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Direct optical density determination of bacterial cultures in microplates for high-throughput screening applications

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ABSTRACT

A convenient and most abundantly applied method to determine the growth state of a bacterial cell culture is to determine the optical density (OD) spectrophotometrically. Dilution of the samples, which is necessary to measure within the linear range of the spectrophotometer, is time-consuming and not compatible with high-throughput applications. Here we present a direct approach to estimate the OD at 578 nm (OD_{578}) of bacterial cultures in microplates without the need for sample dilution. This could be advantageous for high-throughput analysis of bacterial cells in microplates for example when optimizing growth conditions, screening for new substrates of a bacterial strain or monitoring enzymatic activity after enzyme evolution. *Pseudomonas putida* cells were grown in shake flasks. The OD_{578} was determined in parallel in a microplate directly without dilution and in a spectrophotometer cuvette after dilution. The resulting data set was used to identify a conversion formula, which enables direct and reliable transformation of OD measurements of undiluted samples into the corrected OD values as would have been obtained for diluted samples measured in a standard spectrophotometer.

Subsequently we could show that just a few OD calibration points are required to adjust this conversion formula and make it suitable for other suspensions or cultures of bacterial strains different than *P. putida*. The OD calibration points can be obtained by any combination of microplate reader and cuvette spectrophotometer. For this purpose, conversion formulas for a formazine standard suspension and a suspension of *Escherichia coli* BL21(DE3) cells were successfully generated. The OD values calculated by both conversion formulas turned out to be identical with the values as obtained by the control measurements in the spectrophotometer. This indicates the general applicability of the conversion formula as described.

1. Introduction

While investigating different promoters for the biosynthesis of the green fluorescent protein (GFP) in *Pseudomonas putida* KT2440, we were confronted with the - trivial but bothersome - problem to monitor both, the optical density at 578 nm (OD_{578}) and the fluorescence intensity (FI) at 510 nm of bacterial cell cultures grown in shake flasks.

Although the Beer-Lambert law allows the spectrophotometric quantification of substances, if the absorbance of the substance is proportional to the concentration of the absorbing species, it is strictly valid only in homogenous solutions but not in suspensions. In suspensions, for example bacterial cultures, the light with a wavelength in the range of the particle's size (i. e., a bacterium) is scattered (Mie scattering) [1]. When the light is scattered by a single particle in a suspension with many particles, the attenuation of light and hence the measured extinction is proportional to the number of particles [2]. At higher particle concentrations, the incident beam of light is scattered by more than one particle ('multiple scattering'). This phenomenon results in an apparently lower optical density (OD), because multiple scattered light could reach the detector of a spectrophotometric device [3]. Therefore, monitoring the OD of a bacterial culture directly in a microtiter plate, for example during cell growth or as a control in advance of enzymatic assays, is inaccurate.

To avoid 'multiple scattering' in OD measurements, suspensions of bacterial cells usually have to be diluted. In diluted suspensions, 'multiple scattering' plays a minor role and the extinction, which is defined as the sum of scattering and absorbance in this case [2], and which is measured by the spectrophotometer, can be correlated in a linear way with the number of cells in the culture.

Due to the non-linearity of the OD at higher cell densities, we were forced to use two different devices, a standard spectrophotometer (SP) device for determining the OD_{578} as well as a microplate reader (MPR)

Abbreviations: OD, optical density; OD_{578}, optical density at 578 nm; GFP, green fluorescent protein; FI, fluorescence intensity; FAU, formazine attenuation units; SP, spectrophotometer; MPR, microplate reader

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for measuring the FI. This resulted in a time-consuming process, in which the bacterial cultures first had to be diluted to an OD_{578} within the linear range of the spectrophotometer, and additionally pipetted into a 96-well plate to be analyzed in a microplate reader for FI. Such a procedure is - besides being prone to errors - not compatible with high-throughput applications.

A computational conversion of undiluted OD values measured in a microplate into OD values as would have been obtained for diluted samples measured with the spectrophotometer would allow a considerable simplification of cell growth monitoring, but was not available so far. Wind and Szymanski introduced a correction factor to the Beer-Lambert law, which allows transmittance measurements in aerosol media [4]. The authors restrict this solely on single forward scattering because the effects of multiple scattering are much more complicated to consider. This limitation to single scattering does not reflect the reality of a bacterial cell culture and therefore the correction factor, which was introduced into the Beer-Lambert law by Wind and Szymanski, could not be used to correct for the non-linearity of OD measurements at higher cell densities.

Warringer and Blomberg did find a third-order polynomial correlation between SP and MPR measurements [5], but it was developed for yeast species and the applicability to bacterial species was not shown. Dalgaard *et al.* described an approach to correct for the non-linearity of the OD at higher cell densities. Herein, OD values measured with a spectrophotometer without dilution were converted into OD values measured with the same device after dilution [6]. Compared to our work, the approach by Dalgaard *et al.* is restricted in two respects: Firstly, the conversion formula did not deal at all with the utilization of a combination of two spectroscopic devices and therefore did not enable the transformation of OD values from undiluted microplate culture to those expected from dilution and subsequent spectrophotometry. Secondly, the authors did not show the general applicability of their OD-correction approach by a standardized suspension.

