Comparison of Two Optical-Density-Based Methods and a Plate Count Method for Estimation of Growth Parameters of *Bacillus cereus*¹

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Quantitative microbiological models predicting proliferation of microorganisms relevant for food safety and/or food stability are useful tools to limit the need for generation of biological data through challenge testing and shelf-life testing. The use of these models requires quick and reliable methods for the generation of growth data and estimation of growth parameters. Growth parameter estimation can be achieved using methods based on plate counting and methods based on measuring the optical density. This research compares the plate count method with two optical density methods, namely, the 2-fold dilution (2FD) method and the relative rate to detection (RRD) method. For model organism *Bacillus cereus* F4810/72, the plate count method and both optical density methods gave comparable estimates for key growth parameters. Values for the maximum specific growth rate (*μ*<sub>max</sub>) derived by the 2FD method and by the RRD method were of the same order of magnitude, but some marked differences between the two approaches were apparent. Whereas the 2FD method allowed the derivation of values for lag time (λ) from the data, this was not possible with the RRD method. However, the RRD method gave many more data points per experiment and also gave more data points close to the growth boundary. This research shows that all three proposed methods can be used for parameter estimation but that the choice of method depends on the objectives of the research.

Food products are required to be safe and sufficiently stable within their given shelf-lives. For this reason, generally some kind of preservation is applied. An often-used preservation method is mild heat treatment, such as pasteurization, which inactivates vegetative microbial pathogens and spoilage microorganisms. *Bacillus cereus* is a Gram-positive, facultative anaerobic, spore-forming rod (17) that can be found in, for example, soil, food, and the human gastrointestinal tract (23). Viable spores present in a food product may germinate, and the vegetative cells may grow if conditions are favorable. In order to delay or prevent this, one or more hurdles for germination and outgrowth need to be present in the food or food environment. Examples of such hurdles are the acidification of food and addition of salt (12). When a combination of hurdles is used, generally the intensity of the hurdles may be lower to show a preservative effect comparable to the level of those hurdles when they are used individually (24).

In this investigation, the use of acidification as a hurdle to prevent growth of *B. cereus* was studied. The amount of acid to be added to the food product is of importance for both safety reasons and organoleptic properties. *B. cereus* will not be able to grow at low pH values (pH 5 to 6, depending on the acidulant) (1), but, on the other hand, the food product should not be too acid, given consumer preferences. Research showed that children do not like orange juice with concentrations of citric acid above 0.02 M; for adults this value is 0.04 M (25).

The lowest pH value allowing growth of *B. cereus* can be investigated by experiments culturing the microorganism in a suitable growth medium or food product with different pH values and using the viable plate count method for enumeration (33). Using such an approach to generate biological data for safety or shelf-life evaluations is considered both slow and human resource-intensive since experiments have to be repeated for every new condition. Quantitative microbiological modeling can speed up experimentation and reduce the resources required. Should modeling make it possible to predict the behavior of *B. cereus* over a wide range and a variety of conditions, then it would help to limit the need for experimentation to the point where just validation of predictions is needed.

For successful use of modeling, the maximum specific growth rate (*μ*<sub>max</sub>) and, preferably, also the lag time (λ) have to be known. These parameters can be determined using different techniques. Plate counting can be used to generate the data points *μ*<sub>max</sub> and λ by fitting of the growth curve (15, 33). Often, automated optical density (OD) measurements are used to determine parameters for growth as they allow quicker data generation with less need for human resources (22). It is not possible, however, to directly translate OD values to *μ*<sub>max</sub> values due to the high detection limit of OD readers of ~10<sup>7</sup> viable cells. When cells reach their maximum cell density, they proliferate at a progressively slower rate. Consequently, values for *μ*<sub>max</sub> would be consistently underestimated by using OD values. For this reason, OD measurements are often used in combination with time-to-detection (TTD) measurements (5). Values for *μ*<sub>max</sub> can be derived from OD measurements and TTD measurements by the 2-fold dilution (2FD) method (5,
28, 36). The 2FD method uses TTD and inoculum size variations to obtain values for both \( \mu_{\text{max}} \) and \( \lambda \). The relative rate to detection (RRD) method also uses OD measurements and TTD measurements to obtain parameters for growth. This method uses the ratio between TTD at the tested and the optimal conditions and can compute \( \mu_{\text{max}} \) but not \( \lambda \) (20, 21, 37). Notably, plate count data allow both \( \mu_{\text{max}} \) and \( \lambda \) to be derived, and this method is required as an addition to the RRD method.

