from the disk. If the antimicrobial has an effect on the bacteria, a clear zone of inhibition will form around the disk. The larger the zone, the more effective the chemical is at preventing bacterial growth. This is known as the Kirby-Bauer method of anti-microbial testing. In this lab you will test the antimicrobial effect of a number of chemicals on a gram-negative and a gram-positive microbe.

D. Sterile technique

The handling of bacterial cultures requires aseptic (sterile) techniques in order to avoid contamination of your experimental bacterial cultures from the millions of microorganisms present in the surrounding environment and to prevent contamination of you and your lab space by the culture you are using. These procedures (Appendix 2) should ideally be done in a bacteriological hood, but in this lab, since we are starting out with a huge inoculum, the experiment should be fine if the procedure is closely followed, without the use of a hood.

Laboratory Procedure

A. Growth Curve

1. Each group of students will receive a 125 ml flask containing 50 ml of nutrient broth (pre-warmed to 37°C--why?). The lab instructor will then add *E. coli* that is in "log phase" (exponential growth phase) to each flask.
2. Swirl the flask so there is an even suspension of bacteria. Pipette 3.0 ml out of the flask into a cuvette (this will be your 0 time point, be sure to record the time). Replace the cap and put flask back in 37°C incubator (the culture is shaken to keep it mixed and aerated). Dispose of pipets in the appropriate container.
3. Read absorbance of 0 time point –
 - a. Set the wavelength at 660 nm.
 - b. "Blank" the spectrophotometer with a cuvette containing nutrient broth (why this?)
 - c. Read the absorbance of the culture. Record. Discard the material in the cuvette in the appropriate container.
4. In order to obtain a good growth curve you should **take 4 more turbidity (absorbance) measurements, roughly one every 20 minutes** following steps 3a-c above. Try to do them as quickly as possible. To avoid cooling the culture take out the 3 ml needed for an absorbance reading and immediately return the stoppered flask to the 37°C incubator, then take reading.

B. Plate Count – Serial Dilution

Look at the flow sheet (Appendix 3) before proceeding with the dilution.

1. At each table will be a set of four dilution tubes along with a flask of sterile saline (0.85% NaCl). Using one pipette tip for the series, add 9.9 ml of saline to tubes #1 and # 2, and 9.0 ml to tube #3 and #4.
2. Label the bottom of 3 petri dishes with the date, some identifying name, and A,B or C.
3. At one of the time points for the turbidity measurements (*in this case where the O.D. is between 0.08 and 0.1*) remove **0.1** ml from your growing culture and add it to the first tube of your dilution series. Thoroughly mix.
4. Remove **0.1** ml from dilution tube #1 and deposit it into tube #2. Mix thoroughly.
5. Remove **1.0 ml** from tube #2 and deposit the entire 1.0 ml into tube #3. Thoroughly mix. With the same pipette tip deposit **0.1 ml** from tube #2 onto the appropriately marked agar dish. Spread the bacteria evenly over the entire surface of the agar with a sterile loop and replace the cover. Dispose of loops in designated container.
6. Remove **1.0 ml** from tube #3 and deposit the entire 1.0 ml into tube #4. Mix. With the same pipette tip deposit **0.1 ml** from tube #3 onto another petri dish. Spread as directed above.
7. Deposit **0.1 ml** from tube #4 onto a third plate. Spread. Dispose of all pipets and loops in appropriate containers.
8. **If reading through this has confused you, look at the flow sheet - Appendix 3**
9. After approximately 10 minutes turn the petri dishes upside down to prevent condensation from falling on the agar. Why? Place plates in 37°C incubator.
10. The following day come to lab and count the bacterial colonies on the **one** plate with 30-300 colonies. **Do NOT open plates.** Dispose of plates in appropriate container.

C. Gram Stain

1. Obtain a clean glass slide
2. Prepare a smear of each organism to be stained (*Escherichia coli* and *Bacillus cereus*) by taking a loopful of the bacteria (with a sterile yellow loop) and spreading it over a small area in the center of the slide. Be sure you keep track of which organism you put on each slide.
3. Allow the smear to air dry and then heat fix by passing the slide quickly through a flame.
4. Place the slide on paper towels and add a drop or two of crystal violet to the smear, let set 1 minute
5. Gently wash the stain off with tap water, being careful not to wash off bacteria.
6. Apply Gram's iodine, let set 1 minute
7. Gently wash the iodine off with tap water and then add the decolorizing agent (95% EtOH) drop by drop until it runs clear
8. Wash off the decolorizing reagent with tap water
9. Counterstain with safranin by adding 1-2 drops and let it set for 45 seconds

