

# *Escherichia coli* viability determination using dynamic light scattering: a comparison with standard methods

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**Abstract** To determine the concentration of bacteria in a sample is important in the food industry, medicine and biotechnology. A disadvantage of the plate-counting method is that a microorganism colony could arise from one cell or from many cells. The other standard methodology, known as optical density determination, is based on the turbidity of a suspension and registers all bacteria, dead and alive. In this article, dynamic light scattering is proposed as a fast and reliable method to determine bacterial viability and, consequently, time evolution. *Escherichia coli* was selected because this microorganism is well known and easy to handle. A correlation between the data from these three techniques was obtained. We were able to calculate the growth rate, usually determined by plate counting or optical density measurement, using dynamic light scattering and to predict bacterial behavior. An analytical relationship between the colony forming units and the light scattered intensity was also deduced.

**Keywords** Total viable count · Dynamic light scattering · Plate count method · Optical density · Master curves · *Escherichia coli*

## Introduction

To determine the concentration of microorganisms in a sample and their evolution in time, plays numerous and important roles in microbiological characterization and experimentation. Particularly, determining viability of pathogen and non-pathogen bacteria is important in the food industry, medicine and biotechnology: It allows the evaluation of important parameters like the shelf life of certain products, the nutritional properties of food, the bacterial growth rate, etc. Standard methods for determining bacterial populations are the plate count method and the spectrophotometric (turbidimetric) analysis, i.e., optical density (OD) determination.

The viable count, which can be obtained using different techniques, is an indirect method that estimates the number of colony forming units per milliliter (CFU/ml) in a bacterial population, i.e., only living organisms are counted (Madigan et al. 2000). To obtain confident results, prior to plate counting, the culture must have the right bacterial concentration and the methodology must be repeated several times. Furthermore, the presence of clumps from various numbers of bacteria in the original suspension may alter the results, i.e., a colony could arise from one cell or from many cells. OD determination is faster than plate counting; however, since it is based on turbidity it registers all bacteria (cell biomass), dead and alive.

Even if the plate count method is well established and accepted for most applications, more reliable, faster and easier methodologies are still desired to determine the viability of bacteria. In this article, we describe the use of dynamic light scattering (DLS) as an alternative to plate counting and OD. DLS allows determination of the particle size as well as of the total scattered light intensity, which is proportional to the number density

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of the scattering particles. This means that the slope of the scattered intensity profile is directly related to the growth rate of viable bacteria. During DLS determinations, the particles (bacteria) contained in a solution inside a transparent cuvette are exposed to a monochromatic and coherent light source (He–Ne laser). A fast photon counter records the intensity of the light scattered by the particles as a function of time. If the particles are moving, the motion shifts the frequency of the scattered light (Doppler effect), resulting in a light intensity correlation function which depends on the type of motion of the particles. If the temperature is constant, the correlation function of the scattered light is only related to the size and number concentration of the particles. Filtration of the solution contained inside the cuvette is critical to remove dust and non-desired particles.

As mentioned above, a potential source of error in the plate count method is that a colony can arise from one cell or from many cells. The situation is similar for DLS, because this technique cannot differentiate whether the scattering particles arise from one cell or from many cells. DLS determines the motion of the scattering particles and consequently their sizes by using the Doppler effect (Berne and Pecor 1976). In addition to obtaining information on the particle size, with DLS it is possible to know whether bacteria are dead or alive, i.e., living bacteria reproduce, increasing the number concentration of scattering particles and consequently the total scattered intensity. This is obtained using the zero-time correlation function (Berne and Pecor 1976).

Each technique has its own characteristic concentration range. To obtain the ranges commonly accepted for countable numbers of colonies on a plate (30–300), typical concentrations are in the range from  $10^{-7}$  to  $10^{-9}$  CFU/ml, while for DLS and OD the values only range from  $10^{-1}$  to  $10^{-2}$  CFU/ml. Due to these large differences in concentrations, it is desirable to have alternative methods to determine the total viable count of bacteria. Additionally, DLS allows building master curves to predict the number of microorganisms by simple methods. Master curves were first employed in rheology in the time–temperature equivalence principle (Andrews and Tobolsky 1951; Struik 1978; Rongzhi 2000; Hiemenz and Lodge 2007; van Gurp and Palmén 2007). The use of these curves allows the prediction of the behavior of a system in one variable by changing another variable.

