Kinetic analysis of microcalorimetric data derived from microbial growth: basic theoretical, practical and industrial considerations

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Highlights

- A mathematical framework for quantitative assessment of bacterial exponential growth measured with isothermal microcalorimetry is introduced

- Analysis of growth curves can be automated with a coding routine

- The coding programme is made publicly available

- Various parameters, characteristic to growth, were easily determined; rate constant, doubling time, initial bacterial concentration and enthalpy of growth

Abstract

We report here a mathematical framework for the quantitative interpretation of exponential bacterial growth measured with isothermal microcalorimetry. The method allows determination of many parameters that define the exponential growth phase. To automate the analysis, we also wrote a coding program, so that the approach could be embedded in a commercial setting. As an exemplar, we apply the method to a commercial probiotic product. The outcome was that we could identify characteristic parameters of growth (including rate constant and doubling time), and hence authenticate product quality, within 15h. This compares favourably with the current 7 – 10 days required for conventional microbiological assessment (to allow release of product for bottling and marketing) via plating methods. The method would lend itself to growth analysis of single and mixed bacterial cultures.

Key words

Exponential growth; Lactobacillus; Probiotic; Isothermal Microcalorimetry; Kinetic analysis

1. Introduction

Microbial growth is critical to the production of numerous products (for instance, beer, probiotic supplements and all fermented foods); quantification of growth (in terms of growth kinetics and identification of species) is essential to ensure consistency of product. Hence, methods that can identify and quantify microbial growth are of considerable commercial interest. Many such methods are available, including those based on nucleic-acid analysis (polymerase-chain reaction amplification), biosensing (optical, electrical or mass) and immunological (enzyme-linked assays and lateral flow tests) (Law et al, 2015). A range of metabolic analyses may also be performed (Braissant et al, 2020). Such tests allow rapid (on the order of hours) analysis of the presence (or absence) of particular species and, in some cases depending on the sampling frequency, determination of growth kinetics.

An alternative, and under-used, method is isothermal microcalorimetry (IMC). IMC has long been used to study microbial growth because where there's life, there's heat. For instance, as long ago as 1856, calorimetry was used to study the fermentation of large volumes (21,400 L) of wine (Dubrunfaut, 1856) and its use over the past century for studying bacterial growth has been extensive (see, for instance, the text by Battley, 1987). There have also been studies of the industrial applications of IMC and thermodynamics (Letcher, 2004).

IMC measures the cumulative heat evolved from all metabolic processes, irrespective of sample homogeneity or medium, and on almost any scale, with no need for optical clarity of the medium (Maskow, 2013). In this regard, IMC is perfectly suited to the quantification of growth kinetics, but is less suited to identification of particular species compared with the analytical methods noted above. Given this versatility, it is somewhat surprising that the technique has not found more widespread acceptance as a standard microbiological tool in the life sciences. In part, the fact that IMC measures *all* heat outputs is also a hindrance; ascribing the heat outputs from specific events is tricky ('Heat does not come in different

colours', Cooper et al, 2001) and so experimental design often aims to reduce a sample to a single reacting component. While this reductionist approach is very successful for chemical samples, it is not suited to quantitative interpretation of microbiological samples. For these more complex systems underpinning mathematical models are required.

Recently, a number of groups have developed mathematical models to obtain information on bacterial systems from IMC data. For instance, Ying et al (2017) used a simple exponential model to analyse growth of *Escherichia Coli*;

$$P_t = P_0 e^{kt}$$

Equation 1

Where P_0 represents the power output from a sample at time 0, P_t is the power output at any other time *t* and *k* is a growth rate constant. Since the growth of *E. coli* gives a complex calorimetric trace, they determined growth rates and times for distinct exponential phases and combined these values with power and heat data to create a data matrix which they interrogated with chemometric software. They were able to show that by co-culturing with *Salvia miltiorrhizae,* an antibacterial effect on *E. coli* growth was seen. Bonkat et al (2012) used the same equation, but cast in terms of heat rather than power, to measure growth rates and doubling times of four common pathogens in urine.

