From Erlenmeyer flask to microplates – lessons learned from measuring *Escherichia coli* growth

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Abstract

Growth curve measurements are one of the fundamental operations in microbiology and molecular biology. Modern microplate readers have the advantage of high-throughput and ease of automation. This allows for real-time monitoring of many small bacterial cultures and a significant decrease labor and materials costs. Using these devices, tens to hundreds of absorbance readings can be collected simultaneously for hundreds of samples. The small volume and the geometry of a well from a microplate implies that calibration and comparative studies must be performed before switching from one cultivation and growth monitoring system to another. Here, *Escherichia coli XL1* Blue was used as a reference strain in order to test the feasibility of using a common, filter-based microplate reader for recording growth curves when performing antibiotic and toxicity testing. Two different cultivation conditions (flask cultivation and microplate cultivation) and three inoculum/media ratios were tested. The recorded growth curves indicated that the growth of the bacteria is much slower, the turbidity levels are lower, and reading are more variable in the microplate compared with the Erlenmayer flask. Several major technical difficulties must be carefully dealt with when measuring bacterial growth curves in microplates: efficiency of oxygenation that leads to slow growth rates and low densities, condensation on the microplate lead that leads to interference and evaporation of the growth media.

Keywords: *E.coli*, growth curve, microplate;

Introduction

Growth curve measurements are one of the fundamental operations in microbiology and molecular biology, many studies starting with a culture of bacterial cells and the assessment of the bacterial cells proliferation (Kurokawa and Ying 2017). Hence, most of knowledge on bacterial life cycle is derived from monitoring cell growth over time in liquid media. Analytical data on bacteria proliferation can be obtained by timed sampling of a bacterial culture followed by determination of the cells number by either measuring optical turbidity or performing colony-forming unit (CFU) assays (Kurokawa and Ying 2017). Due to its simplicity, the most routinely used method is measuring optical turbidity or optical density (OD). Applications of OD measurements range from studying cellular metabolism or physiology, preparing competent cells for cloning, heterologous overexpression of proteins, monitoring biomass accumulation during fermentation processes or determining growth rate for antibiotic resistance studies (Stevenson et al. 2016).

Modern microplate readers have the advantage of high-throughput and ease of automation (Zimmermann et al. 2004). This allows for real-time monitoring of many small bacterial cultures and a significant decrease labor and materials costs. Using these devices, tens to hundreds of absorbance readings can be collected simultaneously for hundreds of samples and the data can be processed with freely available software packages that allow modeling and interpretation (Hall et al. 2014; Sprouffske and Wagner 2016).

Commonly, bacterial cultures in liquid media are performed in Erlenmeyer flasks (baffled or not) or Falcon tubes with a ratio of media volume: flask volume anywhere between 1/10 to 1/4 depending on the size of the culture (Kram and Finkel 2014). The most common practice is to use a ratio of 1/5 for 100 ml to 1L media. This, combined with proper agitation ensures the formation of a thin film of culture media which is essential for optimum aeration and fast growth rates (Somerville and Proctor 2013). The small volume and the geometry of a well from a standard 96 well microplate is far from achieving the ideal conditions for maximal growth. Hence, correct calibration and comparative studies must be performed before switching from one cultivation and growth monitoring system to another. This is especially important when microplate readers are to be incorporated into routinely used experimental designs and data comparability is essential.

Here, *Escherichia coli* XL1 Blue was used as a reference strain in order to test the feasibility of using a common, filter-based microplate reader for recording growth curves when performing antibiotic and toxicity testing. Two different cultivation conditions (flask cultivation and microplate cultivation) and three inoculum/media ratios were tested. The recorded growth curves were compared in terms of lag phase length, growth rate and cultivation time required to reach stationary phase. The direct comparation of the growth curves was preferred instead of a CFU assays as these assays have been reported as being unsuitable for calibrating OD readings when antibiotics are tested (Stevenson et al. 2016).