Although both the 2FD method and the RRD method make use of OD measurements, it is not known whether the two techniques result in comparable estimates of growth parameters and whether these values match values obtained with plate counts. Growth parameters determined by plate count and the 2FD method were previously compared in several studies (5, 27). The present study set out to compare the three different methods for the assessment of parameters for growth on the basis of the following, mainly measurable, criteria: the number of data points obtained per experiment, the measuring intensity, the time consumption per experiment, the reproducibility of the method as determined by comparing the standard deviations of the average \( \mu_{\text{max}} \) at pH 7, the number of parameters obtained, and the number of criteria necessary for data analysis. \( B.\ cerus \) F4810/72 was used as the model organism. It was cultured at different pH values, a common hurdle in food products, to obtain a variety of growth rates.

**MATERIALS AND METHODS**

**Bacterial strain and preparation of the standardized bacterial suspension.** \( B.\ cerus \) F4810/72, an emetic toxin producer, was originally isolated from human vomit (34). This strain is also known as \( B.\ cerus \) NCTC 11143, DSM 4312, and PAL 25 (35; Health Protection Agency Culture Collections [www.nctc.org.uk]). The culture was stored frozen at −80°C in cryovials (Greiner Bio-one GmbH; Frickenhausen, Germany) containing 0.3 ml of glycerol (87%; Fluka-Chemica GmbH, Buchs, Switzerland) and 0.7 ml of bacterial culture in brain heart infusion (BHI) broth (Becton Dickinson and Co.; Le Pont de Claix, France). For every experiment, a loopful of microorganisms was inoculated in a 500-ml physiological salt (PPS) solution. The cell suspensions were then pooled and fractionated to obtain a variety of growth rates.

Establishing experimental conditions. Initial estimates for the pH range for growth of \( B.\ cerus \) and the shape of its specific growth rate curve were obtained using the model of Presser et al. (30) (equation 1):

\[
\mu_{\text{max}} = \mu_{\text{opt}} \times (1 - 10^{-0.01\text{pH}_{\text{max}} - \text{pH}})
\]

where \( \mu_{\text{max}} \) is the maximum specific growth rate (h⁻¹), \( \mu_{\text{opt}} \) is the growth rate under optimal growth conditions (h⁻¹), \( \text{pH}_{\text{max}} \) is the minimum pH value required for growth, and pH is the pH value of the broth used for testing. The initial estimates from literature of \( \text{pH}_{\text{max}} \) and \( \mu_{\text{opt}} \) were 4.6 (4) and 2 h⁻¹, respectively (38). Based on these parameter estimations and the resulting \( \mu_{\text{max}} \) values of this curve, the pH values for the experiments were chosen in such a way that the increases in \( \mu_{\text{max}} \) between consecutive pH values were of the same order of magnitude.

Three methods were applied to investigate the effect of pH on \( \mu_{\text{max}} \) and, where possible, \( \lambda \); the plate count method (8), the 2FD method (5), and the RRD method (20).

**Estimating \( \mu_{\text{max}} \) and \( \lambda \) as a function of pH using the 2FD method.** The 2FD method uses the time to reach a predefined OD level (reflective visible growth) of 2-fold inoculum size variations to obtain \( \mu_{\text{max}} \) and \( \lambda \) according to equation 2 (5):

\[
TTD = \frac{1}{\mu_{\text{opt}}} \times \ln \left( \frac{N_{\text{max}}}{N_0} \right)
\]

where TTD is the time to detection (h) of the inoculum level (i), chosen as the time at which a well reaches an \( \text{OD}_{\text{max}} \) of 0.2 (20), \( \lambda \) is the duration of the lag phase (h), \( N_{\text{max}} \) is the number of organisms per ml at which an \( \text{OD}_{\text{max}} \) of 0.2 is observed, \( N_0 \) is the number of organisms per ml of the inoculum, and \( \mu_{\text{opt}} \) is the maximum specific growth rate (h⁻¹). \( N_{\text{max}} \) was determined in a separate experiment where broth at nine different pH values was sampled as soon as some wells had reached an \( \text{OD}_{\text{max}} \) of 0.2, and the content was plated onto BHI agar to determine cell numbers.

