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Comparison of microbiological and physicochemical methods for enumeration of microorganisms*

Porównanie metod optycznych oznaczania liczby bakterii

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Summary

Determination of the number of cultured bacteria is essential for scientific and industrial practice. A spread plate technique is the most common and accurate method for counting of microorganisms. However, time consuming incubation does not allow for a quick estimation of the number of bacteria in a growing culture. In the present study, the results of photometric measurements: direct optical density method (OD at 585 nm), UV absorbance at 260 and/or 280 nm of separated and lysed bacteria by sodium hydroxide and surfactant with the spread plate technique were compared. The linear regression model for bacterial strains *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* was used to compare these three methods. The UV measurement method enabled determination of the number of bacteria with similar precision. The procedure for solubilized bacteria UV measurement is robust, and is not influenced by dispersions in the original culture medium.

Keywords: spectrophotometry • turbidimetry • spread plate technique • bacterial enumeration • optical density

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Introduction

Among microbiological methods routinely used to isolate, propagate, and enumerate microorganisms [24], a spread plate technique is the most common method for aerobic microorganisms. For enumeration by this method, a bacterial sample is spread on an agar plate and after incubation the colonies are counted. Each bacterial cell should produce a single colony (colony-forming units, CFU). To achieve this effect, samples must be diluted to obtain reasonable counts. The number of bacterial colonies up to 250 CFU can be separated on a typical petri dish [12]. The spread plate technique is commonly used for isolation and identification of different bacterial strains, for example *Actinobacteria* from the soil [19]. Although scientists continue to work on new methods for bacteria enumeration, the spread plate technique still serves as a reference method [3] *e.g.* in investigations of different bacterial mutations associated with drug resistance [1], in detection of urinary track infection [21], and in the assessment of enteric bacteria [18]. The major disadvantage of this method is the long time needed to achieve bacterial growth on a petri dish. Nevertheless, bacterial growth kinetics provides additional information on microorganisms and their behavior in different environmental conditions. In an alternative total cell count method, a microscope is used to determine the number of cells in a liquid culture. The main advantage of this method is that it allows to obtain the results quickly [12,15]. Indirect quantification with optical density method (OD) is another common alternative. However, the relevant physical phenomenon is not the absorption of light but scattering (turbidimetry). The light passing through a sample is scattered on the bacteria particles, and less of it reaches in line detector optics [4]. With a different instrument setup, a direct measurement of scattered light can also be performed (nephelometry) [17]. Light scattering is directly proportional to the size and number of particles [12]. The turbidimetric method is considered fast, but applicable only in certain concentration range. Modern approaches for determination of the number of bacteria are based on flow cytometry [9,13] and DNA quantification. For example real-time quantitative PCR (RTQ-PCR) was used [11,20,22]. Amongst the methods for determining the number of viable bacteria in a culture are the luminescent tests based on quantitation of ATP as an indicator of metabollically active cells [10,26]. For investigating the microbial populations at a single cell level, a well-established technique to be used is fluorescence *in situ* hybridization (FISH) [23]. Mandal and Parvin [16] used fluorescence measurements with water soluble carbon quantum dots specific for cell walls (CQDs) as a fluorescence marker for rapid detection of bacteria and their count.

The aim of the present study was to compare the accuracy and precision of three simple techniques for bacterial enumeration and culture monitoring. These include (i) spread plate technique, (ii) optical density of bacterial culture, and (iii) UV measurements of solubilized bacteria.

Materials and methods

Media

Nutrient broth (pH 7.2 ± 0.2) was obtained from the Laboratory of General Chemistry (Institute of Immunology and Experimental Therapy (IIET), Polish Academy of Sciences (PAS) and consisted of 0.4 g beef extract (Biocorp, Poland), 5.4 g enzymatic digest of casein (BTL, Poland), 1.7 g yeast extract (Biocorp, Poland), 4 g peptone (BTL, Poland), 3.5 g NaCl (Avantor, Poland) and 1% anhydrous glucose (Avantor, Poland), per liter. The components were dissolved in distilled water and sterilized in an autoclave.

Bacterial strains

Escherichia coli PCM 1630, *Pseudomonas aeruginosa* PCM 2058 and *Staphylococcus aureus* PCM 2054 strains were obtained from Polish Collection of Microorganisms, at Institute of Immunology and Experimental Therapy, Polish Academy of Sciences. *E. coli* was maintained on MacConkey agar (BTL, Poland) at 4°C. *P. aureginosa* and *S. aureus* were maintained on blood agar (BTL, Poland) at 4°C. All the bacteria were subcultured monthly.

Culture

E. coli, P. aeruginosa and *S. aureus* were cultured in a nutrient broth. Inoculum was prepared by overnight incubation at 37°C. OD (565 nm) of inoculum was in the range 3.0 - 3.2 McFarland units. The medium was inoculated with the bacterial culture in 1:10 (v/v) ratio.

