Background and References

Bacterial concentration can be measured by several methods, all of which you have studied previously in lecture or in lab. In this lab, you will be measuring bacterial concentration by four methods:

- **Turbidity:** Turbidity, or light scattering, is measured in a spectrophotometer such as a Spectronic 20. This method has the advantage of being the quickest method at the lab bench.
- **Viable cell count (colony count, or CFU/ml):** This is measured by serially diluting a culture, and then spread-plating the dilutions. The concentration of viable cells in the original culture (expressed as colony forming units per ml, or CFU/ml), is calculated from plates counts on the spread plates.
- **Wet mass:** A measured volume of a culture is centrifuged to pellet the cells, and then the mass (weight) of the cells is determined.
- **Dry mass:** A measured volume of the cells is centrifuged to pellet the cells. The pellet is dried, and the mass (weight) of the dry pellet is determined.

For your background, references, and your basic standard procedures you can refer to Unit 8 of the 3340 lab manual (*Basic Concepts of the Microbiology Laboratory*). You can also reference your textbook (Prescott, Harley, and Klein). Be aware that a thorough and well-written background and procedure is expected as part of your grade for this class.

**Background: Making a corrected absorbance curve for *Escherichia coli***

In the background section of your lab writeup, you will review the basic theory of the spectrophotometer and how it can be used to measure bacterial concentration (from the 3340 lab manual). In this lab, we are extending our use of turbidity by correcting for deviations from linearity at high absorbance values. You may paraphrase, summarize, and include the following discussion in your background.

Central to the idea that the spectrophotometer can be used to measure bacterial concentration is this relationship:

\[ \text{Bacterial concentration} \propto \text{Absorbance measured with the spectrophotometer} \]

This says that the bacterial concentration is directly proportional to the absorbance read on the machine. It means that if the bacterial concentration is doubled, then the number on the machine should double as well. That relationship hold true only up to a particular absorbance value. After that, the culture becomes so turbid that the relationship between bacterial concentration and absorbance begins to deviate from linearity. That means that if you read the absorbance at a value higher that the linear range, the actual bacterial concentration is higher than what you are measuring. This is shown in the data listed in the following table and plotted in the following graph. The data were graphed and analyzed using the Microsoft Excel spreadsheet.
<table>
<thead>
<tr>
<th>Dilution #</th>
<th>Volume of Turbid Culture, ml</th>
<th>Volume of Sterile Broth, ml</th>
<th>Dilution Factor</th>
<th>Measured Absorbance at 425 nm</th>
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</thead>
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<tr>
<td>1</td>
<td>10.0</td>
<td>0.0</td>
<td>1</td>
<td>1.092</td>
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<tr>
<td>2</td>
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<td>1.0</td>
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<td>0.998</td>
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<td>21</td>
<td>0.0</td>
<td>10.0</td>
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<td>0.000</td>
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</tbody>
</table>
Absorbance Correction

In this experiment, a turbid broth culture of *E. coli* was diluted by varying amounts. The dilution factor equals the volume of the culture in the dilution divided by the total volume of the dilution. For example, 8 ml of the culture mixed with 2 ml of sterile broth gives a dilution factor of 0.8. The “0” dilution was the sterile broth with which the spectrophotometer was blanked. The absorbance at 425 nm of each dilution was measured. *If the bacterial concentration were proportional to absorbance for the entire range of concentrations in this dilution series, then the relationship would be linear for the entire range. Instead, linearity is only seen up to an absorbance of approximately 0.5.*

From this data, it is possible to derive a correction factor to mathematically correct for the deviation from linearity. The correction factor will allow us to make measurements at absorbance values above the linear range, then calculate a corrected absorbance value that is truly proportional to the bacterial concentration. To do this, we first need to fit an equation to the entire data set. A “best-fit” trendline can be added to the data using Microsoft Excel. In Windows, this is accomplished by right-clicking on the data points and selecting “Add Trendline,” then selecting “Polynomial” under “Type” of trendline. This data fits well to a second order polynomial equation (a good old quadratic equation). The equation for this curve (also shown on the graph) is

\[ y = \text{Uncorrected absorbance} = A_U = -0.449x^2 + 1.5305x \]

\[ R^2 = 0.9994. \]