Twenty target pH values were prepared, and the experiment was repeated twice, giving three data sets. For each experiment, 20 bottles containing 50 ml of BHI broth were pH adjusted, with values ranging from pH 4.4 to pH 7, using sucrose acid. The liquids were filtered sterilized (Stepton-Sterilip; Milipore Corporation, MA), and the standardized bacterial suspension was diluted 10,000-fold in pH-adjusted BHI broth using a new dilution series for every pH value and aimed for an initial cell concentration of approximately \( 10^5 \) CFU/ml. The pH 7 solution was spiral plated onto BHI agar plates in duplicate for enumeration. In both repetitions of the experiment, slight deviations of the target pH value occurred, resulting in a total number of 60 pH values for further data analysis.

All wells of the 100-well honeycomb plate (Oy Growth Curves AB Ltd; Helsinki, Finland) were filled with 150 µl of pH-adjusted BHI broth, with five wells filled per pH value. Every pH value was investigated in duplicate using a second honeycomb plate for the duplicate. Every first well of a pH series was inoculated with 150 µl of a particular target pH value-adjusted bacterial culture. Twofold dilutions were made up to the fifth well for that pH value. Both honeycomb plates were incubated in the Bioscreen C (Oy Growth Curves AB Ltd; Helsinki, Finland) at 30°C for 3 days with continuous, medium shaking, and the \( \text{OD}_{\text{max}} \) was measured every 10 min. The \( \text{OD}_{\text{max}} \) data obtained from the Bioscreen were imported in Microsoft Excel for data capturing and processing. Rarely, wells showed an initial \( \text{OD}_{\text{max}} \) above the detection limit due to pipetting errors or air bubbles in the liquid; when this occurred, such wells were removed from the data set. For all other curves the TTD, defined as the time to reach an \( \text{OD}_{\text{max}} \) of 0.2, was determined. The OD values were not corrected for the background color of the broth (−0.1 at \( \text{OD}_{\text{max}} \)). For wells not reaching an \( \text{OD}_{\text{max}} \) of 0.2 within the time frame of the experiment, viability of bacteria was determined by plating the content of the well. In the event that plates did not show colonies, bacteria were considered dead, and the \( \mu_{\text{max}} \) was set to 0 h⁻¹.

For processing of data regarding the 2FD method, wells of the same target pH were included, and \( \mu_{\text{max}} \) and \( \lambda \) were calculated using equation 2. Excel solver add-in, and a linear fit, minimizing the residual sum of squares (RSS). The mean square error (MSE) between the data and the fit was calculated using equation 3:

\[
\text{MSE} = \frac{\sum_{i=1}^{n} (\text{OD}_{\text{observed}} - \text{OD}_{\text{model}})^2}{n - s}
\]

where \( \text{OD}_{\text{observed}} \) (h) is the TTD when the well reaches \( \text{OD}_{\text{max}} \); \( \text{OD}_{\text{model}} \) (h) is the fitted TTD, and DF is the number of data points (3).

The following rules were applied for data exclusion: MSE > 5, \( \lambda < 0 \), \( \mu_{\text{max}} < 0 \), and \( \mu_{\text{max}} > 3.5 \). If any of the parameters was out of range, both resulting parameters \( \mu_{\text{max}} \) and \( \lambda \) for the same experimental condition were excluded from further data processing.
conditions (TTD_{opt}), in this case, at pH 7. The \( \mu_{\text{opt}} \) value was estimated independently by plate counting and fitting of the Gompertz model to the counts. Equation 4 was used to derive RRD values and to calculate \( \mu_{\text{max}} \) values for various test conditions from RRD and \( \mu_{\text{opt}} \):

\[
\mu_{\text{max}} = \frac{\mu_{\text{opt}}}{\text{RRD}} = \frac{\text{TTD}_{\text{opt}}(\text{OD}_{600} = 0.2)}{\text{OD}_{600} = 0.2}
\]

(4)

With respect to evaluating both OD methods, for the RRD method the well with the highest inoculum level for a particular target pH value was selected, and the RRD was calculated according to equation 4, using the TTD of the well with pH 7 as TTD_{opt}.

