Technical Overview

Monitoring Growth of Suspension Cultures in Microplates

Using light scatter to kinetically record changes in cell number

Introduction

Bacteria and yeast are microorganisms of great economic and medical importance. Much of our understanding of bacterial and yeast life cycles stems from monitoring their proliferation over time. Typically, this is accomplished using optical density (OD) measurements (Figure 1). The applications of such measurements range from routine checks for the generation of competent bacteria for cloning[1], studying cellular physiology and metabolism[2,3], determining the growth rate for antibiotic resistance[4,5], and monitoring of biomass accumulation during fermentation[6], to name a few.

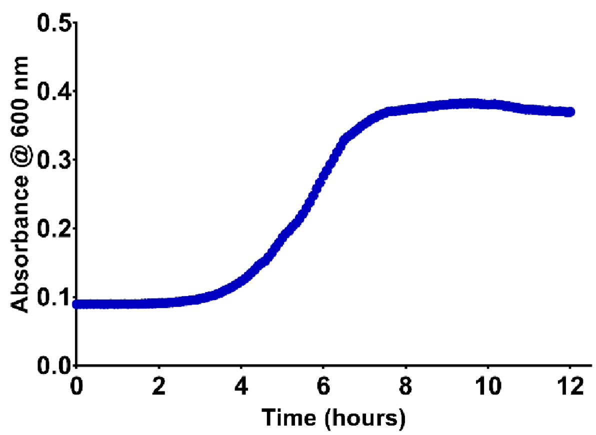

Figure 1. Typical absorbance curve of bacterial growth. Media conducive to growth, when inoculated with a low concentration of bacteria, will demonstrate an increase in measured absorbance with time as a result of an increase in the number and size of the bacteria. When nutrients become exhausted, growth slows and eventually stops. Data were generated using an Agilent BioTek LogPhase 600 microbiology reader, measuring absorbance at 600 nm every 2.5 minutes for 12 hours with constant shaking between reads.

Growth of single-cell organisms in suspension culture can be monitored using turbidity or light scattering measurements. As the number of cells increases, the solution becomes increasingly cloudy or turbid because light passing through it is scattered by the microorganisms present.[7] While not obeying Beer's law, as light scattering increases, the percentage of the total light beam reaching the detector diminishes and is recorded as absorbance (Figure 2).

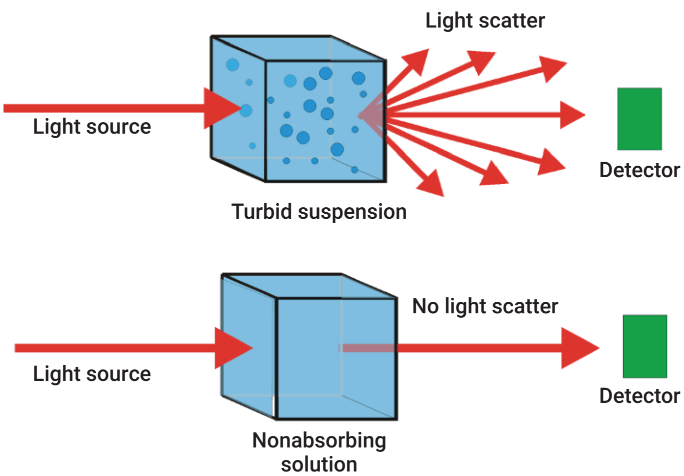

Figure 2. Basis of 600 nm turbidimetry measurements. Unlike true absorbance, the light is not absorbed, but rather the molecules within the cell diffract incident light as a result of changes in refractive index and density as light passes from the buffer to the cells. Some of the diffracted light will be deflected away from the optical path to the detector and be recorded as optical density by the reader. The degree of light loss due to light scatter is influenced by both the suspended particle, as well as the configuration of the instrument optics.

