DRUG DISCOVERY
AND GENOMIC TECHNOLOGIES

Product Application Focus

Determination of Growth Rate of Microorganisms in Broth from Oxygen-Sensitive Fluorescence Plate Reader Measurements

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ABSTRACT

A novel method utilizing the BD Oxygen Biosensor System has been developed to rapidly, simply, and accurately determine the growth rate of microorganisms in broth, with no need for plate counts, standardized inocula, or technically difficult manipulations. The BD Oxygen Biosensor System incorporates an oxygen-sensitive material into the wells of standard Falcon® microplates. The time response of this sensor monitored in a fluorescence plate reader can be used to quantitate microbe growth. The method entails seeding a dilution series of microorganism onto the plate and reading at regular intervals for 3–10 h. As the organisms grow and consume oxygen, the fluorescence intensities increase over time to form a family of sigmoidal growth curves. A simple mathematical analysis of the time intervals between the curves yields the doubling time, which is independent of the initial concentration of organism. The method is ideally suited as a screening tool for assessing the impact of culture conditions, media composition, or added compounds on growth kinetics.

INTRODUCTION

Measurement of the growth of microorganisms remains a central activity in a wide variety of academic, clinical, and industrial pursuits, including the development and testing of the efficacy of antibiotics (18), the preservation of food (1,4,5,16), and the optimization of cell culture (6) or fermentation conditions for food or pharmaceutical applications (2). Other applications include media development (7,14), recovery of injured organisms (10,19), optimization of waste treatments (12), and disinfectant testing (9).

Traditional methods for assessing microbial growth rate involve either optical enumeration methods (e.g., microscopy, light scattering, or cell counters) or viable-counting methods, such as colony counting and most-probable-number (8). The former group often lacks the ability to distinguish live cells from dead cells, whereas the latter requires that cultures be diluted to the point where cells do not interact with each other, which makes them technique dependent and time consuming. Growth can also be measured indirectly via an increase in biomass, nucleic acid content, or carbon dioxide concentration as the cells respire; however, these methods often use radioactivity or are destructive (8).

The BD Oxygen Biosensor System (OBS) (BD Biosciences, Bedford, MA, USA) offers a microplate-based assay platform for assessing growth kinetics without these disadvantages. The OBS consists of a standard microplate containing an oxygen-sensitive film that may be read in a standard fluorescence plate reader (20). The luminescence of this film is reversibly quenched by oxygen (3) and will increase as the oxygen concentration decreases. Oxygen concentration in the film is a function of the rate of oxygen consumption by the cells and the rate of oxygen influx from the atmosphere. Signal increases once the rate of oxygen consumption exceeds that of influx and will increase until the equilibrium oxygen concentration at the bottom of the well reaches zero. All else equal, it requires a certain rate of oxygen consumption, and hence a given number of organisms, to effect an increase in signal. By comparing the time required for various seed densities to reach a chosen threshold signal, it is a straightforward mathematical exercise to compute doubling times, with no need to manipulate the cells or add reagent.

MATERIALS AND METHODS

Organisms

Salmonella typhimurium 14028 was obtained from the ATCC (Manassas,

VA, USA) and was grown on BBL® Trypticase Soy Agar with 5% Sheep blood for 18–24 h at 35°C. *Pseudomonas aeruginosa* (ATCC 9027) and *E. coli* (BL21-SI; Invitrogen, Carlsbad, CA, USA) were grown on LB agar. Several colonies of the test organism were removed from solid agar and used to prepare a suspension in BBL Trypticase Soy Broth (TSB) for seeding the 96- or 384-well Oxygen Biosensor plates.

Oxygen Biosensor Systems

OBSs of both 96- and 384-well format were used. Test organism was added to the plate via serial dilution in TSB to a final volume of 180 µL/well for the 96-well format or 80 µL/well for the 384-well format. A polystyrene lid or gas-permeable adhesive membrane was used to cover the plate. Fluorescence was measured from the bottom using temperature-controlled fluorescence plate readers, equipped with a 485-nm bandpass excitation filter and either a 612-nm (*S. typhimurium*) or 595-nm (*E. coli* and *P. aeruginosa*) bandpass emission filter (comparable results may be obtained using any emission filter in the range of 590–640 nm).