Hernández Garcia et al (2017) developed an empirical model to correlate the relationship between initial viable cell number and the time taken for the cells to produce a certain heat value for growth of *Lactobacillus reuteri*;

$$t = \frac{1}{0.434\mu}\log X_{v0} + \frac{1}{0.434\mu}\log\left(\frac{\mu Q}{k_c V}\right)$$

Equation 2

Where μ is a specific growth rate, X_{v0} is the cell concentration (cfu/mL) at time 0, Q is the heat produced by the cells in volume V of medium and k_c is the heat production rate per cell. A linear relationship between the time taken to reach a particular power and logarithm of viable cells was seen and the model has subsequently been applied to other species (Nykyri et al, 2019).

These models are empirical, and so function well for determining variance in growth for specific cultures, but as they are not derived from models of bacterial growth, their more widespread versatility is limited. Braissant et al. (2013) described some earlier mathematical models for the analysis of microbial growth and extended the analysis through use of simple exponential models to "more complex models using heat data". We have used somewhat similar approaches (described below) to analyse the exponential growth periods of the study organisms. These are based on (1) the power-time output from IMC and (2) upon the power and its integral over the exponential period.

As a test system, to which we apply the models, we have used a commercially available probiotic supplement (Symprove™). Symprove is an aqueous probiotic suspension containing *Lactobacillus acidophilus* NCIMB 30175, *Lactobacillus plantarum* NCIMB 30173, *Lactobacillus rhamnosus*[1] NCIMB 30174 and *Enterococcus faecium* NCIMB 30176 and provides an ideal example of an industrial manufacturing process where monitoring of bacterial growth during fermentation is critical to ensuring final product quality (both in terms of making sure the four probiotic species are present in the correct ratios and concentrations and ensuring no contaminant species have proliferated). During production, the probiotic cultures ferment a barley-substrate in pairs; *L. rhamnosus* and *L. acidophilus* in fermentation

[1] We note that recent taxonomic revisions mean that *L. rhamnosus* is now called *Lacticaseibacillus rhamnosus* and *L. plantarum* becomes *Lactiplantibacillus plantarum*

vessel 1 and *L. plantarum* and *E. faecium* in fermentation vessel 2. Once fermentation is complete, the contents of the vessels are mixed to give the final product.

Symprove has been shown to be effective in reducing clinical symptom severity scores in IBS (Sisson et al, 2014) and reducing abdominal pain scores and significantly reducing constipation, diarrhoea and mucorrhoea in diverticular disease (Kvasnovsky et al, 2017). Symprove has also shown anti-pathogenic activity against various common gut pathogens, including *Clostridium difficile* (Fredua-Agyeman et al, 2017), *Escherichia coli*, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Shigella sonnei* (Dodoo et al, 2019). The product showed good tolerance to acid exposure during *in-vitro* testing (Fredua-Agyeman and Gaisford, 2015) but while this study incorporated comparison with the performance of other commercially available probiotics it did not include determination of (relative) cell numbers surviving after acid exposures. Note here that there is, in the acid tolerance test (conducted over different time periods of exposure), a requirement also to determine the effect of such exposure to acid on any change in the metabolism of the sample organisms.

We speculated, therefore, that some of the characteristics required to establish a sound production and achieve the desired product could be achieved through quantifying IMC data through direct observation of the active processes. An additional benefit is that product quality (in terms of the correct mixture of probiotic species and assurance of the absence of contaminating species) could be made more rapidly than is presently possible (using microbial culture techniques). Moreover, as we propose using quantifiable parameters derived from IMC measurements and we suggest that such data outcomes could be used in an industrial context then it is essential that the analysis process itself be automated, rapid and independent of operatives i.e. essentially a “black box” analysis. To this end we have written a code that performs the necessary data analysis automatically.

To demonstrate that our analysis is a plausible method to evaluate both production and product we deemed it essential that the method should be carefully evaluated in controlled

experiments (that is, in a non-production study). Consequently, we report here experiments conducted with pure samples of one of the constituent organisms (*L. rhamnosus*) in a standard medium (MRS broth). The data collected were subjected to analysis via the developed equations manually and with the coding software. The resulting data produced "best outcome" results for this IMC-based approach and allowed judgement of its suitability for application in real (here industrial although this approach may offer insights into more fundamental and exploratory studies) situations to be made.

2. Materials and Methods

De Man, Rogosa and Sharpe (MRS) agar, from Sigma-Aldrich, was prepared according to the manufacturer's recipe and used in all experiments. Phosphate buffered saline (PBS) tablets and glycerol were purchased from Sigma-Aldrich. *L. rhamnosus* NCIMB 30174 was used in all experiments reported in this paper and was grown overnight in MRS broth for 16 h at 37 °C. Cells were then centrifuged, washed in PBS, resuspended in 15 %v/v glycerol at an organism density of 10^8 cfu/mL and frozen in aliquots (1 mL) at -80°C.