The value \( R^2 \) is the “correlation coefficient,” a statistical term that tells how “good” the fit is; if the data fits the curve perfectly, then the \( R^2 \) value is 1. An \( R^2 \) value of 0.9994 indicates an excellent fit, as the picture of the graph also shows.
The next step in deriving our correction factor is to get a second equation for the linear part of the graph. The linear equation will serve as the basis of our corrected absorbance value. To do this, we plot the linear portion of the data on a separate graph.

The data are plotted in Microsoft Excel and a trendline is determined. In this case, we determine the best linear trendline to the data as the basis for our corrected absorbance:

\[ y = \text{Corrected absorbance} = A_C = 1.3807 \times \]

\[ R^2 = 0.9984 \]

Now we have two equations (a set of “simultaneous equations,” as the mathematicians say):

\[ A_U = -0.449 x^2 + 1.5305 x \]

\[ A_C = 1.3807 x \]

What we do now is to take the first equation and solve for \( x \) as a function of \( A_U \). Then, we plug \( x \) into the second equation and we have our correction formula.
To solve the first equation, we use the quadratic formula $\Theta$:

$$A_U = -0.449 \, x^2 + 1.5305 \, x$$

$$0 = -0.449 \, x^2 + 1.5305 \, x - A_U$$

$$x = \frac{-1.5305 \pm \sqrt{(1.5305)^2 - 4(-0.449)(-A_U)}}{2(-0.449)}$$

$$x = \frac{-1.5305 \pm \sqrt{2.3424 - 1.796 \, A_U}}{-0.898}$$

$$x = 1.7043 \pm \frac{\sqrt{2.3424 - 1.796A_U}}{0.898}$$

Like most calculations with a quadratic equation, we have to figure out whether the “plus” or the “minus” is the correct sign in front of the radical. This is easily done by taking a measured $A_U$ from the data at a specific $x$ value (dilution factor), plugging the $A_U$ into the equation, doing the calculations with the “plus” and the “minus” signs, and seeing which one gives the correct $x$ value (dilution factor). At a dilution factor ($x$ value) of 0.1, the measured $A_U$ was 0.145. Entering the value 0.145 for $A_U$ into the equation and calculating possible $x$ values using the “plus” and “minus” signs, we get:

Using “plus” sign: $x = 3.311$ (not close to 0.1)

Using the “minus” sign: $x = 0.0975$ (almost equal to 0.1).

Therefore, the correct sign in our calculation is the “minus” sign.

$$x = 1.7043 - \frac{\sqrt{2.3424 - 1.796A_U}}{0.898}$$

Having solved for $x$ as a function of $A_U$, now we can enter this into the second of the two equations to get our correction formula.

$$A_C = 1.3807 \, x$$

$$A_C = 1.3807 \left( 1.7043 - \frac{\sqrt{2.3424 - 1.796A_U}}{0.898} \right)$$

$$A_C = 2.3531 - 1.5375 \sqrt{2.3424 - 1.796A_U}$$

The graph on the next page shows the data set of measured absorbance values ($A_U$) plotted together with the corrected absorbance values determined from the formula.
There are several points to note in this analysis.

- In the range of linearity, there is no significant difference between the measured (uncorrected) absorbance values and the corrected absorbance values, as we would expect.
- The corrected absorbance values give an excellent linear fit ($R^2 = 0.999$) with the same slope as the linear region of the uncorrected absorbance values, again as we would expect.
- The formula is only valid for calculations on uncorrected absorbance values up to 1.3. At $A_U$ values just over 1.3, the term under the radical becomes negative, so an $A_C$ value cannot be calculated. This is not an issue, because a broth culture will rarely reach an absorbance as high as 1.3. Furthermore, an absorbance of 1.3 represents a percent transmittance of about 5%; in other words, a suspension so turbid that it is almost opaque. If one had to make an optical measurement of such a suspension, there would be no choice but to dilute the suspension, measure the absorbance (and correct if necessary), and multiply the absorbance times the dilution factor.
- The correction formula may be different for different bacterial species, or for the same species grown in different media or grown under different conditions. The data presented above were collected on *E. coli* grown in Minimal Davis broth medium. In this laboratory exercise, you will be determining a similar correction factor for *E. coli* grown in tryptic soy broth.
**Procedure: Making a corrected absorbance curve for *Escherichia coli***