RESULTS AND DISCUSSION

A suspension adjusted to 50% transmittance using sterile TSB was made from 18-20 h growth of *S. typhimurium*. The density of the starting suspension was determined by the spread plate method (15) to be about 1×10^8 cfu/mL. The salmonella was seeded via 4-fold serial dilution across one row of a 96-well biosensor plate, yielding initial seed densities ranging from 6×10^6 down to approximately 1.4 cfu/mL. The plate was placed into a fluorescence plate reader at 37°C and read every 10 min. The resulting curves are shown in Figure 1. Fluorescence values were normalized to the maximum signal.

The curves from each well in the series show the same shape but take progressively longer to achieve the threshold rate of oxygen consumption. Wells with no cells show no such increase in signal (not shown). Since the only difference between the wells in the series is the starting concentration of salmonella, the time differences for the onset of fluorescence must be those required for the organism's growth to make up the difference.

A simple mathematical algorithm allows one to calculate the doubling times from such proliferation data. Figure 2 shows the relationship between the $\log_2$ (i.e., logarithm to the base 2) of the seed density of organism and the time to reach the midpoint of fluorescence intensity. In this example, the midpoint of the growth curve was chosen because it was easier to measure than the onset of the increase in fluorescence, although in theory any normalized fluorescence value could be used as the threshold. The absolute value of the slope of the line (0.33 h in this case) is the doubling time for the organism under the test conditions. The mathematical interpretation of the Y-intercept as the time required