In this study, three different characterization techniques were compared to determine the number of viable bacteria: DLS, plate counting and OD. The comparison revealed that DLS can be used as an alternative method to determine the viable count of bacteria. Bacterial growth rate can be deduced from the slope of the profiles obtained using this technique. A non-pathogenic strain of *Escherichia coli* was

selected for this study, because it is easy to handle, safe and grows rapidly (doubling time 20 min at 37 °C).

## Materials and methods

### Bacterial strains and sample preparation

A strain of *Escherichia coli* (*E. coli*) ATCC 25,922 was obtained from the culture collection of the Universidad Autónoma de Querétaro, Qro., Mexico, stored in nutrient agar (Merk, Darmstadt, Germany) at 4 °C and transferred monthly. Microorganisms were activated by incubation on trypticase soy broth supplemented with 0.6 % (w/v) yeast extract (TSBY) at 36 °C/24 h. Two Erlenmeyer flasks, containing 4 ml of the initial 24 h culture, inoculated into 16 ml fresh TSBY at 36 °C were prepared. Serial dilutions were obtained from the suspensions contained in these two identical flasks. From one of them, a small sample was taken and diluted to obtain concentration  $c_1$ . A small sample was taken from this dilution ( $c_1$ ) and diluted to obtain concentration  $c_2$  and so on. The samples for plate counting and OD determination were taken from flask 1, and the samples for DLS were obtained from flask 2. The whole experiment, i.e., plate counting, OD determination and DLS was repeated three times. In this article,  $c_0$  refers to the original bacterial suspension and  $c_{-n}$  to a concentration of  $c_0 \times 10^{-n}$ . Using a lower inoculum size would result in a long lag phase, significantly extending the duration of the experiment.

### Plate count method

Cells suspensions were diluted with 9 ml isotonic saline solution (0.9 % NaCl w/v) to obtain serial dilutions ( $10^{-1}$ – $10^{-9}$ ) from bacterial suspensions. The procedure was repeated taking cells from the Erlenmeyer flask (maintained at 36 °C) from  $t_1 = 0$  to  $t_9 = 240$  min, i.e., every 30 min, to fill nine test tubes (one for each dilution). Cell counts were determined by pour plating on TSBY. Agar plates were incubated at 36 °C/24 h and CFU counted using a colony counter (Darkfield Colony Counter, Leica Inc., NY, USA). Only the last three suspensions ( $10^{-7}$ ,  $10^{-8}$  and  $10^{-9}$ ) were counted. Plate counting, known as the “total viable count”, is the method of reference to determine the number of microorganisms in a sample.

### Optical density measurement

The absorbance technique, also called optical density measurement, determines the fraction of electromagnetic radiation absorbed by a sample at specific wavelengths (Ingle and Crouch 1988; Zitzewitz 1999; Hollas 2004). The

absorbance  $A_\lambda$  is expressed as a logarithmic ratio between the incident radiation  $I_o$  on a material and the transmitted radiation  $I_t$  through a material:

$$A_\lambda = \log_{10} \left( \frac{I_o}{I_t} \right) \quad (1)$$

where  $A_\lambda$  is the absorbance at a specific wavelength. It is important to mention that this technique assumes that the scattered light is negligible as compared with the transmitted light. OD measurements have limitations when the sample shows a relatively high turbidity, due to the presence of a large amount of individual microorganisms that scatter light. The average cell mass in a culture must first be determined whether the turbidity of a culture is to be used to estimate cell count. Additionally, transmitted light depends on the concentration of particles in the sample and on their sizes. All OD measurements were made on an Uro-spec 3,300 pro UV/visible spectrophotometer (GE Healthcare Life Sciences, PA, USA) at 600 nm. To determine the number of bacteria by OD, samples of the first two dilutions ( $10^{-1}$  and  $10^{-2}$ ) were placed inside the spectrophotometer, starting at  $t_1 = 0$  and finishing at  $t_9 = 240$  min, i.e., absorbance was recorded every 30 min with one cuvette per dilution.