10. Rinse with tap water and look at under the microscope. Determine if bacterium is Gram + or-.

D. Antimicrobials

1. Obtain two nutrient agar plates. Label bottoms with date, name, and type of bacteria (use the same two types of bacteria as you used for the Gram stain, *E. coli* and *B. cereus*).
2. Add 0.5 ml of bacteria to the agar plate and spread with a sterile loop. Be sure to dispose of pipet tip and loop in the appropriate container.
3. Determine which antimicrobials you want to test. There are disks that have already been saturated with antibiotics. If you want to use antiseptics and disinfectants you will need to soak sterile disks in the material you want to test (pick up a disk with forceps and place into liquid to be tested until it is saturated). **Use the same antimicrobials for both types of bacteria so you may do a comparison between gram positive and gram negative bacteria.**
4. Number either on the bottom of the plate or the side 1-6.
5. Place saturated disks onto agar plate (fig 6)
6. Place plates into 37^o incubator.
7. Come in *tomorrow* and record the zones of inhibition for each substance tested (fig 7). **Do NOT open plates.** When all data are collected dispose of plates in appropriate container.

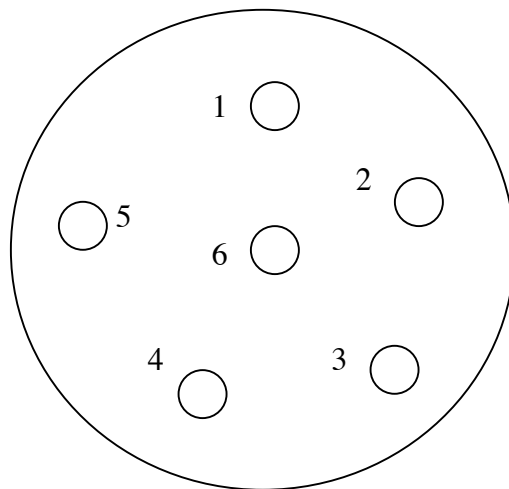


Figure 6. Place 6 disks (each containing a different antimicrobial) evenly spaced on the agar as above.