Note: This may be used as written, except that you should include the table with the volumes in your lab notebook. I just didn’t want to have to put it down twice.

1. Be sure to wear gloves during this procedure, to avoid getting culture on your hands while transferring liquid from tubes.
2. Turn on a Spectronic 20 spectrophotometer and set the wavelength to 425 nm. Let it warm up for at least 15 min.
3. Obtain a turbid broth culture of *E. coli*, tryptic soy broth (TSB), 21 clean culture tubes (they do not need to be sterile), a clean Spectronic 20 tube, three 10-ml pipets, and a pip-pump.
4. Label the culture tubes 1 – 21.
5. Using different pipets, add turbid *E. coli* culture and TSB to each tube in the volumes shown in the table on page 2.
6. Mix each tube thoroughly by vortexing for 1 – 2 sec with a vortex mixer.
7. With no tube in the spectrophotometer, set the 0%T setting.
8. Transfer 3 – 4 ml of broth from tube 21 (TSB with no *E. coli*) to the Spectronic 20 tube, carefully wipe the outside of the tube with a kimwipe, place the tube in the machine, and set the 100%T setting (blank the spectrophotometer). Switch the spectrophotometer setting to “Absorbance.”
9. Remove the Spectronic tube and pour the broth back into tube 21. Carefully drain the last few drops from the lip of the Spectronic tube into a kimwipe. **Put the contaminated kimwipe in the biohazard bag.**
10. Starting with tube #20 and going backwards to tube #1, transfer 3 – 4 ml of each dilution to the Spectronic tube, wipe the tube with a kimwipe, place the tube in the spectrophotometer, and read the absorbance. After each tube, pour the broth back into its numbered tube and drain the last few drops into a kimwipe, as described earlier.
11. Place caps on the culture tubes, remove the labels, and place the tubes in the proper area for cleanup.
12. Transfer the data to a Microsoft Excel spreadsheet. The spreadsheet should contain the following columns: volume of *E. coli* culture, volume of broth, dilution factor, and measured absorbance.
13. In the spreadsheet, create an X-Y scatter plot of dilution factor vs absorbance. Note: All charts must be added as separate sheets in the workbook, not pasted into the worksheet with the data columns.
14. Add a second order polynomial trendline to the plot, and set the options on the trendline to show the equation and the correlation coefficient.
15. From the plot, identify the range in which absorbance is proportional to bacterial concentration.
16. Create a second plot, plotting only the linear range of dilution factor vs absorbance.
17. Add a linear trendline to the second plot, and set the options for the trendline to show the
equation and the correlation coefficient.

18. Using the equations from the first and second plot, derive a correction formula to correct for linearity at high absorbance readings.

19. Add another column to the spreadsheet: Corrected absorbance. The values in the “Corrected absorbance” column are calculated from the “Measured absorbance” column using the correction formula, entered in Microsoft Excel notation. For example, if the correction formula is

$$A_c = 2.3531 - 1.5375\sqrt{2.3424 - 1.796A_U}$$

then the formula entered in the spreadsheet cell is

$$=2.3531-(1.5375*SQRT(2.3424-(1.796*D7)))$$

where “D7” is the reference to the cell containing the “Measured absorbance” ($A_U$) value.

20. Create a third plot with two data series: dilution factor vs. measured absorbance, and dilution factor vs. corrected absorbance. The easiest way to do this is to copy the first chart (by right clicking on its tab), going to the copy of the chart, selecting the data points (by right clicking on them), selecting “Source data – Series,” clicking “Add series,” and setting the Y-value ranges on the new series to correspond to the “Corrected absorbance” column.