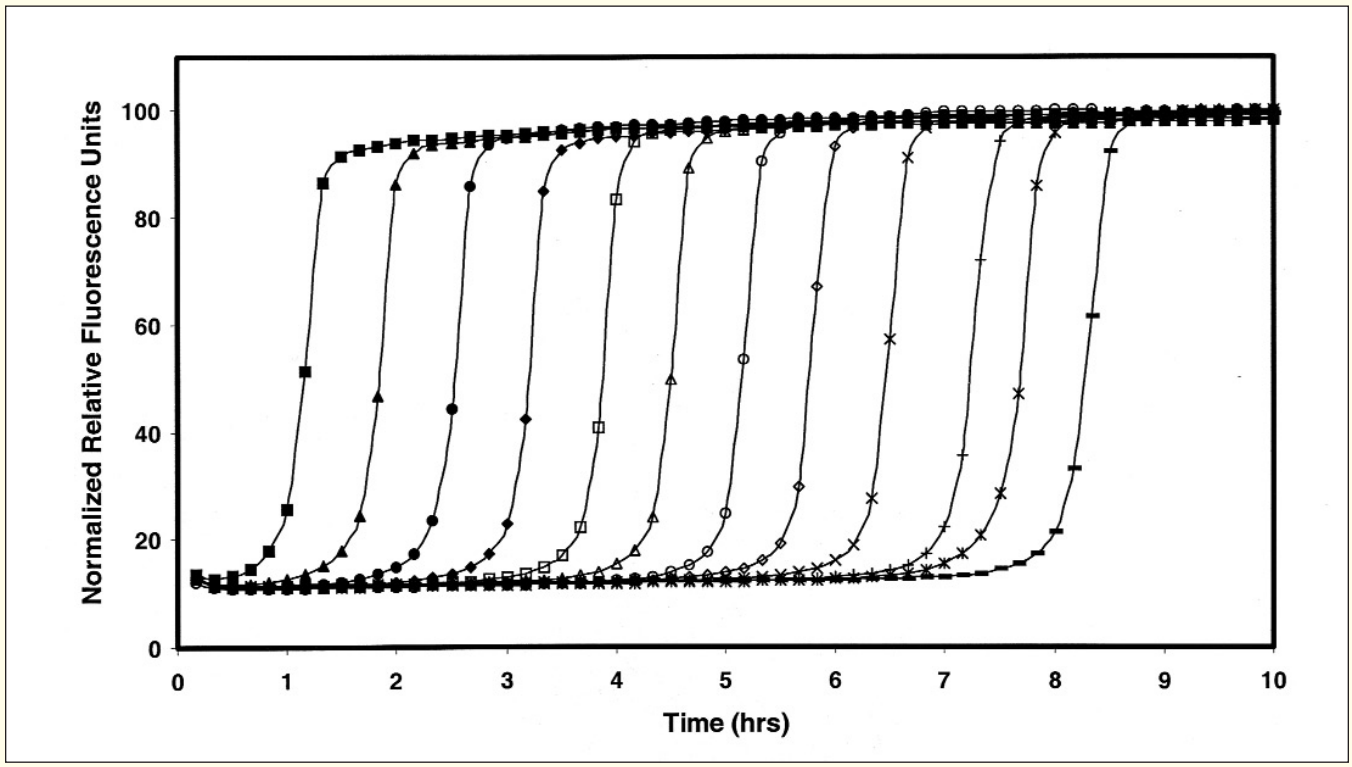

Figure 1. Percent maximum relative fluorescence intensity versus time for a series of 4-fold dilutions of *S. typhimurium*. Wells in column 1 (■) contained 180 µL TSB containing 6×10^6 cfu *S. typhimurium*. Columns 2 (▲), 3 (●), 4 (◆), 5 (□), 6 (Δ), 7 (O), 8 (◇), 9 (×), 10 (+), 11 (✱), and 12 (–) contain 4-fold dilutions of the previous column in the series. The fluorescence intensity from each well is normalized to its maximum intensity, and the average such value from each dilution is plotted versus time.

for a single organism to reach the threshold signal intensity is borne out by the experimental result of the lowest seed density. The protocol is most powerful when absolute seed densities are known, since it can be used to determine the effective seed density of any unknown well cultured under identical conditions. The method may also be used when only relative seed densities are known because the slope on such a semilog plot will be the same for absolute or relative densities.

Because it supports homogenous assays, this method is ideal for use in high-throughput systems (21) that look for growth inhibitors, stimulants, or chemicals and conditions to affect doubling time and/or lag time before growth. It has been used in our laboratory to reveal how differing broths impact both the lag time and the doubling time of several

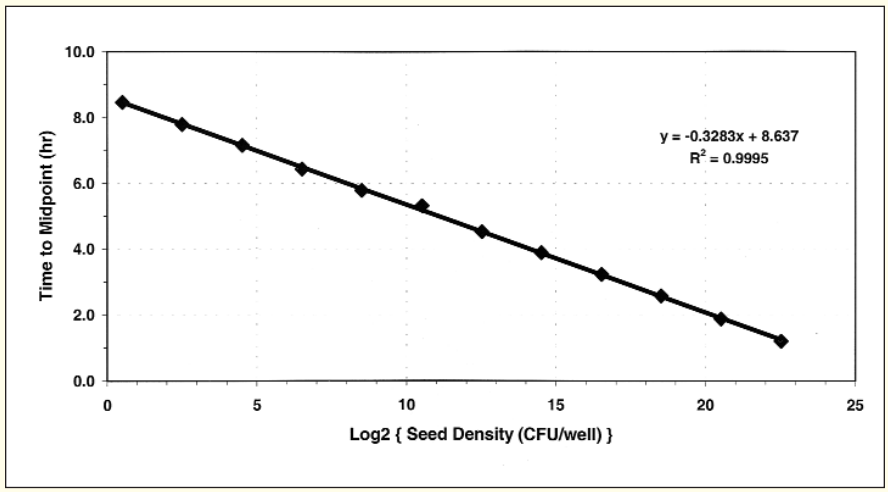

Figure 2. Time to detection versus the Log_2 of the seed density. Log_2 of the seed density of each data point from Figure 1 was calculated via the formula: $Log_2(x) = Log_{10}(x)/Log_{10}(2)$. The absolute value of the slope of the least squares line drawn through the points equals the doubling time. Seed density is reported as cfu/well.