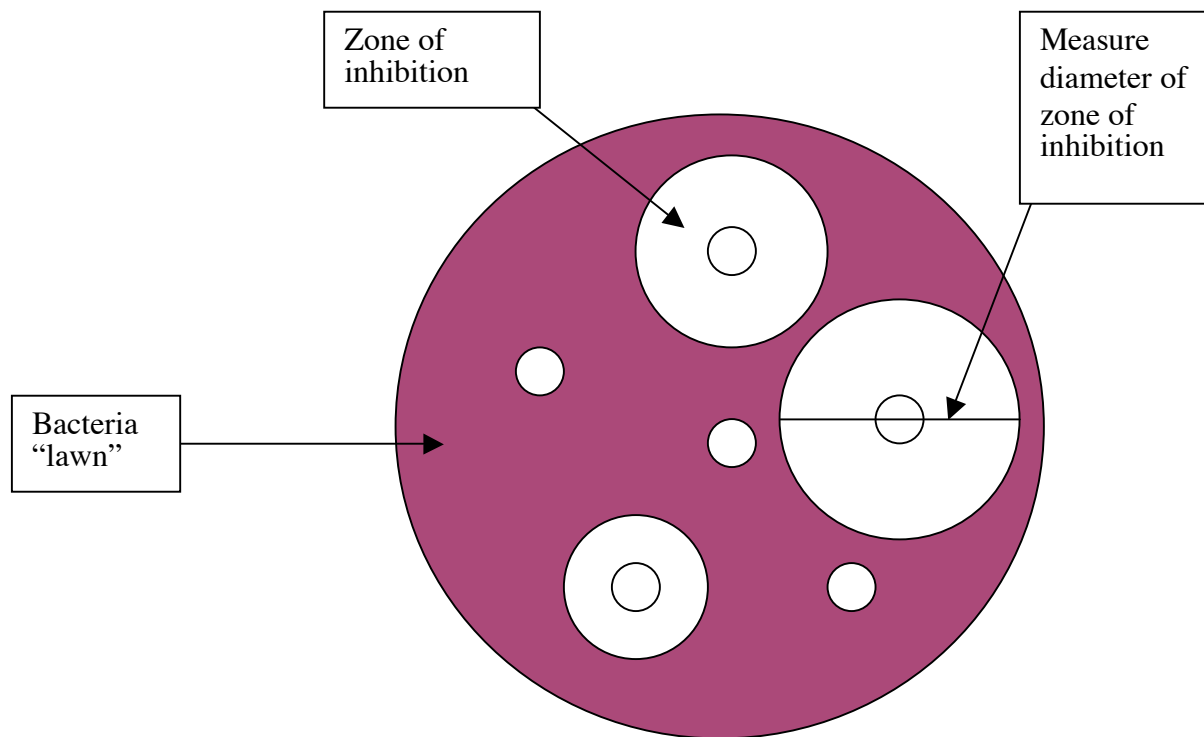


Figure 7. Collecting data from Kirby-Bauer antimicrobial experiment

E. Data Work-Up

For this lab you will need to write only a data sheet. Be sure to include the following, appropriately labeled:

1. Growth curve of *E. coli*. Use Excel or another computer graphing program (plot the absorbance on the y-axis vs. time on the x-axis, this needs to be a semi-log plot to get a straight line).
2. Doubling time of *E. coli*. Determine the doubling time from your graph
3. Plate count of *E. coli*. Be sure to state which plate in the dilution series you counted
4. Determine the concentration of bacteria in your culture flask at the time your sample for the serial dilution was taken. **Show all calculations.**
5. Gram stain results
6. Results of your antimicrobial tests on *E. coli* and *B. cereus* - include a table or figure and briefly **state** your results

Appendix 1.

Agents Used to Control Microbial Growth

A. Antiseptics and Disinfectants

1. Phenols and phenolics - these compounds inactivate proteins, denature enzymes, and injure plasma membranes and should only be used on surfaces. Examples include Lysol, hexachlorophene, and pHisoHex.
2. Halogens – may be used on surfaces either alone or as components of organic or inorganic solutions to inactivate enzymes and other cellular proteins. Tend to be strong oxidizing agents. Iodine combines with the amino acid tyrosine, chlorine when added to water forms hypochlorous acid. Betadine is another example often used instead of iodine.
3. Alcohols – denature proteins and dissolve lipids. Examples include ethanol and isopropanol.
4. Heavy metals – such as silver, mercury, copper, and zinc exert their influence through oligo-dynamic action such as combining with the sulfhydryl (-SH) groups and denaturing proteins. Examples include silver nitrate, mercurochrome, and copper sulfate.
5. Surface active agents – soaps and detergents decrease the tension between molecules that lie on the surface of a liquid
6. Quaternary ammonium compounds (quats) – cationic detergents attached to NH_4^+ disrupt plasma membranes, denature proteins, and inhibit enzymes. Examples include Cepacol and Zephran.
7. Organic acids – used in the food and cosmetic industry to prevent growth of microorganisms. Examples include sorbic acid, benzoic acid, and propionic acid.
8. Aldehydes – formaldehyde and glutaraldehyde attach methyl or ethyl groups to DNA and proteins making them nonfunctional.