#### Dynamic light scattering

DLS allows determination of the particle size, the particle size distribution and the number concentration of scattering particles, dissolved or suspended in a liquid (Berne and Pecor 1976). This technique is based on the inelastic scattering of photons from a laser, upon the interaction with the scattering particles. In this way, it is possible to determine the diffusion coefficient that is directly related to the particle sizes through the Stokes–Einstein relationship:

$$D = k_B T / 6\pi \eta_0 R \quad (2)$$

where  $k_B$ ,  $T$ ,  $\eta_0$  and  $R$  are the Boltzmann constant, the absolute temperature, the liquid viscosity and the radius, respectively. In this particular case, a He–Ne laser emitting at 632.8 nm was used. Additionally, from the total scattered intensity ( $I_s$ ), it is possible to obtain information on the growth rate of bacteria during the incubation process (Berne and Pecor 1976):

$$I_S = I_0 K c_m M \quad (3)$$

where  $I_0$  is the incident light intensity,  $K$  is a constant that depends on several parameters (the refractive index  $n$ , the rate  $n$  changes with concentration  $dn/dc$ , the wavelength of light  $\lambda$  and the structure factor which depends on the particle shape),  $c_m$  is the mass concentration and  $M$  the molecular weight of the scattering particle. The former equation can also be written as:

$$I_S = I_0 K' c_n R^6 \quad (4)$$

being  $K'$  a constant,  $c_n$  the number concentration and  $R$  the particle radius. Then, the scattered intensity depends linearly on the concentration  $c_n$  of bacteria and other scattering particles. Salts and nutrients have significant smaller sizes than bacteria and, consequently, have a negligible contribution to the scattered intensity.

As mentioned,  $c_n$  is the sum of the number concentration of bacteria and other scattering particles:  $c_n = c_n^{(\text{bac})} + c_n^{(\text{other particles})}$ . Since only viable bacteria reproduce, they change their population as a function of time, so that

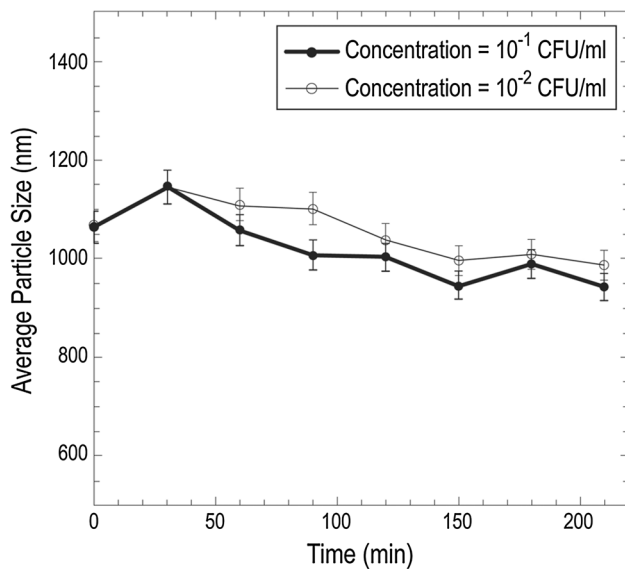
$$\frac{dc_n}{dt} = \frac{dc_n^{(\text{viable bac})}}{dt} \quad (5)$$

Taking the temporal derivate of Eq. (4) we obtain:

$$\frac{dI_S}{dt} = I_0 K' R^6 \frac{dc_n^{(\text{viable bac})}}{dt} \quad (6)$$

The slope of the scattered intensity profile ( $dI_S/dt$ ) provides a measurement proportional to the growth rate of viable bacteria.

Dynamic light scattering measurements were performed on a research goniometer and laser light scattering system, model BI200SM (Brookhaven Instruments Corp., NY, USA), equipped with a high-speed digital correlator (PCI-BI9000AT), a solid-state light detector and a 35 mW He–Ne laser model 9,167 EB-1 (Melles Griot, Albuquerque, NM, USA); the scattering angle was set to  $90^\circ$  in all cases. Samples for DLS were taken from flask number two, which was prepared at the same moment, with the same strain and following the same methodology as for flask 1, i.e., samples in both flasks were equally prepared. All vials were thoroughly washed with filtered (0.2 microns, Cole Parmer Instrument Company, IL, USA) distilled water, sterilized and filled with 9 ml 0.9 % (w/v) NaCl filtered (0.2 microns) solution. The cuvettes for the DLS system were washed, filled with filtered (0.2 microns) distilled water and placed inside the DLS equipment to assure that no particles were registered. After this, the mother culture was added to the distilled water inside the cuvettes, to obtain dilutions as described above; however, in this part of the experiment only the first ( $10^{-1}$ ) and second ( $10^{-2}$ ) dilutions were needed to determine particle size and scattered intensity. The total incubation time during DLS determination was 210 min. Every 30 min, two cuvettes (one for each dilution) containing 5 ml of the bacterial suspension were placed inside the light scattering system to record scattered intensity and particle size; this measurement took about 10 s. The average of five scattered intensities and five measured particle sizes per cuvette was recorded.