Estimating \( \mu_{\text{max}} \) and \( \lambda \) as a function of pH using the plate count method. Erlenmeyer flasks (500 ml) containing 100 ml of BHI broth were adjusted to pH 5 or pH 7 using sulfuric acid and inoculated with the standardized bacterial inoculum size (\( \ln N_{\text{inoc}} \)) in BHI broth at pH 6.93, linear fitting (solid line) of the data points to estimate \( \mu_{\text{max}} \), and estimation of \( \lambda \) using the theoretical TTD (dashed line) assuming \( \lambda = 0 \), according to the 2FD method of Cuppers and Smelt (5).

**RESULTS**

**Growth boundary.** The lower limit for growth of B. cereus F4810/72 in BHI broth acidified with sulfuric acid was pH 4.8, which is 0.2 above the published estimated growth boundary for B. cereus NCIMB 11796 (4). The initial estimate of the shape of the growth-pH curve (the pH model of Presser et al. [30]) (equation 1) is included in Fig. 1 for illustration purposes. The estimated \( \mu_{\text{max}} \) values are higher than the \( \mu_{\text{max}} \) values obtained using the 2FD method, but this is mainly caused by the difference in growth boundary, which caused a shift of the curve. The estimated \( \mu_{\text{max}} \) values for optimal growth conditions (pH 7) are equal.

**2FD method.** Figure 2 shows for one pH value (6.93) how \( \mu_{\text{max}} \) and \( \lambda \) were obtained using the 2FD method. The negative reciprocal slope of the curve fitting the data points equals \( \mu_{\text{max}} \) 2.05 h^{-1}, whereas \( \lambda \), which equals 0.80 h, is derived from the deviation from the theoretical TTD for a \( \lambda \) of 0. For every \( \mu_{\text{max}} \) and \( \lambda \) data point obtained with this method, five wells were used, limiting the number of data points to 20 per honeycomb plate. Figure 1 shows \( \mu_{\text{max}} \) and \( \lambda \) for 57 pH values out of the total of 60 pH values tested. All wells not showing growth (pH < 4.8) failed to generate colonies on the plate, indicating no surviving microorganisms. Two data points, pH 4.9 and pH 4.88, both close to the growth boundary, had to be excluded because of the MSE was >5. One data point (pH 4.83) had to be excluded because of an optical failure in the Bioscreen toward the end of the experiment. Figure 3 shows that at pH 7, the OD_{600} threshold value of 0.2 \( (N_{\text{turb}}) \) was approximately equivalent to 7.5 log CFU/ml. The exact value for \( N_{\text{turb}} \) is required for the 2FD method to calculate \( \lambda \), and this value was determined for nine different target pH values by enumerating the number of microorganisms in each well when the OD was different for every well, and no trend was visible. \( N_{\text{turb}} \) had an average value of 7.3 (±0.31) log CFU/ml. The 95% confidence interval of \( N_{\text{turb}} \) was calculated, and the highest and lowest values of the interval were used to determine the effect of various \( N_{\text{turb}} \) values on the calculated \( \lambda \). Two pH values were selected, the highest tested pH value (pH 7) and the lowest
tested pH value showing clear growth (pH 4.93). The value of \( \lambda \) for \( B.\ cerus \) in BHI broth of pH 6.93 varied between -0.5 and 0.86, whereas \( \lambda \) varied between -1.26 and 4.66 h for broth of pH 4.96, showing that \( \lambda \) cannot be estimated with reliability due to the variability in the microbial numbers at the TTD.

**RRD method.** For each Bioscreen run, 100 data points could be obtained since no inoculum size variations were necessary. In this investigation the same data set was used for testing both the RRD method and the 2FD method. Only the wells with the highest inoculum levels were selected for further investigation, and therefore RRD values were calculated as a function of pH for 59 different pH values obtained from three separate Bioscreen runs. The RRD values were multiplied by \( \mu_{\text{opt}} \) (2.42 h\(^{-1}\)) to obtain \( \mu_{\text{max},\text{RRD}} \) (Fig. 5).