Quantitation of cell suspensions based on light scattering was first described by Lord Rayleigh around 1900. The light is not absorbed, but rather the molecules within the cell diffract incident light. Much of the diffracted light will be deflected away from the optical path to the detector and be recorded as optical density by the reader. The degree of light loss due to light scattering is influenced by both the suspended particle, as well as the configuration of the instrument optics. Cellular-specific effectors include: cell size, membrane make-up, interior cellular organelle anatomy, and cell density.[8] Instrument optical dimensions such as the distance between the absorbing material and the detector, the presence or absence of focusing lenses, and beam size all influence the "light scatter" signal.[9]

These light scatter measurements are typically made using a light source on or near 600 nm. The use of 600 nm wavelength is based to some extent on a historical wavelength, when microbiologists used the simple KLETT-Summerson colorimeter developed in 1939 and popular into the 1960s with fixed filters (red, green, and blue) without having the possibility to adjust the wavelengths.[10] The blue (600 nm) filter had the added advantage of providing low energy light that was not deleterious to the biologic sample and having low interference in most bacterial broth mixtures.

Most bacteria and yeast scatter light only a few degrees[11], so the distance from the cell to the detector and the radius of the focusing aperture will determine the degree of light loss at the detector (Figure 2). As the number of cells increases, the probability of incident light being scattered by particles multiple times also increases, often referred to as a multiple scattering regime. With this phenomenon, the Beer-Lambert law is no longer a suitable approximation, and OD curve is expected to flatten (Figure 3). For most yeast and bacteria applications, OD_{600} readings above 0.5 are no longer truly linear with respect to concentration. Comparison of different samples on the basis of OD values can easily be performed in relative terms, but to calculate cell numbers for organisms some sort of calibration is required.

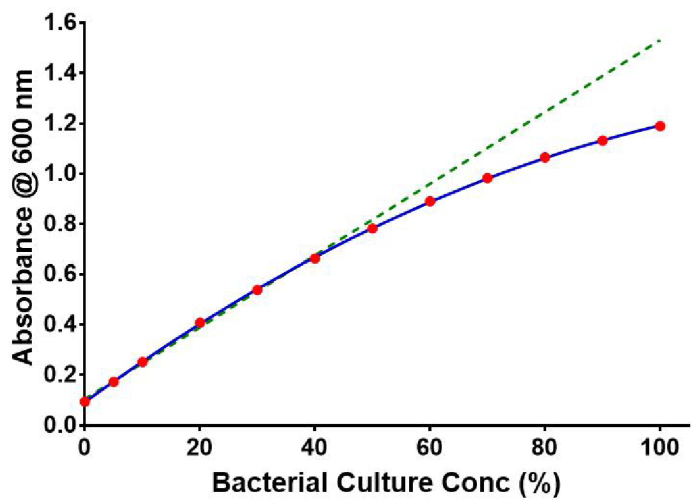

Figure 3. Absorbance measurements of *E. coli* bacterial dilutions. An overnight culture of *E. coli* grown in 2XYT media was diluted in fresh 2XYT media and the absorbance was determined at 600 nm using an Agilent BioTek LogPhase 600 microbiology reader. Data reflect the mean of eight replicates at each dilution.

There are a number of ways to calibrate OD_{600} values from suspension cultures to determine actual cell concentration. The most accurate method uses culturing of dilutions of the suspension onto agar plates. After plating, each colony formed on the solid substrate is the clonal expansion from a single cell. The physical number of colonies formed for a volume of suspension plated is equivalent to the cell concentration. While quite accurate, this method is extremely labor-intensive and other methods to estimate cell number, particularly bacteria, have been developed. One such estimate are McFarland standards. McFarland standards were originally made by mixing specific amounts of barium chloride and sulfuric acid to form barium sulfate precipitate.[12] Currently, latex beads serve the same purpose with a considerably longer shelf life. The standards, designated 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 correspond to bacteria concentrations of 1.5, 3.0, 6.0, 9.0 12, and 15×10^8 cells/mL, respectively (Figure 4). These standards are primarily used to adjust bacterial suspensions to within a given range for standardized bacterial tests, such as antibiotic susceptibility testing by measurement of minimum inhibitory concentration (MIC). In these tests, if a suspension used is too heavy or too dilute, an erroneous result (either falsely resistant or falsely susceptible) for any given antimicrobial agent could occur.