Material and Methods

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Strains, media and growth conditions. *Escherichia coli* XL1 Blue (Stratagene, endA1 gyrA96(nalR) thi^{-1} recA1 relA1 lac glnV44 F'[::Tn10 proAB^{+} lacI^{q} Δ(lacZ)M15] hsdR17(r_K^- m_K^+) were kept as glycerol stocks at -80^{0}C and revived by plating on LB plates supplemented with 10 microg/ml tetracycline (Sigma Aldrich, Germany). Plates were incubated overnight at 37^{o}C and one colony was used to inoculate 10 ml LB broth, 10 microg/ml tetracycline in a 50 ml Falcon tube. After 16 hours at 37^{o}C, 190 rpm in a GFL-3031 (GFL, Germany) orbital shaker, the preculture was used to inoculate the main cultures. Flask cultivation was performed in a 500 ml not baffled Erlenmeyer flask containing 200 ml LB broth, 10 microg/ml tetracycline. Microplate cultivation was done in standard 96 well microplates, each well containing 200 microL LB broth, 10 microg/ml tetracycline. Each culture was started by adding the inoculum in various ratios and incubated at 37^{o}C, 190 rpm. For shaking the microplates, an microplates adapter was printed using a Creality3D Ender 5 printer (Figure 1). A model of the adapter and instructions are available on NIH 3D Print Exchange server (Coakley et al. 2014), Model ID 3DPX-015527. LB medium (Sambrook et al. 1989) was prepared as following: 10 g NaCl (Chemical Company, Romania), 5 g yeast extract (Merck, Germany), 10 g Peptone from casein (Carl Roth, Germany) were dissolved in 1 L distilled water and autoclaved at 115^{0}C for 20 min using a AES-50 (Raypa, Spain) autoclave. For solid medium, 16 g/L of microbiology grade agar-agar (Roth, Germany) was added prior to sterilization.

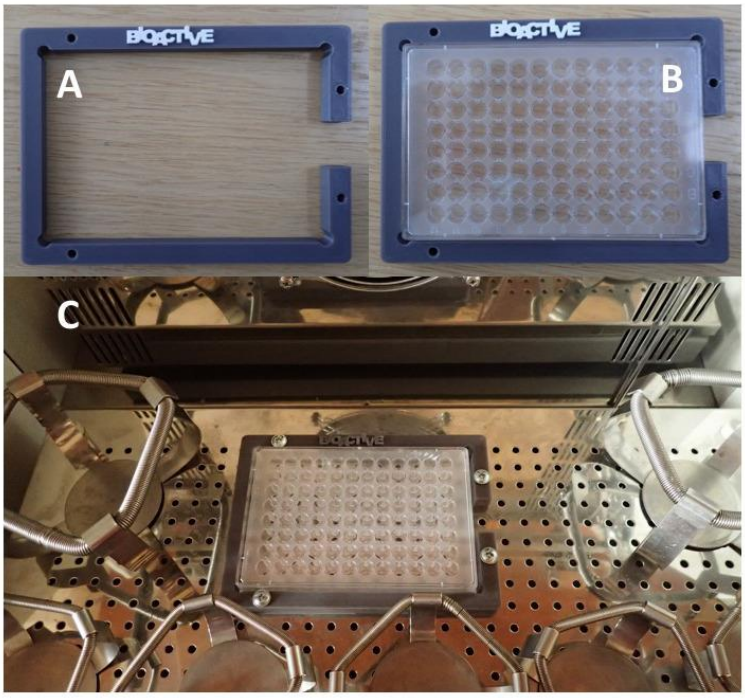

Figure 1. Adapter for secured fixation of the microplates to the GFL-3031 shaking platform fabricated using 3D printing (**A** and **B** adapter ready to be mounted; **C** adapter attached to the shaker platform)

OD measurements. 0.8 ml samples were taken every hour from the Erlenmeyer flasks and OD at 590 nm were recorded against a blank with LB medium using a DU 730 UV/VIS Spectrophotometer (Beckman Coulter). Plates were removed from the shaker and OD were read using a Tristar^2 Multimode Reader LB942 (Berthold Technologies). The reading program consisted of 2 s shaking (orbital motion, normal speed) fallowed by the endpoint measurement (counting time 0.2 s) with a "by well" operation mode. A microplate adapter was fabricated to allow the reading of the microplates with the leads on. The adapter was printed using a Creality3D Ender 5 printer. A model of the adapter and printing instructions are available on NIH 3D Print Exchange server (Coakley et al. 2014), Model ID 3DPX-015703.