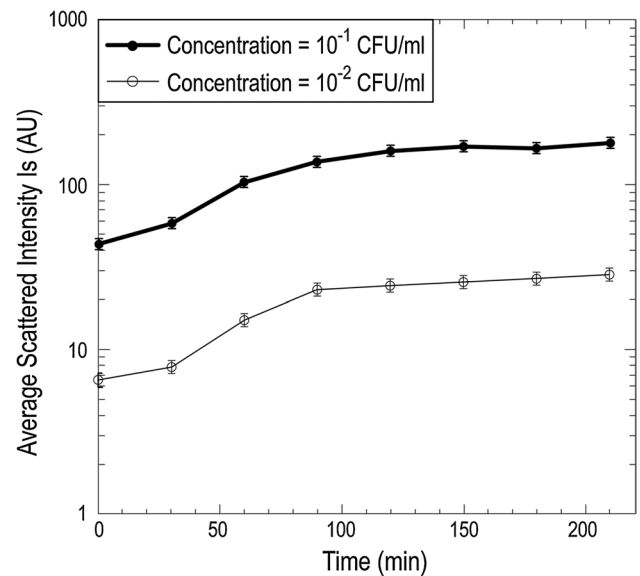
**Fig. 1** Average particle size as a function of the incubation time obtained from dynamic light scattering at concentrations  $c_{-1}$  and  $c_{-2}$

## Results and discussion

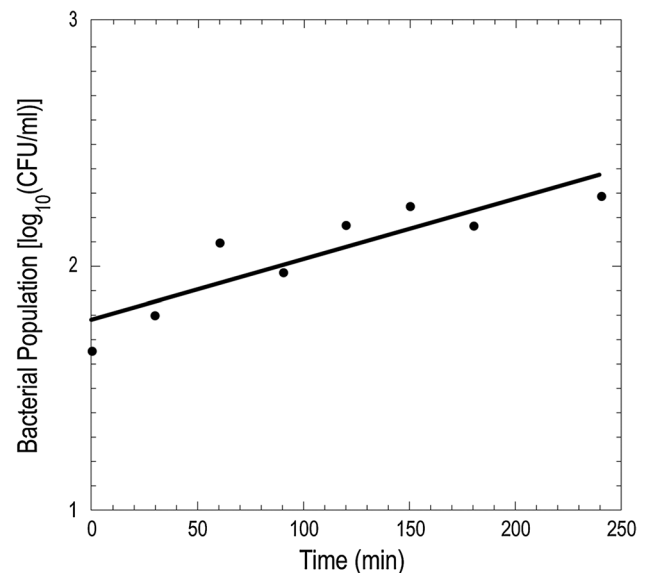
Figure 1 shows the average particle size profiles obtained from the DLS equipment for two different concentrations:  $c_{-1}$  and  $c_{-2}$ . At the beginning of the incubation process, there is a slight increment in particle size, probably due to improved conditions of food and space at the appropriate temperature that render in a fast bacterial growth with possible aggregation processes which increase the measured particle size moderately. However, after about 30 min, the size starts to decrease slowly from 1,150 nm to about 900 and 1,000 nm for  $c_{-2}$  and  $c_{-1}$ , respectively. This may be due to a reduction in the concentration of nutrients, and the available space that renders in bacteria with small size. It is possible to observe that, within the experimental error (standard deviation), the particle size profiles are statistically the same for the two concentrations. This is an expected result because the particle size is independent of the concentration.

Figure 2 shows the average scattered intensity profiles obtained from DLS for the three equally prepared replicas, at concentrations  $c_{-1}$  and  $c_{-2}$ , plotted on a semi-log scale. These profiles reveal that the scattered intensity  $I_s$  grows monotonically with the incubation time, showing three different regimes according to bacterial rate of growth: the first one (from 0 to 30 min) characterized by a small slope, the second one (from 30 to 90 min) has a large slope, and the third regime (from 90 to 240 min) shows a slope close to zero.