Since the calculation of RRD does not require fitting of data points and therefore MSE values were not relevant, no data points needed to be excluded on the basis of MSE values. The data point at pH 4.83 was excluded because of equipment failure. The RRD data were transformed to \( \mu_{\text{max}} \) values by multiplying RRD by \( \mu_{\text{opt}} \), which was estimated at pH 7 by plate count to be 2.42 h\(^{-1}\). All calculated values for \( \mu_{\text{max}} \) met the criterion of a \( \mu_{\text{max}} \) of <3.5 h\(^{-1}\).

**Plate count method.** The \( \mu_{\text{max}} \) values for the individual experiments are displayed in Fig. 5. The mean \( \mu_{\text{max}} \) value for growth of \( B.\ cerus \) F4810/72 in BHI broth adjusted to pH 7 was 2.42 h\(^{-1}\) (±0.35). The best overall fit of the same data using the Gompertz equation was calculated to be 2.39 h\(^{-1}\) (±0.10). The mean \( \mu_{\text{max}} \) value in BHI broth adjusted to pH 5 was 0.63 h\(^{-1}\) (±0.08). The average value of \( \lambda \) for BHI broth of pH 7 was 2.41 h and varied between 1.87 h and 2.76 h. The average \( \lambda \) for cells cultured at pH 5 was found to be 7.62 h and varied between 7.02 h and 8.16 h. Values obtained by plate counting did not significantly differ from either of the other methods.

**DISCUSSION**

**Maximum specific growth rate determination.** The classical method to determine \( \mu_{\text{max}} \) is based on plate counting, where samples are taken in time and plated on agar. In this study the parameters \( \mu_{\text{max}} \) and \( \lambda \) were obtained by fitting the experimental data using the Gompertz model (39). The model of Rosso (32) returned \( \mu_{\text{max}} \) values which were 6% lower than the values obtained using the model of Gompertz when both models were fitted to the same data set. The Gompertz model was selected since it showed the lowest RSS value and also because of its smooth transition from the lag phase to the exponential phase. The choice for the Gompertz model is in line with a comparison made by other authors (39). The model of Presser et al. was used to predict the pH-growth rate curve and the range of pH values for the experiments to be performed. It proved to be useful to use a model and parameters from literature to design the experiment and select pH values to collect data points (Fig. 1).

Figure 3 shows an example of a growth curve for growth at optimal conditions, namely, pH 7. The derived \( \mu_{\text{max}} \) value (2.6 h\(^{-1}\)) fits within the range of 2.3 h\(^{-1}\) to 3.33 h\(^{-1}\) from published data for growth in laboratory media (10, 19). Both OD methods tested, the RRD method and the 2FD method, derived similar \( \mu_{\text{max}} \) values from the TTD data, so these alternative methods to classic plate counting could confidently be used. The variability between the TTD data for equal experimental conditions was minimal.

Various other studies showed that \( \mu_{\text{max}} \) can be derived from turbidimetric measurements (2, 6, 7); Lindqvist (26) compared TTD methods and turbidimetric methods and considered the TTD methods best to estimate the growth rates. None of the above studies, however, considered the use of the RRD method (20).

When the RRD method is used, \( \mu_{\text{opt}} \) has to be estimated in a separate experiment to obtain \( \mu_{\text{max}} \). If \( \mu_{\text{opt}} \) is wrongly estimated, the resulting \( \mu_{\text{max}} \) value will be heavily affected. Estimating \( \mu_{\text{opt}} \) can be done by plate counting of a growing culture of a particular strain using optimal growth conditions or by using the 2FD method. Multiple repetitions of this experiment are necessary to establish a reliable value for \( \mu_{\text{opt}} \). Once established, this same value for \( \mu_{\text{opt}} \) can be used for every experiment. The \( \mu_{\text{opt}} \) value used to establish \( \mu_{\text{max},\text{RRD}} \) is the average of the five \( \mu_{\text{max}} \) values obtained by plate count at pH 7. Since the plate count method at pH 7 shows a large variability in \( \mu_{\text{max}} \) (Fig. 5), the data points do not adequately
reflected a best fit of the growth rate. Since the \( \mu_{\text{max,RRD}} \) data are based on \( \mu_{\text{opt}} \) obtained by plate count, these two methods are related, and both therefore show the same conservative estimate. Differences between the methods might be caused by the difference in aeration levels between the Erlenmeyer flask and the microtiter plate (9), although they were assumed to be equal. Since use of a Bioscreen and microtiter plates is a convenient method to allow high-throughput screening of different growth conditions, this assumption is unavoidable. Considering the difference in aeration between plate counts and Bioscreen experiments, the RRD method is favored over the 2FD method. For the RRD method the relative difference between two test conditions is determined, so possible oxygen effects are considered to be equal and do not contribute to the final RRD value. When \( \mu_{\text{opt}} \) is determined using plate counts, without any negative growth effects due to low aeration, the \( \mu_{\text{max}} \) value obtained with the RRD method is considered equal to the values obtained using plate counts.