21. On the third plot, add a second order polynomial trendline to the dilution factor vs measured absorbance plot, and a linear trendline to the dilution factor vs corrected absorbance plot.
**Background: Calibrating absorbance measurements with viable cell counts, wet mass, and dry mass measurement**

In the background section of your laboratory writeup, you will review the basic concepts of the “viable cell count” technique, given in your 3340 laboratory manual (*Basic Concepts of the Microbiology Laboratory*), your textbook, and the lecture notes from this class. In this lab, we will determine the viable cell count (CFU/ml) of a turbid broth culture of *E. coli*, making the following modifications to the procedure from the 3340 lab manual.

- We will measure the absorbance of the *E. coli* culture before making the serial dilutions and plates. The measured absorbance will be corrected using the correction formula. After determining the CFU/ml from the plate counts, we will have established a “calibration factor” to convert absorbance ($A_C$) into CFU/ml.

- In two separate centrifuge tubes, we will centrifuge 10 ml of the turbid culture to pellet the cells. After discarding the broth, we will determine the wet mass (wet weight) of the cells, then we will allow the cells dry out to determine the dry mass. After determining the CFU/ml from the plate counts, we will have established a “calibration factor” to convert absorbance ($A_C$) into wet mass (gm wet cells/ml) or dry mass (gm dry cells/ml).

- We will plate duplicate plates of each dilution.

- Plates will be counted with valid counts in the range of 25 – 250 colonies per plate, according to the *FDA Methods of Bacteriological Analysis*. Also, we will round the counts to two significant figures after making our calculations.

- The CFU/ml in the broth culture will be determined by the formula

$$\frac{\text{CFU}}{\text{ml}} = \frac{\sum C}{[(1* n_1) + (0.1*n_2) + ...]*d_1* V}$$

Where:

- $C = \text{Sum of all colonies on all plates between 25 – 250}$
- $n_1 = \text{number of plates counted at dilution 1 (least diluted plate counted)}$
- $n_2 = \text{number of plates counted at dilution 2 (dilution 2 = 0.1 of dilution 1)}$
- $d_1 = \text{dilution factor of dilution 1}$
- $V = \text{Volume plated per plate}$
Procedure: Calibrating absorbance measurements with viable cell counts, wet mass, and dry mass measurement

Materials
1. Obtain a turbid broth culture of *E. coli*, 16 sterile culture tubes with caps, sterile tryptic soy broth, 32 tryptic soy agar plates, two Sorvall centrifuge tubes, a Spectronic 20 tube, three sterile 10-ml pipets, a sterile 1-ml pipet, pi-pumps, vortex mixer, and 16 sterile plastic disposable inoculating loops.

Determining the corrected absorbance of the *E. coli* culture
2. Warm up a Spectronic 20 spectrophotometer for at least 15 min and set the wavelength to 425 nm. With no tube in the machine, set 0%T. Blank the spectrophotometer (100%T) with sterile tryptic soy broth.
3. Transfer 3 – 4 ml of the *E. coli* culture into the Spectronic 20 tube, and measure the absorbance. Record the absorbance in your lab notebook.
4. Discard the used culture from the Spectronic tube into a waste container, and repeat the procedure to get a replicate reading. Record the absorbance, and average the two readings together to get the uncorrected absorbance ($A_U$) for the culture.
5. Using the correction formula determined in the earlier procedure, calculate a corrected absorbance ($A_C$) value for the culture.