IMC data were recorded with a Thermal Activity Monitor (TAM) 2277 (TA Instruments Ltd., UK). Sterile glass ampoules (3 mL) were filled with pre-warmed (37°C) sterile MRS (2.97 mL) and inoculated with *L. rhamnosus* (30 μL - an aliquot of frozen organism was thawed in a water bath at 40 ^{o}C for 3 minutes, then vortexed for 1 minute before inoculation). The ampoules were immediately sealed under aseptic conditions then inserted into the thermal equilibration position for 30 minutes before data capture began. Data were collected using the software package Digitam 4.1. Digitam 4.1 collects data at 4 Hz; here, once 40 data points were collected, the average value was recorded, giving a final data set comprised of 1 data point every 10 s (this method has the effect of smoothing the power data).

2.1. Manual data analysis

Manual data processing and analysis was performed using OriginPro 2020b. The raw data were uploaded and converted to ln plot. The best linear part on the first slope of the log plot was selected and linear fit applied. This operation yielded three basic parameters of the slope, intercept and R-square. All manually processed data were compared with coding data.

2.2. Coding

The design challenge was to produce an efficient and valid reverse-engineering solution suitable for deployment with large data sets and for embedding in an industrial process. As usual in computer science there is a trade-off between exhaustive recursive searches to compute all possible paths versus following clear signs to take short cuts. A structural decision was made to pre-process data to store results of elemental mathematical operations in database tables where efficient summing and other operations would be efficient and robust. In examining datasets one can easily locate maxima and minima and positive/negative changes but other features are less apparent. Submitting data triggers a first analysis of slope between the bottom and top of the first slope, and the tool allows adjustment for re-analysis. A batch version of the tool for intensive analysis targets exactly average values as seed points for searches and this is delivering results at least matching that of human operatives (see results). The system instantly generates a line of best fit for any selected portion of a submitted data set, and can algorithmically identify candidate portions with a close match to the best manual selection. It does this line of best fit calculation repeatedly and stores values for bits of the calculation which can be used to tune the data selection to get a best fit with the widest range.[2]

2.3. Theoretical analysis

[2] The coding programme is given in the SI and a link to the coding process can be found here; https://ubiubi.org/beezer.html

The output from an isothermal calorimetric experiment is a plot of power (W, or J s^{-1}) as a function of time (s). The area under the curve is thus equal to the cumulative heat released (J). The power output from a growing bacterial cell is very small (for instance, *Escherichia coli* produces between 1.4-3.5 pW $cell^{-1}$ depending on the growth medium, Higuera-Guisset et al, 2005) so assuming an average cell produces ca. 2 pW (Braissant et al, 2010), 10^4 cells will produce a power of 20 nW, just within the measurement capability of a modern IMC instrument. By extension, 10^5 cells will produce ca. 200 nW and 10^6 cells will produce ca. 2 μW of power. In the experiments reported here, the initial inoculum density was 10^6 cells per mL.

Growth of bacterial cells proceeds with synthesis of cellular constituents (biomass, such that all cell components are duplicated) followed by cell elongation and splitting (binary fission). If all the cells in a culture are growing in this fashion, then the number of cells (*N*) will increase exponentially with time, described by the geometric series $2^0 \rightarrow 2^1 \rightarrow 2^2 \rightarrow 2^3 \rightarrow 2^n$.

Assuming growth is in a medium with excess nutrients and that environmental factors (such as temperature, pH etc) are constant, cell division should proceed at a constant rate (*k*). (Alternatively, the time taken for cell division will be constant, the doubling time, t_d, or generation time, *g*). In which case,

$$n = k.t = \frac{t}{g}$$

Equation 3

In either event, a logarithmic plot of cell number versus time will be linear (and if $\log_2$ is used, the y-axis will represent the number of generations). This phase of growth is termed ‘constant exponential’ or ‘logarithmic’. Attempts to model the kinetics of bacterial growth are usually based either on increase in cell number or increase in biomass (Egli et al, 1993), and in either case the equations can be constructed in calorimetric form.