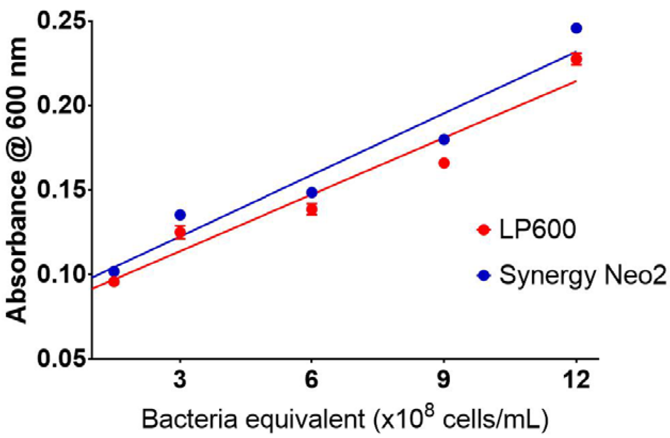

Figure 4. Differences in absorbance signal of McFarland standards with two different microplate readers. McFarland light scatter standards were aliquoted (150 µL) into clear flat-bottom microplates and measured kinetically every 2.5 minutes for one hour (25 determinations) and the mean for each well determined. The mean and standard deviation of eight replicate wells was plotted as a function of the bacterial equivalent reported for the standards. The same plate was measured on two different Agilent BioTek microplate readers.

Nephelometric standards are used to adjust measurements between different instruments, rather than estimating cell concentration. Nephelometry is also based on the light scattering of microorganisms, but contrary to OD_{600} measurements where the loss of transmission due to scattering is measured, nephelometry directly detects the scattered light at an angle to the light source rather than directly in its path, so only diffracted light will reach the detector. The specific turbidity standard formazin, is made from the reaction of hydrazine sulfate and hexamethylenetetramine in water, which forms a poorly soluble suspension.[13] Depending on the stoichiometry of the reaction, specific amounts of turbidity are produced with the defined nephelometric turbidity units (NTU). These commercially available colloidal solutions can be used to compare results between two types of spectrophotometers. As mentioned earlier, dissimilar optical arrangements among two instruments would provide different OD results when measuring the same sample for scattered light.

Results and discussion

A typical misconception is that changes in OD at or near 600 nm are a measure of bacterial absorbance. This value, often referred to as A_{600} or OD_{600}, is really a combination scattered and absorbed light. Because most bacterial cultures do not strongly absorb visible light, the changes at the detector are primarily light scattering, especially when the sizes of the particles (e.g., bacterial cells) are close to the visible wavelength of the light.[14]

An OD_{600} measurement is dependent on the type of instrument configuration being used, and also differs when comparing among different microbial organisms. For example, larger organisms tend to scatter more light than smaller ones (yeast versus bacteria), and minor differences in light paths produce different measurement results. Using the proper measurement technique allows the user to produce results that are comparable among different instrument configurations and that can be trusted as a repeatable method to qualify a particular process. Scientists often rely on published OD_{600} data to take different actions during the course of an experiment. Since all the variables related with the instruments being used and the processes being characterized are rarely known, it is recommended that empirical concentration measurements be determined by the use of growth curves correlating OD_{600} values against plate counts for any particular cell type.

For reproducible results, always use the same photometer for repetitive OD_{600} experiments. When using the same photometer, some factors still influence each measurement and have to be considered when the results of the turbidity measurement vary strongly. Calibration of OD typically changes with growth conditions and cell size. As the culture growth rate begins to slow, many organisms will grow more in size rather than divide and increase in number. Likewise, changes in the refractive index of the growth media can take place over time. For example, cell lysis can introduce membrane lipids, which can act as surfactants. The addition of supplements, such as sugars, to the growth mixture can alter the refractive index in addition to promoting microbial growth.

Monitoring microorganism growth in a microplate reader requires that two parameters be tightly controlled: temperature and aeration. The optimal temperature for microorganisms to grow varies, but it is often approximately 30 °C for yeast and 37 °C for many bacteria. The LogPhase 600 microbiology reader provides temperature control from 30 to 45 °C in 1 °C increments. In addition, a slight temperature gradient can be applied (software selectable) such that the top of the microplate is slightly warmer than the bottom, preventing condensation on the inside of the lid or plate seal, which would lead to aberrant results. For aeration, setting up the best parameters may be more challenging. Aeration is needed for bacterial growth, because oxygen typically does not dissolve well in liquids.

Unless oxygen can be supplied directly into the cultures, and the best way to provide aeration is by shaking the microplate. The LogPhase 600 provides orbital shaking, which has been optimized in regards to rotational speed and amplitude to ensure that suspension cultures remain suspended.[15]

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