Wet mass and dry mass measurements
6. With a laboratory marker, label the Sorvall tubes “1” and “2.” Note: the tubes may have already been permanently numbered; if so, just use the numbers on the tubes and make note of the numbers in your lab book.
7. Weigh each of the Sorvall tubes, and record their weights in your lab notebook.
8. Carefully measure 10 ml of the *E. coli* culture into each Sorvall tube.
9. Centrifuge the tubes at 5000 rpm for 10 min in the Sorvall RC-5 centrifuge.
10. Decant the supernatant into the waste container. Carefully blot the last few drops from the lip of the tube with a kimwipe. **Discard the kimwipe in the biohazard bag.**
11. Weigh the tubes with the wet cell pellets. Subtract the weight of tube+pellet minus tube alone to get the weight of the wet cell pellet.
12. Divide the wet weight of cells by 10 ml to get the concentration of *E. coli* in the broth culture, expressed in gm wet cells/ml. Do this for each tube, and average the two values.
13. Divide the value obtained in step 12 (gm wet cells/ml) by the value obtained in step 5 ($A_C$) to obtain the calibration factor to convert $A_C$ into gm wet cells/ml.
14. Place the open tubes, in a test tube rack, in a biosafety hood with the UV lamp turned on. Let the tubes sit opened for 2 days to dry out in the hood.
15. Weigh the tubes with the dry cell pellets. Subtract the weight of tube+pellet minus tube alone to get the weight of the dry cell pellet.
16. Divide the dry weight of cells by 10 ml to get the concentration of *E. coli* in the broth culture, expressed in gm dry cells/ml. Do this for each tube, and average the two values.
17. Divide the value obtained in step 16 (gm dry cells/ml) by the value obtained in step 5 ($A_C$) to obtain the calibration factor to convert $A_C$ into gm dry cells/ml.
**Viable cell count measurements**

18. Label the sterile culture tubes $10^{-1} - 10^{-16}$.
19. Carefully add 9 ml of TSB to each culture tube.
20. Label two TSA plates $10^{-1}$, two $10^{-2}$, etc., through $10^{-16}$.
21. Using a 1-ml pipet, transfer 1 ml of the *E. coli* culture to the $10^{-1}$ tube. Do not discard the pipet.
22. Mix the dilution thoroughly by vortexing 1 – 2 sec.
23. Rinse the pipet 3 – 4 times in the $10^{-1}$ dilution, then carefully withdraw 1 ml of the $10^{-1}$ dilution with the pipet and transfer it to the $10^{-2}$ tube. Note: the purpose of the “pipet rinsing” step is to completely “coat” the inside of the pipet with the current dilution so the same pipet can be used for each dilution in the series with negligible error. Otherwise, the inside of the pipet would be coated with the previous dilution, which is tenfold higher than the dilution we are pipetting.
24. Mix the dilution, rinse the pipet, and continue the procedure until all the serial dilutions have been made.
25. Discard the pipet in the appropriate used glass container.
26. Using a new 1-ml pipet and beginning with the $10^{-16}$ dilution, pipet 0.1 ml of the dilution onto an appropriately labeled TSA plate (do not discard the pipet), and immediately spread the drop with a plate spreader formed from a plastic inoculating loop. Note: the spreader can be made by bending the needle portion of the loop into an “L” shape in the sterile lid of the petri dish.
27. Repeat this procedure with the duplicate $10^{-16}$ plate. Do not discard the pipet. Note: the reason for starting with the $10^{-16}$ dilution is so the same pipet can be used for all the dilutions.
28. Rinse the pipet 3 – 4 times with the $10^{-15}$ dilution, and repeat the plating procedure (steps 26 – 27).
29. Incubate the plates at $37^\circ$C for 48 hr.
30. Count the colonies on plates having 25 – 250 colonies.
31. Determine the CFU/ml in the broth culture by the formula

$$\frac{\text{CFU}}{\text{ml}} = \frac{\sum C}{[(1 \ast n_1) + (0.1 \ast n_2) + \ldots] \ast d_1 \ast V}$$

where:
- $C =$ Sum of all colonies on all plates between 25 – 250
- $n_1 =$ number of plates counted at dilution 1 (least diluted plate counted)
- $n_2 =$ number of plates counted at dilution 2 (dilution 2 = 0.1 of dilution 1)
- $d_1 =$ dilution factor of dilution 1
- $V =$ Volume plated per plate
32. Divide the value obtained in step 31 (CFU/ml) by the value obtained in step 5 ($A_c$) to obtain the calibration factor to convert $A_c$ into CFU/ml.