Data processing. Flask cultures were performed in triplicate, while microplates cultures were repeated at least 12 times (one row of wells in the microplate). OD readings from the spectrophotometer and multiplate reader were imported into Microsoft Excel spreadsheet program. Means and standard error were calculated using the Data Analysis function from the Analysis ToolPak and plotted using the same program.

Results and Discussions

General aspect of the growth curves. *Escherichia coli* XL1 Blue strain was grown in both classic shaken Erlenmeyer flask and a 96 well microplate using the same batch of LB media. For each cultivation system, three ratios of the same inoculum have been used: 0,5%, 1% and 2%. The growth of the cultures was recorded at 590 nm using either a spectrophotometer or multiplate reader and the data is plotted in figure 2.

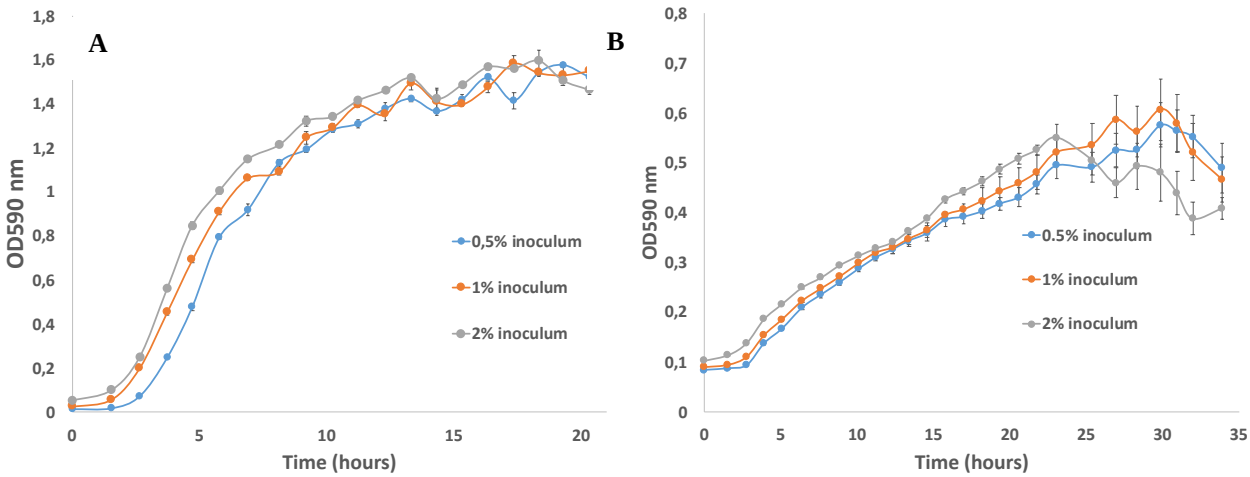

Figure 2. Growth curves of *Escherichia coli* XL1 Blue when cultivated in (**A**) Erlenmeyer flask and (**B**) 96 well microplate, both incubated at 37°C and 190 rpm. Each point is a mean of 3 measurements for the Erlenmeyer flask cultivation and at least 12 measurements for the microplate. Vertical error bars represent standard error.

A striking difference between the two cultivation systems can be easily observed. Disregarding the amount of inoculum used, the growth of the bacteria is much slower in the microplate compared with the Erlenmayer flask. This is also clearly demonstrated by the calculated slope of the exponential phase. In the case of the microplate culture, the calculated slopes are 0,0169 OD units/hour (R^2=0.947) for 0.5% inoculum, 0,0175 OD units/hour (R^2=0.9899) for 1% inoculum and 0,0187 OD units/hour (R^2=0.9963) for 2% inoculum, while for the Erlenmeyer flask culture the calculated slopes are 0,2197 OD units/hour (R^2=0.970) for 0.5% inoculum, 0,1934 OD units/hour (R^2=0.987) for 1% inoculum and 0,1808 OD units/hour (R^2=0.960) for 2% inoculum.

