Week 5 : Exercise JS1

Growth of *E.coli* at 30°C and 37°C in nutrient broth in a closed system: use of turbidity and viable counts to measure growth: (Read introduction to section 6 and Exercise 6.1 from LeBoffe and Pierce (customized for UTSC) as well as chapter 5 Custom Pearson text .before coming to lab.

- You require a flow chart outlining (preferably diagrammatic) this laboratory. It must be initialed by your TA to enter the lab..
- Your TA will indicate whether you will be collecting data for growth at 30°C or 37°C. You will be provided with the other temperature data for your write up.

Concepts:

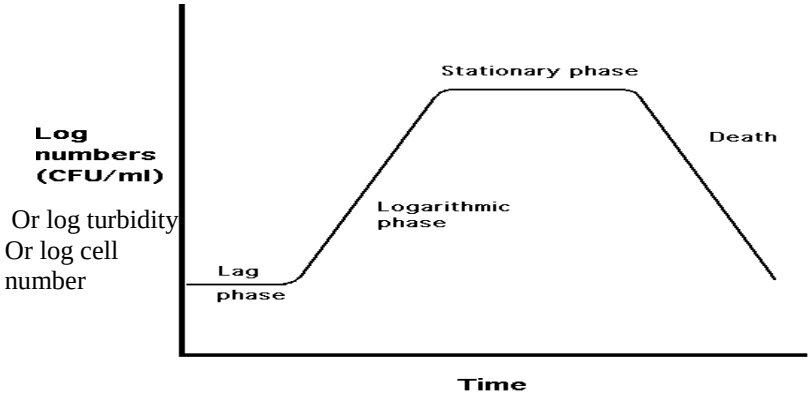
The bacterium *E. coli* can be easily grown and maintained in liquid or solid medium. Under condition of optimal growth, these bacteria may reproduce by binary fission at an average rate of once every 20 minutes. This rate can be maintained for only a short period (up to approximately 3 hrs). When the bacteria are inoculated into fresh culture medium these cells pass through four phases of growth

Lag phase: initial phase during which there is intense metabolic activity but little of no cell division (multiplication). The length of the lag is dependent on the age of the starting culture, the source (temperature and media conditions) of the culture and the new media conditions

Logarithmic phase: period when the organisms grow at a maximum rate, increasing by geometric progression as a result of reproduction by fission medium. At this time, if the logarithms of the number of bacteria present are plotted against time, a straight line is obtained

Stationary phase: the plateau period during which time the bacteria are dying at the same rate as they are dividing

Decline phase: when the growth rate declines steadily and cell death exceeds the rate of cell division



Hypothetical bacterial growth curve.

Most experiments including those used in recombinant DNA experiments are carried out on log phase bacteria. Therefore the investigator must know the concentration of bacterial cells per ml at a given time (i.e. must know the rate of growth of the bacterial culture). The **two basic methods** used to determine using a direct method the number of cells in a culture are by:

1) **total count (direct count or microscope count which measures all the cells in the culture both viable and non-viable)** and by **viable (plate) count**. The viable (a viable cell is actively dividing) count is determined by spreading appropriate dilutions of a bacterial culture onto solid medium and allowing the colonies to grow at the appropriate temperature. Since each **viable cell** will produce **one colony**, the concentration of bacteria in the original culture can be readily calculated. However, since it takes 8 to 16 hrs to form a visible colony this method is impractical for rapid determination of cell concentration. Therefore, it is routine to relate viable counts and/or direct counts to turbidity, which is determined in a spectrophotometer. Direct methods can determine cell number on culture concentrations below 10^7 cells/ml.