Spread plate technique

During bacterial culture that lasted 7 hours, samples were collected every hour, then diluted and inoculated on agar plates in the volume of 0.1 ml. Petri dishes with appropriate dilution of bacterial culture were incubated for 24 hours at 37°C. Following incubation, the colonies were counted and the number of bacteria in the original solution was calculated. The experiment was performed in six replicates.

OD measurement

The measurement of bacterial culture samples was performed using a densitometer (DEN1, BIOSAN) adapted to measurements in the range of 0.3 - 15 McFarland units at 565±15 nm (Capin 2003). During the bacterial culture, samples were collected every hour for 7 hours and measured directly. The experiment was performed in six replicates.

Solubilized bacteria UV measurement

During 7 hours of the experiment bacterial culture samples were collected every hour and centrifuged at 14 000 x *g* for 3 min.The precipitates were washed with ultrapure water, centrifuged at 14 000 x *g* for 3 min. The precipitated bacteria were solubilized in 0.5 ml of 0.1 M NaOH with

2% sodium dodecyl sulfate (SDS) at room temperature (or briefly heated up to 60°C) to achieve a clear solution. Then, either (i) 0.1 M NaH_2PO_4, (ii) 0.09M acetic acid, or (iii) water were added in 1:1 (v/v). Absorbances were measured at 260 and 280 nm in 1 cm cuvettes using Specord 250 (Analytik Jena AG). The experiment was performed in six replicates.

Statistical analysis

A linear regression model was used to describe the relationship between the three predictors (OD result, absorbance 260 nm, absorbance 280 nm) and the number of bacterial cells obtained by the spread plate technique (response variable). Obtained data were transformed using $\log_{10}$. The quality of correlation were confirmed by both coefficient of determination R^2 and residual standard error ($SE = \sqrt{MSE}$). Based on these statistics, the best method was chosen for which the SE are the smallest, while the R2 value is the highest.

Results and discussion

Here we evaluate single absorption measurements at either 260 or 280 nm for quantification of bacteria after lysis. The procedure entails just a few steps: i.e. the bacteria were separated from the culture medium by centrifugation and subsequently the bacterial sediment is lysed and dissolved in sodium hydroxide. A UV-transparent detergent (e.g. SDS) was required for most of the studied bacterial strains. The optional brief heating of the lysate up to 60°C accelerated the dissolution but did not influence the end result. The UV measurements could be carried out immediately after dissolution. However, when base-sensitive cuvettes were used it safer to neutralize the mixture before measurement. The choice of a neutralizing agent did not disturb the assay. Both the basic and neutral solutions were transparent. The 260 and 280 nm wavelengths corresponded to absorption bands for nucleic acids and proteins, respectively. After neutralization the absorbance values at 260 and 280 nm were by 8 and 4% lower than under basic conditions, respectively, as expected for nucleic acids [6] and proteins [14]. Quantification of nucleic acids by the absorbance measurement at 260 nm is a fundamental tool in molecular biology [7] and can be used to determine DNA quantity alterations during bacterial growth. Absorbance at 280 nm due to aromatic amino acids is used to calculate protein concentrations [25]. Thus the results should reflect the actual concentration of bacterial structural components.

The experiments were carried out using three morphologically and taxonomically different bacteria: *E. coli* B, *S. aureus* and *P. aeruginosa*. Bacterias were cultured in nutrient broth for 7 hours. Samples were collected every hour for determination of the number of bacteria by a set of methods. These included a reference spread plate technique, and spectrophotometric OD method and solubilized bacteria UV measurements. The data obtained from all the techniques were compared for each bacteria separately.

Biesta-Peters et al. [2] compared the OD and the plate count methods for bacterial growth monitoring. Their results showed that both methods are of similar reproducibility. However, the results of bacteria quantification can be influenced by the aggregation of the microorganisms [12], and various other factors causing light scattering, e.g. anti-foam agents, dispersed gases (originating both from fermentation and agitation). Unaccounted light scattering can also arise from dispersed inorganic salts and protein aggregates. Nevertheless, Hernández and Marin [8] used OD to estimate bacterial growth in the presence of suspended solids. The measurements are carried at ca. 560 nm, so as to exclude specific absorption.

The monitoring of the number of *S. aureus* in the growing culture by a spread plate technique is shown in Figure 1. The corresponding measurements of OD and solubilized bacteria UV measurements at 260 and 280 nm are shown in Figure 2.