More important for safety is the behavior of the methods under more growth-inhibiting conditions, and here the difference is significantly smaller than under optimal growth conditions. This difference is supported by the residuals, which showed a consistent difference and were not normally distributed. This difference may be explained by the way the TTD data were analyzed. The inoculum level of the culture was \( 10^4 \) cells/ml, and the detection limit of the Bioscreen (OD\text{\_\_}gal = 0) is \( \approx 10^2 \) cells/ml. TTD is defined as the time until the threshold value of OD\text{\_\_}gal 0.2 is reached. This OD\text{\_\_}gal threshold value was chosen because it is well above the detection limit of the Bioscreen C, and such a threshold helps avoid false-positive growth samples due to fluctuations in optical density close to the detection limit of the Bioscreen C. Considering the inoculum level, the TTD consists of a lag time and the time necessary to reach the OD\text{\_\_}gal threshold value, while the bacterial culture is already exponentially growing. For this reason, the TTD method is not able to distinguish \( \lambda \) separately. The increase of \( \lambda \) around the growth boundary becomes significantly longer than under optimal conditions, as has been reported for Listeria monocytogenes (31) and Pseudomonas fragi (13). The increase of \( \lambda \) closer to the growth boundary is also seen for B. cereus (Fig. 1). The use of the RRD method relies on the assumption that TTD\text{\_opt}/TTD\text{\_l} = \mu_{\text{opt}}/\mu_{\lambda}. Since TTD does not distinguish between \( \mu_{\text{max}} \) and \( \lambda \), this assumption can only be made when the product of \( \mu_{\text{max}} \) and \( \lambda \) is constant for all pH values. If this assumption is not valid, for example, when the RRD is calculated for data close to the growth boundary, the ratio between TTD\text{\_l} and TTD\text{\_opt} changes more than expected since \( \lambda \) contributes more to TTD\text{\_l} than to TTD\text{\_opt} thereby underestimating \( \mu_{\text{max}} \). This difference between the two methods is, however, smaller than the variability in \( \mu_{\text{max}} \) data obtained by plate counting, as can be seen in Fig. 5. Additionally, the products of \( \mu_{\text{max}} \) and \( \lambda \) are in the same order for different pH values, and no clear trend of increasing product (\( \mu_{\text{max}} \times \lambda \)) is visible with decreasing pH toward the growth boundary. It can be concluded that both methods are valid although their results are not identical.

Determining the lag time. Variability in \( \lambda \) is observed for replicates of plate count experiments using the same culture conditions. The values of \( \lambda \) range between 1.78 h and 2.73 h, with an average value of 2.41 h for B. cereus cultured in BHI broth at pH 7. As can be seen in Fig. 1, such a wide variability is not observed when the 2FD method is used (\( \lambda = 0.09 \) to 0.23 h) for the same culture condition. This is in line with Wu et al. (36), who obtained accurate estimation of \( \lambda \) using the Bioscreen C. The \( \lambda \) value obtained using the 2FD method, however, depends on the number of cells estimated to be present at the time TTD is reached (N\text{\_turb}). The value of N\text{\_turb} was tested for nine different pH values, and it was shown that the number of cells in the wells varied, but there was no trend between pH and N\text{\_turb}. For further analysis, the average N\text{\_turb} value (7.3 \pm 0.31 log CFU/ml) was used, but ideally N\text{\_turb} has to be determined for every test condition in order to have a good estimate of \( \lambda \). To determine N\text{\_turb} for every experiment requires extra time when the effect of a lot of different pH values on \( \mu_{\text{max}} \) and \( \lambda \) is tested, and this is not, therefore, convenient.