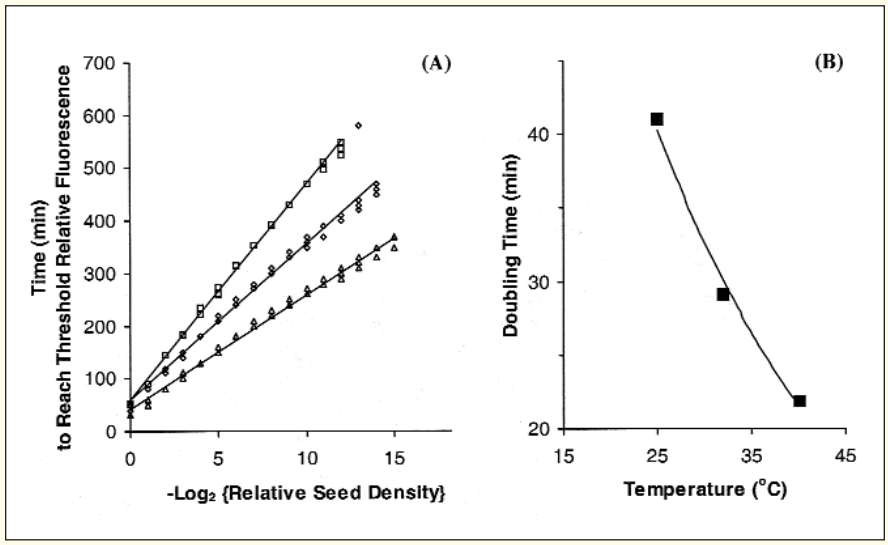

Figure 3. Doubling time plots for *P. aeruginosa* at three temperatures. (A) The time required for the signal to reach the threshold level was plotted against the $-log_2$ of the relative seed density. The same fresh *P. aeruginosa* culture was used to inoculate each plate and was serially diluted across each plate. Plates were incubated in readers at 40°C (Δ), 32°C (◇), or 25°C (□) and read every 10 min. Each of the eight replicates per dilution is shown; times to detection for the replicates were typically within 20 min of each other. The correlation coefficient for each least squares fit was greater than 99%. (B) The doubling times determined from panel A are plotted versus the incubation temperature.

test strains, as well as to optimize broth for certain bacteria (data not shown).

To demonstrate the utility of this method, we investigated the effect of culture temperature on doubling time. The basic procedure was repeated using fresh preparations of *P. aeruginosa* and *E. coli*, each diluted in 8-fold replicates via a 2-fold dilution series in TSB across the columns of a 384-well biosensor plate; wells containing no organism were included as negative controls. Three such plates were prepared, and each was incubated and read in a separate fluorescence plate reader controlled to a different temperature: 25°C, 32°C, or 40°C. For both organisms and at each temperature, a series of nested growth curves (not shown) resembling those in Figure 1 resulted. Figure 3 shows the doubling time plots for the *P. aeruginosa* wells. Across more than a dozen dilutions, straight-line fits were obtained for each temperature. As would be expected, the doubling time varied with the incubation temperature. The *E. coli* wells showed a similar trend (data not shown). Although we took data out to 10 h, it is clear from the data that decent estimates of doubling time could have been obtained in as few as 3 h because we used sufficiently large starting densities.

This procedure may be generalized for any dilution series and has been applied to a number of organisms and cell lines. Most microorganisms that can grow aerobically, including yeasts, other fungi, and mycobacteria, may readily be detected using an oxygen sensor (11, 13). Unlike the technique of biological oxygen demand, the OBS signal is reversible, meaning that the signal will decrease as cells die, which makes the platform suitable for use as a kinetic screen for toxic and/or antibiotic activity (21).

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