2. Material and methods

2.1. Spectroscopic devices

In this study, a GENESYS™ 10S UV–vis spectrophotometer (Thermo Scientific, Waltham, MA, USA) and semi-micro cuvettes (SD10 PS, volume 1.5–3.0 mL, window 4.5 x 23 mm, GML-alfaplast GmbH, Munich, Germany) were used for OD_{578} measurements. To determine the FI of the *P. putida* KT2440 cells, which are synthesizing GFP, an Infinite® M200 PRO (Tecan, Männedorf, Switzerland) microplate reader was used. The samples were measured without dilution in a 96-well plate (Microplate, PS, F-Bottom, clear, Greiner Bio-One, Frickenhausen, Germany).

A serial dilution of bromophenol blue in 20% (v/v) ethanol and NaOH (0.02 M) ranging from 0.5 µM to 125 µM was used to determine the linear ranges of both devices. Both spectroscopic devices were used to measure the absorbance at 578 nm and the resulting OD_{578} values were compared with each other.

2.2. Bacterial cell culture

P. putida KT2440 cells were grown in Luria-Bertani (LB) medium, being composed of 10 g/L tryptone (Carl Roth, Karlsruhe, Germany), 5 g/L yeast extract (Carl Roth, Karlsruhe, Germany) and 10 g/L NaCl (Sigma-Aldrich, Munich, Germany) and adjusted to pH 7.0. For precultures 20 mL LB medium were inoculated with one bacterial clone. The cultures were incubated in shaking flasks for 16–18 hours at 30 °C and 200 rpm. A preculture of *P. putida* KT2440 was used to inoculate 200 mL of LB in a shake flask. *Escherichia coli* BL21(DE3) cells were cultivated similarly, but incubated at 37 °C and 200 rpm. In case of plasmid containing strains, kanamycin (50 µg/mL) (Life Technologies, Darmstadt, Germany) was added.

2.3. OD_{578} determination

The OD_{578} of the bacterial culture was measured every two hours for 34 h. To determine the OD_{578} within the linear range of the spectrophotometer, the bacterial culture sample was diluted. The microplate reader was used to measure the OD_{578} of the same sample, but without dilution. For that, 200 µL of the non-diluted bacterial cell culture were pipetted into a well of a 96-well plate. The OD_{578} of the culture medium was measured both in the microplate reader and the spectrophotometer as well and was in both cases subtracted from the measured OD_{578} values of bacterial samples.

2.4. Data analysis

By measuring the OD_{578} of the bacterial cell culture both with the spectrophotometer and the microplate reader, a dataset of 343 OD_{578} values of the same sample – one measured after dilution within the linear range of the spectrophotometer and the other one measured without dilution in the microplate – was generated. The data was analyzed by the use of the data analysis and graphing program Origin 9 (OriginLab, 2013). The non-linear parameter estimation was performed with the algorithm *BoxLucas1* [7] and the Levenberg-Marquardt iteration algorithm with a confidence interval of 95%.

2.5. Formazine standard

For the verification of the exponential fitting approach, a suspension of a defined turbidity was generated. The suspension, a formazine standard of 4000 FAU (formazine attenuation units), is usually used to determine the turbidity of water during water quality analysis. It was prepared according to the DIN EN ISO 7027:1999 [8]. The formazine standard suspension was used to prepare samples of various concentrations.

2.6. Conversion formula for E. coli BL21(DE3)

The supplemented Origin-worksheet (Supplementary material 1) was used to generate a conversion formula for OD_{578} estimation of *E. coli* BL21(DE3) cells. The cells were diluted serially and the OD_{578} values of the prepared 23 dilutions were determined as triplicates in parallel with the microplate reader without dilution and with the spectrophotometer after sample dilution. The resulting data set was inserted into the Origin-worksheet (Supplementary material 1), which gave a conversion formula with adapted parameters *a* and *b* for the OD_{578} values measured with the microplate reader.

3. Results and discussion

3.1. Comparability of spectroscopic devices and correction for differing layer thicknesses

To guarantee comparability of two spectroscopic devices, both devices require identical linear ranges. If the linear ranges of two spectroscopic devices are identical, the measured absorbance will be independent of the used spectroscopic device. A solution of bromophenol blue was used to determine the linear ranges of spectrophotometer (SP) and microplate reader (MPR) used in this study. By measuring the absorbance at 578 nm, it could be shown that Infinite® M200 PRO and GENESYS™ have identical linear ranges between extinction values of 0.1 and 4.0 (data not shown). This finding guarantees comparability of both spectroscopic devices used in the following. Furthermore, we anticipate the procedure presented hereafter can be used effectively with various MPR-SP device pairings having identical linear ranges.

To determine a correlation between OD_{578} values measured by spectrophotometer (OD_{578}^{SP}) and microplate reader (OD_{578}^{MPR}), cultures of *P. putida* KT2440, containing plasmids with *gfp* regulated by