According to Eqs. (4) and (6), the scattered intensity depends on the particle size ( $R$ ) and the number

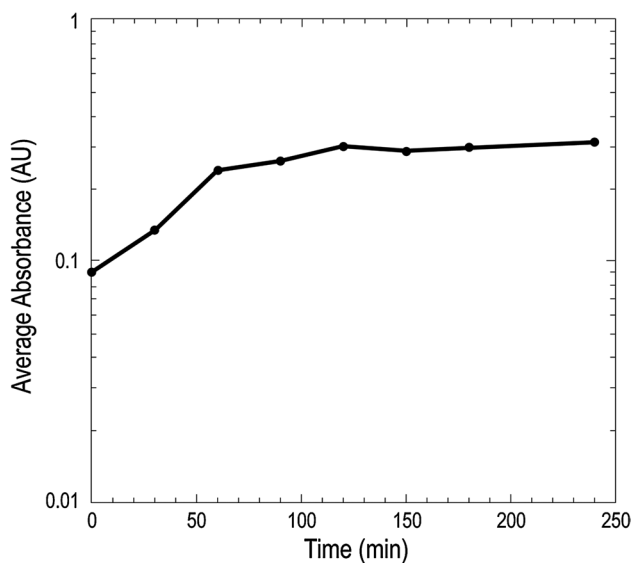


**Fig. 2** Average scattered intensity  $I_s$  as a function of the incubation time obtained from dynamic light scattering for concentrations  $c_{-1}$  and  $c_{-2}$  on a semi-log scale. The error bars are indicated on each plot. AU arbitrary units



**Fig. 3** Bacterial population obtained by the plate count method at concentration  $c_{-7}$  as a function of the incubation time on a semi-log scale. The data were fitted using a linear relationship in  $\log_{10}$  (CFU/ml) and time variables

concentration ( $c_n$ ). Since the variation in particle size is significantly small compared to the average value during the time of the experiment, the scattered intensity is proportional to the number concentration  $c_n$ . The first regime corresponds to the lag phase, characterized by a slow growth of the bacterial population. It is defined as the initial phase in the growth of a population when bacteria are adjusting to



**Fig. 4** Average absorbance versus incubation time at concentration  $c_{-1}$  on a semi-log scale. *AU* arbitrary units

the medium. The physiochemical environment of the original and the new growth medium, as well as the amount and type of microorganisms influence the duration of this period (Swinnen et al. 2004). The second regime (exponential phase) is characterized by a fast growth of the population because bacteria are in appropriate conditions of food, space and temperature. In this phase, also referred to as the logarithmic (log) phase, if nutrients are freely available all cells divide as fast as possible and the number of new bacteria per unit time is proportional to the current population. After certain time, the medium is short of nutrients and full of wastes. This is the beginning of the stationary phase, characterized by saturation of the bacterial number (Madigan et al. 2000). This behavior was observed for both concentrations. It is important to point out that both  $I_S$  and  $\log_{10}(I_S)$  cannot be fitted as a linear function of the incubation time.

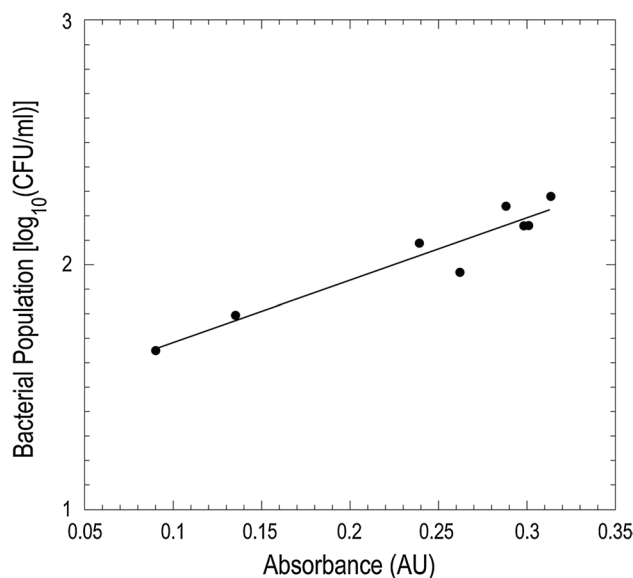
The results for the plate count method are reported in Fig. 3. This figure shows the bacterial population as a function of time on a semi-log scale for concentration  $c_{-7}$ . The bacterial population, i.e.,  $\log_{10}(\text{CFU/ml})$ , grows approximately linearly with the incubation time: the continuous line corresponds to a linear fitting of  $\log_{10}(\text{CFU/ml})$  versus time:

$$\log_{10}(\text{CFU/ml}) = 1.778 + 0.00245t \quad (7)$$

with a correlation coefficient of 0.885. On a semi-log scale, this linear fitting corresponds to an exponential dependence of CFU/ml with time:

$$\text{CFU/ml} = (59.951)10^{0.00245t} = (59.951)e^{0.00564t} \quad (8)$$

As expected, this result reveals that, during bacterial incubation, the population growth rate was not constant.



**Fig. 5** Plot of bacterial population as a function of the average absorbance on a semi-log scale. The continuous line corresponds to a fitting using Eq. (7). *AU* arbitrary units

The OD results, reported in Fig. 4, have a similar behavior as those obtained from DLS (Fig. 2). This figure shows the average absorbance as a function of the incubation time on a semi-log scale. As it can be noticed, it is not possible to fit the absorbance as a function of the incubation time with a straight line.

A plot of bacterial population obtained from plate counting as a function of the absorbance recorded by OD measurements is shown in Fig. 5, where the experimental data were fitted using the linear equation:

$$\log_{10}(\text{CFU/ml}) = a + b(\text{OD}) \quad (9)$$

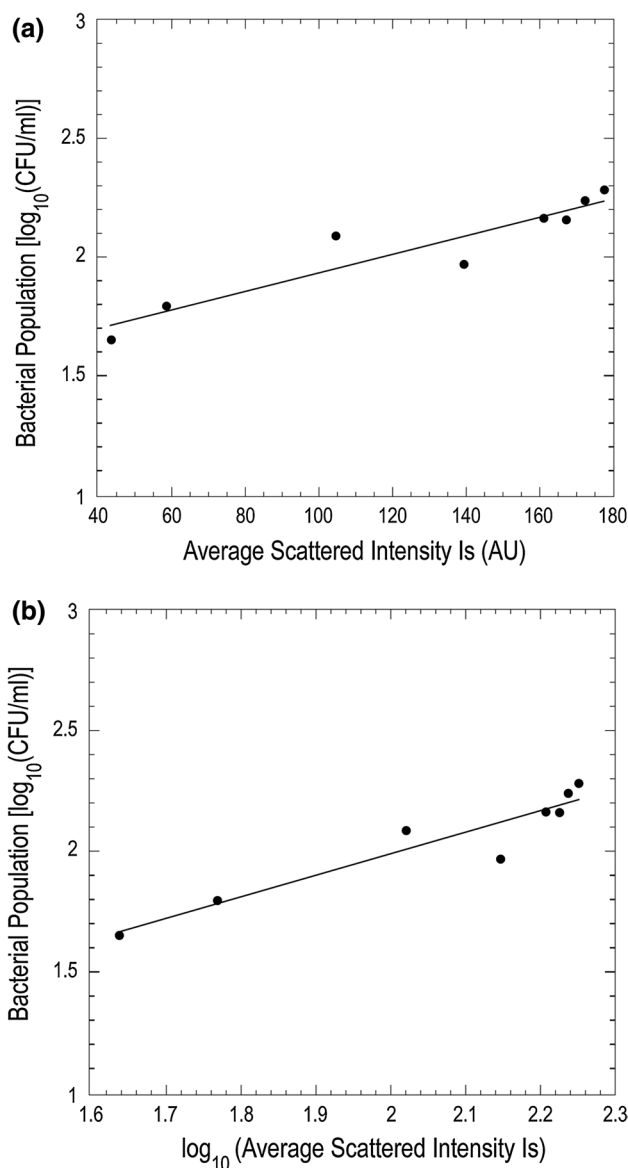
where  $a$  and  $b$  are the fitting parameters. The results of the fitting procedure produce  $a = 1.434$  and  $b = 2.535$ , with a correlation coefficient = 0.955:

$$\log_{10}(\text{CFU/ml}) = 1.434 + 2.535(\text{OD}) \quad (10)$$

This correlation coefficient shows that the linear function fits the  $\log_{10}(\text{CFU/ml})$  versus OD data well. It is important to mention that this linear function fits all the experimental data from the beginning of the lag phase to the stationary phase. Equation (10) can be written as:

$$\text{CFU/ml} = 27.1456e^{5.838(\text{OD})} \quad (11)$$

Figure 6a, b shows plots of the bacterial growth rate obtained by plate counting as a function of  $I_S$  on a semi-log scale and on a log scale, respectively. To obtain Fig. 6a, the data points were fitted, as in the former case, using the linear function:



**Fig. 6** Plots of bacterial population as a function of: (a) the average scattered intensity  $I_S$  and (b)  $\log_{10}(I_S)$ . The continuous lines correspond to fitting using Eqs. (10) and (13). AU arbitrary units

$$\log_{10}(CFU/ml) = a + b(I_S) \tag{12}$$

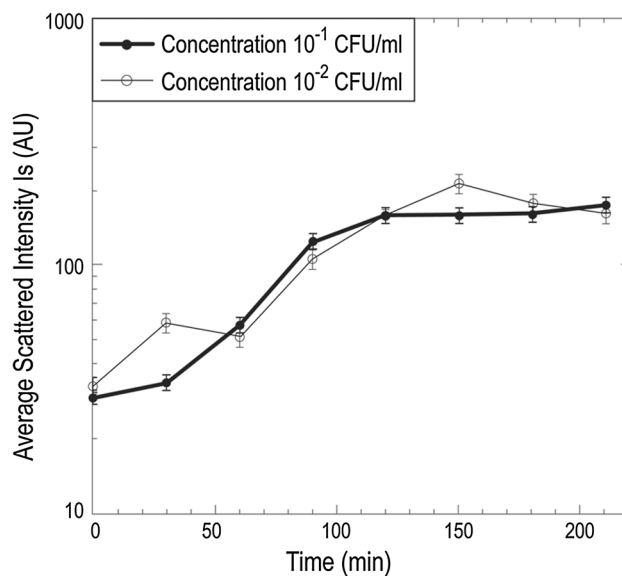
The fitting using this equation produces:

$$\log_{10}(CFU/ml) = 1.543 + 0.004(I_S) \tag{13}$$

with a correlation coefficient of 0.938, indicating that these data were well fitted by this equation. Equation (13) can also be written as:

$$CFU/ml = 34.93e^{0.009I_S} \tag{14}$$

For the plot of  $\log_{10}(CFU/ml)$  versus  $\log_{10}(I_S)$ , shown in Fig. 6b, the fitting using a linear relationship produces:



**Fig. 7** Plot of the averaged scattered intensity  $I_S$  as a function of the incubation time for concentrations  $c_{-1}$  and  $c_{-2}$ . The  $I_S$  profile for  $c_{-2}$  was shifted using Eq. (17):  $I_{S2} = I_{S1} (c_{n2}/c_{n1})(R_2/R_1)^6$  to overlap the curve obtained for concentration  $c_{-1}$ . AU arbitrary units

$$\begin{aligned} \log_{10}(CFU/ml) &= a + b \log_{10}(I_S) \\ &= 0.213 + 0.889 \log_{10}(I_S) \end{aligned} \tag{15}$$

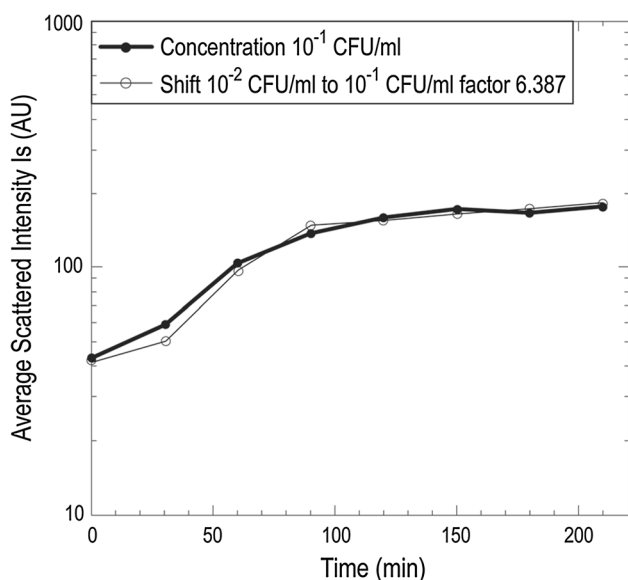
with a correlation coefficient equal to 0.945. This equation can also be written as a power law equation:

$$(CFU/ml) = 1.633(I_S)^{0.889} \tag{16}$$

However, the exponent in this power law equation is close to one, so there is practically a linear relationship between CFU/ml and  $I_S$ . For low values of  $I_S$  (which is our case), Eq. (14) can be written as a linear relationship between CFU/ml and  $I_S$ . This means that both plots shown in Fig. 6 produce the same result: the CFU/ml obtained by plate counting can be linearly related to the  $I_S$  obtained by DLS within the whole time interval of bacterial growth. According to Fig. 6b, the best correlation coefficient (0.945) was obtained plotting  $\log_{10}(CFU/ml)$  as a function of  $\log_{10}(I_S)$ .

Our results support the use of DLS as an alternative to determine the viable count using either Eqs. (14) or (16) to obtain the CFU/ml just by measuring  $I_S$ . DLS is a fast, clean and reliable technique that provides, in addition to  $c_n$ , the particle size  $R$ . The profiles obtained from DLS clearly show the different regimes produced during the incubation process of bacteria. Additionally, it is also possible to detect contamination of the samples, because impurities would affect  $c_n$ ,  $R$ , or both.

Figure 7 shows two  $I_S$  profiles obtained from DLS at two different concentrations:  $c_{-1}$  and  $c_{-2}$ . These two profiles were shifted one toward the other using Eq. (4):



**Fig. 8** Master curve obtained from dynamic light scattering for the average scattered intensity  $I_s$  at different concentrations. The shifting factor is 6.387. Error bars are not shown. AU arbitrary units

$$I_{S2} = I_{S1} \left( \frac{c_{n2}}{c_{n1}} \right) \left( \frac{R_2}{R_1} \right)^6 \quad (17)$$

According to this equation, it is possible to obtain the complete  $I_{S2}$  profile from the  $I_{S1}$  knowing the number concentration ( $c_{n2}$ ) and the particle size ( $R_2$ ) from the unknown sample. This increases the application possibilities of DLS to obtain information about plate counting.

As mentioned before, the graph resulting from overlapping similar profiles obtained at different conditions is called “master curve”. Figure 8 shows a master curve for DLS using two profiles (concentrations  $c_{-1}$  and  $c_{-2}$ ) for DLS. Overlapping of both  $I_s$  curves is good, providing confidence in respect to Eq. (17). According to our results, it is enough to have a complete profile for one concentration and the shifting factors to know the other profiles. The shifting factor required to build this master curve is included in the figure. Since the scattered intensity strongly depends on the particle size when the number concentration is used (Eq. 4), it is important to have a precise determination of the particle size to have a good overlapping.

## Conclusions

The results of a comparative study between the kinetics of bacterial growth, determined by plate counting, dynamic light scattering and optical density, as well as an analytical correlation between the data obtained from these three methodologies were reported in this article. The viable

count, traditionally estimated using the plate count method, was determined by DLS. Compared to plate counting, DLS has the following advantages: (a) It is a faster technique, allowing to follow the kinetics of bacterial population, (b) less dilutions are required, (c) it provides the particle size of the scattering particles (bacteria) allowing the detection of potential problems with the bacterial growth related with changes in size, (d) it is less sensitive to the contamination produced during plate counting, (e) it produces better statistical results, because the number of bacteria counted is significantly larger ( $c_{-1}$ ,  $c_{-2}$ ) compared to plate counting ( $c_{-7}$ ,  $c_{-8}$ ,  $c_{-9}$ ), (f) DLS equipments are common to many laboratories and (g) it is an alternative technique to elucidate the presence of potential problems that may arise during handling of bacteria.

DLS is a powerful technique; however, its main limitation is the possible presence of large particles (dust, contaminants, dirt particles, etcetera), because they would have a strong influence on DLS measurements. It is not possible to determine viable counts in food samples, because the contribution to the scattered intensity due to big particles is extremely large as compared to the contribution from bacteria.

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