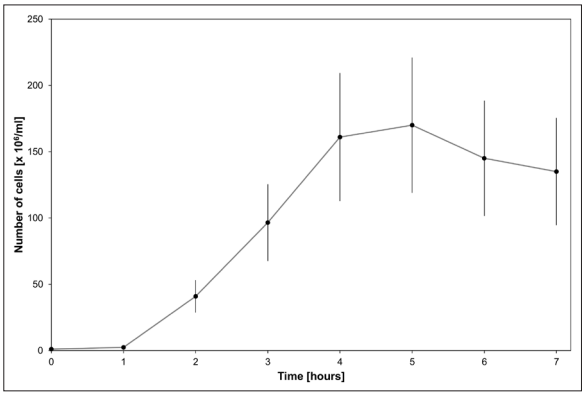

Fig. 1. Growth of *Staphylococcus aureus* determined by spread plate method. Each point corresponds to six independent assays

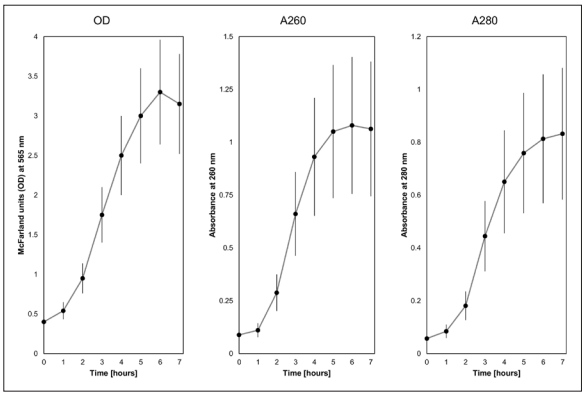

Fig. 2. Growth of *Staphylococcus aureus* determined by optical density method at 565nm (OD) and spectrophotometrical determination (at 260 and 280 nm) of solubilized and neutralized lysate

The linear relations between the logarithms of OD, and solubilized sample absorbances at 260/280 nm vs. bacteria counts for *S. aureus* are shown in Figure 3. Similar relationship was found for *E. coli* and *P. aeruginosa*. The quality of the estimation varied for different bacteria.

Table 1. Statistical analysis of linear relationships between the number of bacterial cells and three predictors: OD, 260 and 280 nm

Bacteria	Predictors	Regression equation		R2	SE
		a	b		
Staphylococcus aureus	OD	0.3727	-2.508	0.9349	0.0730
	A260	0.4747	-3.9308	0.9611	0.0711
	A280	0.4938	-4.2180	0.9486	0.0855
Pseudomonas aeruginosa	OD	0.2366	-2.1241	0.9280	0.0690
	A260	0.3681	-4.0792	0.9435	0.1266
	A280	0.3704	-4.3210	0.9508	0.1180
Escherichia coli B	OD	0.3130	-2.4771	0.9257	0.0474
	A260	0.5342	-4.7844	0.9528	0.0556
	A280	0.5130	-4.8857	0.9465	0.0569

The better agreement of the results was obtained for *Staphylococcus aureus* than for *Pseudomonas aeruginosa*. The choice of the wavelength for the solubilized bacteria UV measurement also depended on the bacteria.

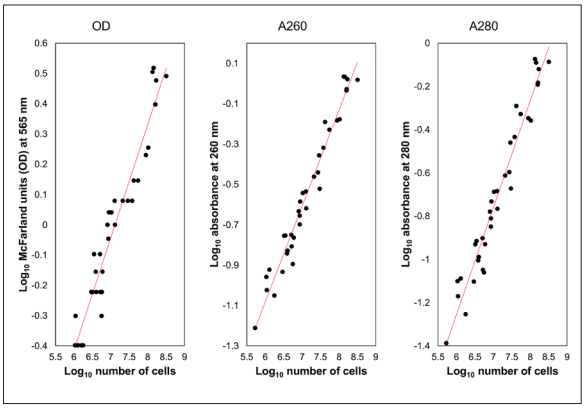

Fig. 3. Correlation between the number of bacteria in the culture of *Staphylococcus aureus* determined by spread technique and optical density method at 565 nm (OD), and absorbance at 260 and 280

The linear relations between the logarithms of bacteria counts vs. OD, and absorbances at 260/280 nm for *S. aureus* are shown in Figure 3. Similar relationship was found for *E. coli* and *P. aeruginosa*. Table I shows the summarized results of two statistical measures used to describe linear relationships between the number of bacterial cells and three predictors: OD, 260 and 280 nm. Statistical relationship is described by coefficient (R^2) and standard error (SE).

OD method and solubilized bacteria UV measurements were used with relatively good accuracy for tracking the growth of the bacterial culture. R^2 were higher for both A280 or A260 predictors. At the same time SE values proved to be comparable for all three methods of cell counting.

Conclusions

Determination of the number of cultured bacteria is essential for scientific and industrial practice. All studied methods anabled to determine the number of bacteria with similar precision. The procedure for solubilized bacteria UV measurement is robust, and is not influenced by dispersions in the original culture medium. The time needed for the tests was about 10 minutes. It can be anticipated that modifications to the method should not compromise the quality of the estimation and these features make it applicable in the industry as well as for tutorial purposes.

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