The cause of the difference in N\text{\_turb} for various pH values is unknown. Literature reveals that environmental conditions might influence the cell length, showing significant elongation of Salmonella enterica serovar Enteritidis when cells are exposed to low water activity (16) and elongation of B. cereus cells from 2 to 5 up to 15 to 40 \( \mu \)m when cells are exposed to a sublethal growth condition of \( \text{pH} \) 5.0 (14). An increase in cell length causes an increase in biomass and, for that reason, an increase in OD but no increase in cell numbers. Another explanation for this variation might be clustering of the microorganism at a lower pH value, which gives an underestimation of the number of microorganisms since a cluster is counted as one CFU even though it originated from more than one individual cell. Microscopic analysis of the cell culture when N\text{\_turb} was reached did not show elongation or clustering of the cells. This suggests that something else is causing variation in N\text{\_turb} values, for example, heterogeneity of the population. Due to heterogeneity of the population, only a small number of cells may actually start growing at low pH values. The dead or dormant cells hardly contribute to the OD compared to the total amount of cells, but they are counted when wells are sampled since dormant cells might recover on plate. This mechanism is not of importance under more favorable growth conditions, resulting in lower cell counts at an OD\text{\_\_}gal of 0.2 for higher pH values.

Efficiency and accuracy of the methods. With respect to the efficiency and accuracy of the different methods, a number of factors can be considered. These are summarized in Table 1 for all three methods in order of appearance in this discussion. First, the number of data points obtained per experiment for all three methods will be discussed. For the 2FD method the data of five 2-fold-diluted wells are necessary to calculate \( \mu_{\text{max}} \) and \( \lambda \), compared to one well using the RRD method. The RRD method therefore retrieves five times more data from one experiment than the 2FD method. Both methods are able to retrieve much more data in one experiment than the widely used plate count method. The use of the latter method enables a maximum of six conditions to be performed in one plate count run, assuming sampling of an Erlenmeyer flask every 20 min and a work load of approximately 3 min to dilute and plate a sample. For this reason, the number of data points per condition is limited to three data points per hour, whereas the number of OD measurements per hour using the Bioscreen can be as high as 12 measurements per well per hour. The
Bioscreen is also able to measure day and night for multiple days, which is not practical using the plate count method. Furthermore, plate counting needs additional labor such as pouring of the plates, plating, and counting. The labor required when the plate count method is used for a 3-day experiment is therefore estimated to be 4 days. Both OD methods are less labor-intensive than the plate count method. Filling of plates when the RRD method is used is less time-consuming than with the 2FD method, and the data analysis is quicker since no fitting of the data points, as shown in Fig. 2, is necessary.

Compared to the plate count method, both the 2FD method and the RRD method are of equal, good reproducibility, as can be concluded by comparing the standard deviations of the mean $\mu_{\text{max}}$ (Table 1), which are considerably larger for the plate count method. Analysis of the standard deviation between the replicate experiments as a function of the pH proved that the within-experiment errors were homoscedastic. Plate counts generally have a limited precision, and the fitting performance of the growth models has influence on the estimation of growth parameters. Since the 2FD method is able to derive both $\mu_{\text{max}}$ and $\lambda$ from the Bioscreen, this method seems for this reason more favorable for research purposes than the RRD method.

Considering inoculum levels, different initial cell numbers may cause different growth behaviors of a microorganism although the same experimental conditions are applied (18). Wells inoculated with assumed equal amounts of cells can consequently show variability in the detection times (11). However, this is only plausible when counts are high enough to allow cell-to-cell communication/quorum sensing. What may be a more plausible explanation is that low densities lead to a less reproducible response. If only a small fraction of the population is able to initiate growth rapidly at boundary conditions, a few more or a few less rapidly growing cells in the population may have a major effect on the outcome of the experiment. This is less likely to happen under optimal growth conditions than close to the growth boundary. The inoculum level of *B. cereus* at concentrations below the detection limit of the Bioscreen was deliberately chosen, and the time until the culture passed a preestablished OD value was measured. Any inoculum level can be chosen as long as it is equal for all wells, and it is better if the level is below the detection limit of the Bioscreen to determine the growth rate before the conditions have changed due to growth. The inoculum size proved to have no effect on experimental outcomes when growth boundaries or MICs were determined (3).