B. Antibiotics

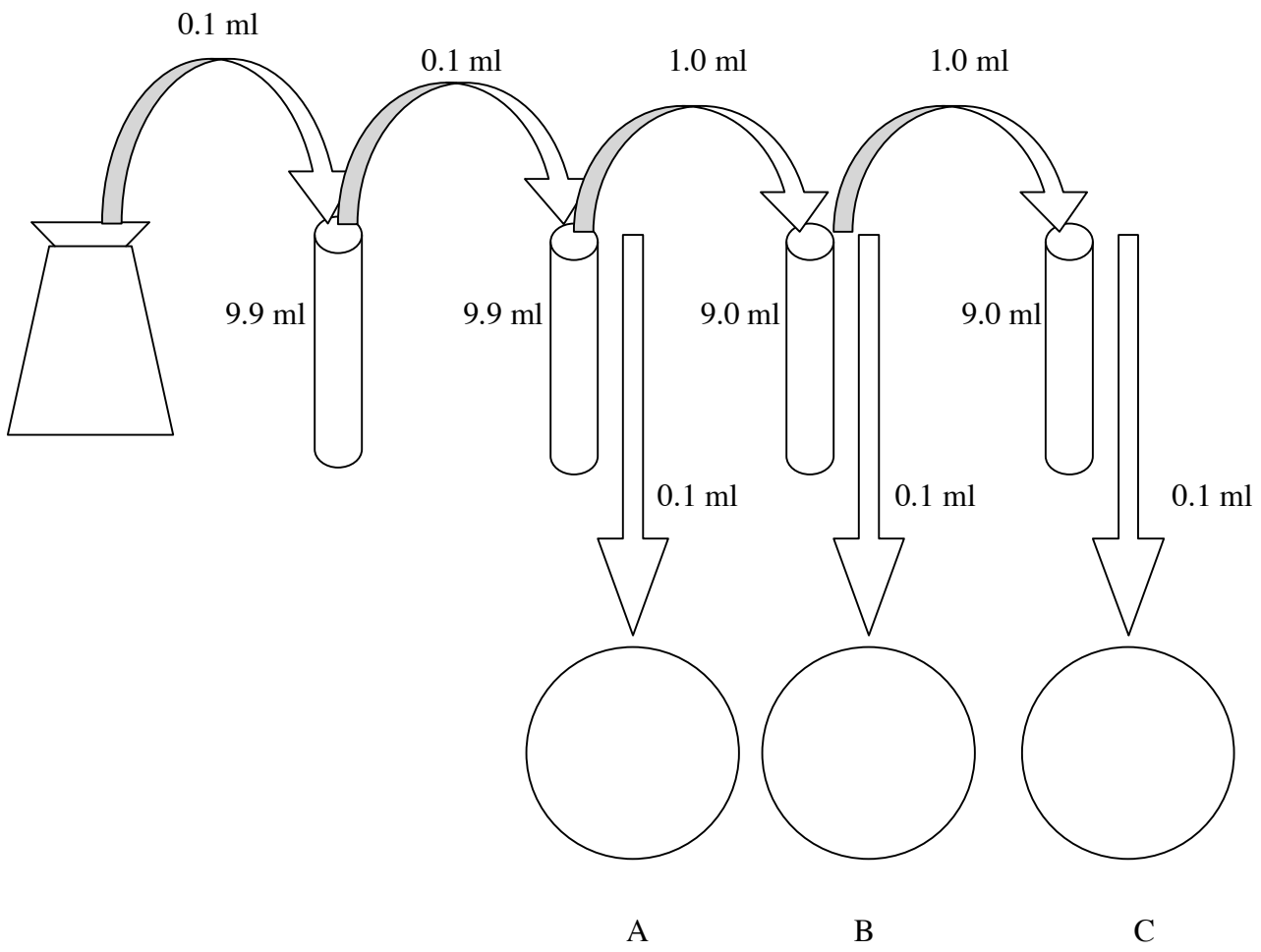
1. Inhibition of cell wall synthesis – may inhibit synthesis of peptidoglycan. Include penicillins, cephalosporins, vancomycin, bacitracin, oxacillin, and nafcillin
2. Damage to plasma membrane – polymyxin B, nystatin, and amphotericin B
3. Inhibition of protein synthesis – streptomycin (causes misreading of codons on mRNA), chloramphenicol (prevents peptide bond formation between amino acids), tetracyclines (prevents hydrogen bonding between anticodon on tRNA-aa complex and codon on mRNA), kanamycin, erythromycin, and gentamicin
4. Inhibition of nucleic acid synthesis – rifamycin, actinomycin D, nalidixic acid, ciprofloxacin, and norflaxacin
5. Structural analogs – such as sulfonamides that are structurally similar to cellular metabolites and compete with these in enzymatic reactions

Appendix 2

Aseptic Techniques to be Used in this Lab

1. Before handling of cultures:
 - a. Remove all unnecessary items from workspace.
 - b. Be sure your hair is tied back
2. Before and after handling cultures:
 - a. Wash your hands
 - b. Disinfect your area
 - c. Sterilize instruments
 - d. Dispose of all contaminated materials in appropriate containers
3. While working with cultures:
 - a. Do not talk
 - b. Never lay caps or covers on the bench tops
 - c. Open petri dishes only when adding and/or spreading bacteria. Tilt the petri dish lid to form a barrier between the culture and you
 - d. Work quickly
 - e. Use sterile pipets right out of the package (don't use one that has been on the lab bench)
 - f. Only take the caps off the dilution tubes or bacterial cultures when pipeting material either into or out of containers. Do not leave caps off for any longer than necessary

Appendix 3 - Serial Dilution



Extensions

Examples of possible experiments/research questions:

1. What is the effect of UV light on microbial growth? The variable could be exposure time, UV wavelength, distance from the UV source, bacterial species, or the SPF of sunscreens.
2. Does a particular substance, such as a spice or other household product, have antimicrobial activity against one of these species of bacteria? Variables could be exposure time, concentration, brand of the same type of antimicrobial product, or different types of spices.
3. Efficacy of various antimicrobials such as antibiotics or household chemicals. The variable could be concentration, brand, or exposure time.
4. Are both species of bacteria (one G+, one G-) equally susceptible to the antimicrobial activity of some substance?
5. What physical factors (temperature, pH, osmotic pressure) affect the growth rate of these bacteria?