Basically, the flask cultures grow at least 10 times faster than the multiplate cultures, this difference in growth rate translating into a delayed stationary phase in the case of the multiplate culture.

Another striking difference between the two cultivation and growth monitoring system is that the recorded turbidity in the stationary phase is less than half in the multiplate compared with the flask cultures. As the relative amounts of nutrients are basically the same in both cultivation systems, these differences must be related to shaking differences and hence oxygen availability. Both the Erlenmeyer flasks and the multiplate were incubated in the same time on the same shaker, so the shape of the multiplate well does not allow for good aeration in our experimental conditions. Reports in the literature agree with our conclusions and recommend a 8-shaped shaking motion as a method to improve aeration in microplates (Kurokawa and Ying 2017) or the usage of a 48-well microplates that provide a better small-scale fermentation systems for microbial cultivation (Zimmermann et al. 2004).

Another particularity of the microplates cultures is a great variability of the OD reads in the stationary phase. A close visual inspection of the microplates indicated the presence of particulate matter in the center of the wells (Figure 3) that would interfere with the path of light in the microplate. This is caused by either improper or low frequency shaking that causes the cells to stack in the center at a high density (Kurokawa and Ying 2017).

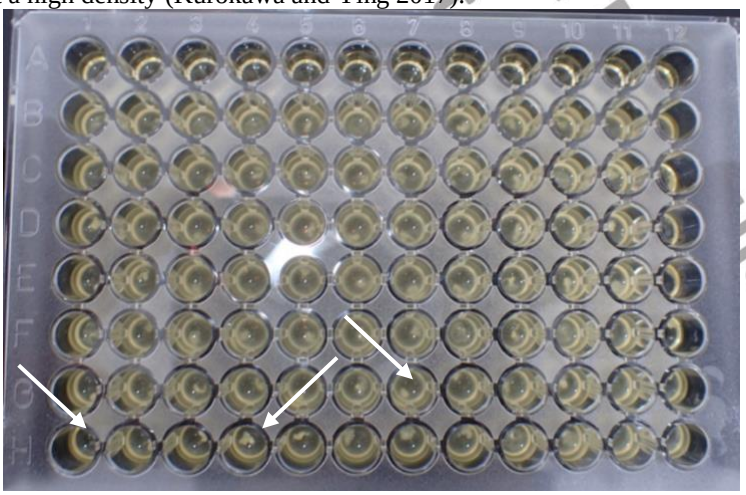

Figure 3. At high densities, bacterial cells tend to stack in the center of the well due to improper shaking causing unreliable OD readings in the microplate reader. Arrows indicate particulate matter in the wells due to cells stacking.

Technical difficulties encountered. During the course of the experiments, several technical difficulties were encountered. First, the wells located on the edges of the microplate suffer from evaporation of the medium that has a great impact on the

reads due to the small culture volume (i.e., 200 microL). Hence, it is advisable that these wells should only contain blank medium and that cultivation time should be less then 48 h (Kurokawa and Ying 2017).
Secondly, as the plate was incubated at 37°C and the multiplate reader was at room temperature, the temperature differences caused extreme condensation on the lead that interfered with the readings. Hence, a lead coating procedure must be used, as the one based on Triton X-100 described by Krishnamurthi et al. 2021 (Krishnamurthi et al. 2021).
Third, repeated loading and unloading of the plate in the microplate reader caused scratches on the lead that interfered with the OD readings, most probably due to an incompatibility between the plates used and the adapter provided with the multiplate reader. As a solution, another adapter was fabricated using 3D printing that placed the plate 2 mm lower, hence removing the scratching and allowing the reads to be performed with the lead on the microplates. A model of the adapter and printing instructions are available on NIH 3D Print Exchange server, Model ID 3DPX-015703.

In conclusion, common filter-based microplate readers can be used for recording growth curves for antibiotic and toxicity testing, but careful calibration and comparative studies must be taken into account before incorporating such devices into routine experimental designs. Several major technical difficulties must be carefully dealt with when measuring bacterial growth curves in microplates: efficiency of oxygenation that leads to slow growth rates and low densities, condensation on the microplate lead that leads to interference and evaporation of the growth media.

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Declarations

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