2)Turbidity of a culture is a function of the cell mass/unit volume of the culture. Approximately **10^7 bacterial cells per milliliter** must be present in a bacterial culture to detect turbidity. Turbidity is due to light scattering and is best measured at wavelengths where the ratio of absorbance to light scattering is low (wavelength between 450 and 600 mμ). Both viable and non-viable and non-viable cells scatter light, so turbidty measures the total cell population. **Turbidity** is considered an **indirect method** as the turibity measurement does not directly measure the number of cells. Changes in cell mass can be followed by measuring turbidity. Since different strains of bacteria have different cell sizes and cell size varies with physiological conditions the turbidity of a culture **can only be used as an estimate of cell concentration after turbidity** has been calibrated against cell number (viable count). The calibration is only valid for a strain in which the culture conditions are identical and only when the culture is in exponential phase. Once turbidity has been calibrated against cell number, turbidity can be used to measure growth (without viable counts) as long as the conditions of growth are identical.

Logarithmic growth

Growing bacteria, dividing by binary fission, exhibit exponential growth in a closed system until the culture reaches saturation at which point the cells enter stationary phase. During logarithmic growth the increase in the number of bacteria (N) per unit time (t) is proportional to the number of bacteria present in the culture. Often we are interested in the generation time of the bacterial culture, which is the time required for the cells to double in number within the logarithmic phase.

To measure growth over time and determine generation time, turbidity measurements or viable counts are made at intervals over several hours. This data is *usually plotted on semilog paper rather than converting each value to log and plotting on l*inear paper to measure growth. In this procedure the point at which the turbidity (read as

absorbance) or cell number doubles (within the log phase) represents the generation time of the culture. Therefore generation time can be read directly off the semilog graph by determining the time period in which cell number or turbidity doubles. **Generation time** can be calculated using the formulas described in exercise 6-5 of Leboffe and Pierce(2002) [provide number of generations per hour] without plotting the data (difficult to see trends without a graph). Alternatively, a derivation of the calculation described in Ex 6-5 can be used to calculate generation time from the plotted data. The slope of the exponential phase of the growth curve can be determined by converting the turbidity or cell number counts to log. Calculation used is **Generation time (GT) = 0.3/slope**.

Experimental procedure

1. Work in benches TA will assign for incubation temperature (30°C or 37°C)
2. Inoculate 80 ml of sterile nutrient broth (in a 250 ml flask) with an aliquot aseptically removed from a mid-log *E.coli* culture (use the canisters of sterile 5 or 10 ml pipettes). The exact volume to use will be provided to you by the TA. The starting OD_{550} should be around 0.05 (no lower than 0.03). Swirl the culture flask containing the freshly inoculated bacterial culture and immediately aseptically remove 5 ml of the bacterial culture and place in a spec 20 tube for the turbidity reading (subsequent procedure described in step 2 under the heading **turbidity).** This is time zero (please tell the TA your turbidity reading as soon as you obtain it so she/he can indicate whether the value is sufficient to continue the experiment). Once you have received the okay from your TA you can remove 0.1 ml from your flask for the zero time viable cell count (see step three under heading **viable count**). As soon as you have removed the samples for the time zero reading place the flask at 37°C or 30C depending on your assigned temperature in the shaking water bath (120 rpm).

Turbidity readings:

a) Prior to your first reading, zero the Spec 20 (550 mμ) using the left hand knob and then blank (using the right hand knob) the Spec 20 with 5 ml of sterile nutrient broth in a sterile Spec 20 tube (keep this tube for the entire experiment to blank the Spec 20). Repeat this procedure prior to reading the turbidity of each bacterial sample.

b) Remember the spec 20 sample tube etched line should line up with the line on the Spec 20 chamber.

c) Wipe the spec 20 tube containing the sterile media used for blanking and the tube containing the bacterial sample with a Kimwipe prior to placing the tube in the machine to remove all fingerprints that will alter the light scattering.

d) Turbidity readings are repeated every 15 min (0, 15, 30, 45, 60, 75, 90, 105, 120 min).

e) Immediately return the flask to 30°C or 37°C (make sure you know which temperature you are using) after removing the sample.