Possible Design Methods:

1. Adding test materials to solid media

You may want to design your experiment to include the use of agar plates containing antimicrobials such as spices or food preservatives. These plates will need to be made up ahead of time (at least several days in advance) to allow for preparation and hardening. I would suggest making up 50 ml of nutrient agar (1.15 g nutrient agar/50 ml dH₂O) and then adding the spices or preservatives. For example, if you wanted to test a 2% concentration of cloves you would add 1.0 g of cloves to the flask containing 1.15 g of nutrient agar. If you weigh out the nutrient agar and spices and also label the plates, I will autoclave (sterilize) the solutions and pour the plates for you. The plates may be kept several days once they've hardened. Don't forget to make up control plates of just nutrient agar.

When you are ready to do your experiment I would suggest diluting the stock bacterial cultures a thousand-fold and then plating 0.1 ml onto the plates. If you decide to test bacteria isolated from food, such as hamburger or fish, I would suggest weighing out 1 g of the foodstuff and adding it to 10 ml of sterile water in a sterile tube, shaking the tube vigorously to dislodge the bacteria, and then plating 0.1 to 1.0 ml onto your plates.

2. Growing bacteria in liquid media or adding test materials to liquid media

If you want to test the effect of temperature, pH, salt or sugar concentration, or some antimicrobial product on microbial growth you may want to design your experiment to include growing bacteria in culture and taking growth curve measurements. Please keep in mind there is limited incubator space so each group may have 4, possibly 5 culture flasks. These numbers include the flasks needed for the appropriate controls. Be sure to think carefully about what the controls should be, what the “blank” for the spectrophotometer should be in each case, and the number of replicates.

3. Exposing bacteria to antimicrobials for short periods of time

If you want to test different concentrations of an antimicrobial, compare the effectiveness of different antimicrobials, or do a time course of exposure to an antimicrobial, you may want to use a microcentrifuge (microfuge) (Figures 1 and 2). I would suggest you pipet 1.0 to 1.5 ml of a concentrated bacterial suspension into microfuge tubes and then spin the tubes for 2-4 minutes at $10 \times 1,000$ rpm. This spinning will produce a pellet of bacteria (a very, very small pellet on the bottom side of the tube) and a supernatant of nutrient broth. The supernatant should be carefully poured out of the tube, taking care not to dump out the bacteria! The antimicrobial(s) can then be added to the bacteria pellets in the tubes and the bacteria re-suspended by vortexing. Don't forget the control (add nutrient broth instead of antimicrobial to one of the tubes). At the end of the exposure time the bacteria should be spun down in the microfuge again. To get rid of all traces of the antimicrobial, the supernatant should be discarded and the bacteria re-suspended in nutrient broth, spun down, the supernatant once again discarded and the bacteria finally re-suspended in nutrient broth. This re-suspended bacteria solution can then be plated out onto nutrient agar.



Figure 1. The types of microfuges available in the lab. The speed and spin time may be programmed into the centrifuge on the left. The centrifuge on the right has one speed and no timer.



Figure 2. A balanced centrifuge. Always be sure there is a tube directly across from each tube and the tubes contain the same volume of liquid.

4. Exposing bacteria to UV light

If you want to use UV light for your experiment we have several UV lamps (UVP, 8W Model UVLMS-38) you may use. Each lamp can be set at one of three different wavelengths – UVA (365nm), UVB (302 nm), or UVC (254nm). You probably should dilute the bacterial cultures around a thousand-fold and plate 0.1 ml of that dilution onto nutrient agar plates. Lids should be taken off the Petri dishes before exposure as the plastic lid blocks the UV rays. Plastic wrap does not block the UV rays so that may be used to cover the plates if your experimental design calls for the plates to be covered. Recommended exposure times would be in the range of 5 seconds to 5 minutes. You should wear safety goggles whenever using the UV light. Your control would be plates that are not exposed to UV light.