Increase in variance of TTD values for equal experimental conditions is especially the case under more severe stress conditions, which in our study are low pH values close to the growth boundary. It becomes harder to fit a linear line through the data points due to the increase in the variability of the detection times, resulting in high MSE values and/or unrealistic values for $\mu_{\text{max}}$ and $\lambda$. The use of the 2FD method will therefore result in fewer data points around the growth boundary than with the RRD method. The effect of inoculum size on the behavior of cells around the growth boundary or under suboptimal conditions was previously studied by Pascual et al. (29), who showed that inoculum size has an effect on the estimated $\mu_{\text{max}}$ value and $\lambda$. Bidlas et al. (3) also discussed the effect of inoculum size on the MIC and the growth–no-growth interface for the 2FD method and the RRD method. Both studies concluded that the effect of a changing inoculum size can be modeled independently of any other factor, suggesting that simple, short experiments measuring the TTDs of various initial inocula can be used as an adjunct to currently available models. This suggestion favors the use of the RRD method, which does not require inoculum size variations. Moreover, apart from variability in inoculum level, variability in cell numbers at TTD ($N_{\text{min}}$) is also possible, resulting in wrong estimates of $\lambda$ for the tested conditions when the 2FD method is used.

The method used to make predictions to evaluate food safety risks depends on the scenario to be tested. For the study of growth boundaries, the RRD method is considered the best to use since it gives more data points at the growth boundary. Information about $\mu_{\text{max}}$ and $\lambda$ is needed to make a prediction when studying growth kinetics, and in this case the 2FD method has to be used since it provides both parameters.

In summary, this research shows that OD measurements can be used to derive growth rates and lag times of bacterial cultures to investigate the effect of a large variety of pH values on growth parameters. Plate counting, however, will always remain a good method to locally investigate growth of cultures and to test the effect of specific growth conditions like modified atmospheres, which cannot be achieved in OD measurements, and will remain necessary for validation of new methods to establish parameters for growth. Notably, it was established that the use of the 2FD method or the RRD method is justified

### TABLE 1. Characteristics for the three methods to estimate growth parameters

<table>
<thead>
<tr>
<th>Characteristic(s)</th>
<th>2FD</th>
<th>RRD</th>
<th>Plate count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Data points per run (no.)</td>
<td>20</td>
<td>100</td>
<td>6</td>
</tr>
<tr>
<td>Time interval between measurements (min)</td>
<td>5–10 (24 h/day)</td>
<td>5–10 (24 h/day)</td>
<td>20 (&lt;24 h/day)</td>
</tr>
<tr>
<td>Labor</td>
<td>5 h</td>
<td>4 h</td>
<td>4 Days</td>
</tr>
<tr>
<td>Reproducibility (SD)</td>
<td>Good (0.085)</td>
<td>Good (0.032)</td>
<td>Less precise (0.346)</td>
</tr>
<tr>
<td>Parameter(s) obtained</td>
<td>$\mu_{\text{max}}, \lambda$</td>
<td>$\mu_{\text{max}}$</td>
<td>$\mu_{\text{max}}$</td>
</tr>
<tr>
<td>Criteria</td>
<td>MSE $&lt; 5, \lambda &gt; 0, 0 &lt; \mu_{\text{max}} &lt; 3.5$</td>
<td>$0 &lt; \mu_{\text{max}} &lt; 3.5$</td>
<td>MSE $&lt; 5, \lambda &gt; 0, 0 &lt; \mu_{\text{max}} &lt; 3.5$</td>
</tr>
<tr>
<td>Factor(s) affecting data</td>
<td>Inoculum levels, $N_{\text{min}}$ data</td>
<td>$\mu_{\text{opt}}$ obtained by plate counts</td>
<td>Choice of fitting model</td>
</tr>
</tbody>
</table>

*a Required for a 3-day experiment.

*b Criteria that were applied to the fitting process and the resulting parameters before parameters were included for further analysis.
as the performances of both methods are comparable. The choice of one method over the other will depend mainly on the experimental setup, the particular objective of the research, and the growth parameters targeted. If $\mu_{\text{max}}$ has to be determined around the growth boundary, the RRD method will be most efficient. If information on $\lambda$ is needed, the 2FD method is the better choice.

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REFERENCES


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