f) The spec 20 tube used for your 5 ml sample does not have to be sterile as you are not keeping this sample. However, you must remove the bacterial sample from the culture flask using aseptic technique. The 5 ml for the blank must remain free of bacteria so it must be removed from the appropriate flask containing sterile media using aseptic technique**.**

g) Record the turbidity readings in a table you construct. Hand in a copy of your readings to the TA before leaving.

h) **Plot turbidity versus time for both growth temperatures** on the semi-log paper provided**. Read generation** time off the graph. **Calculate generation time** from the graph by determining the slope of the line and using the GT calculation described above. These values should identical (or almost identical). Both methods of GT determination are required.

3. Viable counts:

a) Obtain three 9.9 ml sterile saline blanks and one 9 ml sterile saline blank per time point (twelve 9.9 ml tubes and four 9 ml tubes in total). The saline is in screw top sterile test tubes. Obtain 3 nutrient agar plates per time point (12 plates in total). Make sure your bench has canisters of sterile 5 or 10 ml pipettes and sterile 1 ml pipettes**.**

b) Viable counts are carried out every 30 minutes (0, 30, 60, 90 min).

c) Remove 0.1ml at the same time your remove the 5 ml for the corresponding turbidity reading. In this way at time zero and at 30 min intervals there after there will be both a viable count and turbidity reading, which will allow for the calibration of turbidity against cell number. In order to do the viable counts you will carry out a serial dilution of the growing culture.

d) Immediately aseptically place the 0.1 ml into a 9.9 ml saline blank. This is a 10^{-2} dilution. Agitate the 10^{-2} dilution to ensure good mixing.

e) Aseptically remove 0.1 ml of the 10^{-2} dilution into the second 9.9 ml saline blank. Mix by agitation. This is a 10^{-4} dilution

f) Aseptically remove 1 ml of the 10^{-4} dilution into the 9 ml saline blank. Mix by agitation. This is a 10^{-5} dilution.

g) Aseptically remove 0.1 ml of the 10^{-4} dilution into the third 9.9 ml saline blank. Mix by agitation. This is a 10^{-6} dilution

h) Label the bottom of three plates with your name, time point and dilution plated (write down the final dilution on the plate) Aseptically transfer 0.1 ml of the 10^{-4}, 10^{-5} and 10^{-6} dilution to each of three plates (final serial dilution on plates: 10^{-5}, 10^{-6} and 10^{-7}). Use proper aseptic technique and the L-rod with turntable to spread the 0.1 ml on each of the plates. Allow the inoculums to dry and place the plates in the incubator at 37°C for 16 to 24 hrs. The plates will be removed for you and placed at 4°C to prevent further significant growth.

i) **The following week** (or next lab period) you will count the number of colonies on each plate for each time period. Record all readings in a tabular format. When doing the colony counts you only count the agar plates with between 30 and 300 colonies. The plates with less than 30 or more than 300 colonies have to few or too many colonies to count. If your dilutions are done correctly only one plate will have the appropriate number of colonies.

j) **Plot cell number versus time for both growth temperatures** on the semi-log graph provided.

k) Read generation time off the graph. **Calculate generation time** by determining the slope of the exponential phase of the curve and using the GT calculation described above. **You must present GT in both ways**.

<u>Lab assignment</u>. Write an introduction for this lab that explains the term generation time and explains the objective of this lab. Write a brief conclusion comparing the calculated generation time for both temperatures with the expected generation time. Hand in with graphs described in section J and K for viable counts and section H in the turbidity section.

Review questions that you should be able to answer after completing this laboratory:

1. Define generation time
2. Is generation time calculated from any phase of the growth culture? Explain your answer.
3. What is occurring in the bacterial culture during lag phase?
4. What is occurring in the bacterial culture during the growth phase (log)
5. What is turbidity as it relates to a bacterial culture?
6. Does temperature affect growth rate