2.4. Growth based on cell number

The increase in cell number (*N*) of a growing culture can be expressed as;

$$\frac{dN}{dt} = k.N$$

Equation 4

The solution for Equation 4 is;

$$N_t = N_0 e^{kt}$$

Equation 5

The heat produced by the cells (q_N) is given by;

$$q_N = \Delta_N H.N$$

Equation 6

Where $\Delta_N H$ is the enthalpy of cell growth (in J/cell). This means that the heat produced by the cells in time *t* ($q_{N,t}$) is given by;

$$q_{N,t} = \Delta_N H(N_t - N_0)$$

Equation 7

Where N_0 is the number of cells initially and N_t is the number of cells after time *t*. Substitution of Equation 7 into Equation 5 yields;

$$q_{N,t} = (\Delta_N H N_0 e^{kt} - \Delta_N H N_0)$$

Equation 8

Given that $\Delta_N H$ is constant and N_0 is constant for a given experiment, then the differential form of Equation 8 is;

$$\frac{dq_N}{dt} = k\Delta_N H N_0 e^{kt}$$

Equation 9

Equation 9 can be expressed in logarithmic terms;

$$\ln \frac{dq_N}{dt} = \ln k\Delta_N H N_0 + kt$$

Equation 10

Accordingly, a plot of ln (power) versus time should yield a straight line of slope k and intercept ln $k\Delta_N H N_0$. Finally, we note that if Equation 9 is differentiated with respect to t, then;

$$\frac{d^2 q_N}{dt^2} = k^2 \Delta_N H N_0 e^{kt}$$

Equation 11

Hence, dividing Equation 11 by Equation 9 gives k directly.

2.5 Growth based on biomass

The increase in biomass (M) of a growing culture can be expressed as;

$$\frac{dM}{dt} = \mu . M$$

Equation 12

Where μ is the *specific growth rate* (i.e. it is the rate of increase in biomass, normalised by the amount of biomass). Following the same argument as above yields;

$$\ln \frac{dq_M}{dt} = \ln \mu \Delta_M H M_0 + \mu t$$

Equation 13

Where q_M is the heat produced by the biomass, $\Delta_M H$ is the enthalpy of biomass production (in J/g), M_0 is the biomass initially and M_t is the biomass after time t. Accordingly, a plot of ln (power) versus time should yield a straight line of slope μ and intercept ln $\mu \Delta_M H M_0$.

2.6. The link between cell number and biomass

We note that since calorimetric data can be described either by Equations 10 or 13, the values of k and μ for a given data set will be the same and so it is a necessary condition that the biomass is comprised solely of dividing cells, and that those cells remain identical throughout the growth phase, for the models to apply.

2.7. Analysis based on cumulative heat determination over the exponential growth phase

Equation 8 can be rewritten as;

$$q_{N,t} = \Delta_N H N_0 (e^{kt} - 1)$$

Equation 14

Which can be rearranged to;

$$e^{kt} = \frac{q_{N,t}}{\Delta_N H N_0} + 1$$

Equation 15

Substitution into Equation 9 produces;

$$\frac{dq}{dt} = kq_{N,t} + k\Delta_N H N_0$$

Equation 16

Thus a plot of power at time *t* versus the corresponding area (i.e. heat) value will be linear with slope *k* and intercept $k\Delta_N H N_0$.

2.8. Time from inoculation to signal detection in IMC

Let N_s be the number of organisms required (in the sample volume placed into the IMC) to give a specified signal – this will be same for all similar samples independent of the initial inoculum density i.e. enthalpy is taken as a constant for the particular sample constituents. The time from inoculation to a specified signal will be dependent on the initial number of organisms present in the sample. Here we set a reference sample of number N_r at inoculation that takes time t_r to produce the specified signal, N_s. At some other cell number, N_{exp}, the time taken to achieve N_s will be t_{exp}. We can now write;

$$N_s = N_r e^{kt_r}$$

Equation 17

And;

$$N_s = N_{exp} e^{kt_{exp}}$$

Equation 18

Equations 17 and 18 can be set equal;

$$\ln N_r + kt_r = \ln N_{exp} + kt_{exp}$$

Equation 19

After rearrangement;

$$\ln N_r = \ln N_{exp} + k(t_{exp} - t_r)$$

Equation 20

Thus, the relative cell numbers may be determined or, if a cell count has been performed for the reference sample, then cell numbers in the experimental sample may be determined. Note that, importantly, the assumption is made that exponential growth commences at the time of inoculation and persists throughout the period up to the time taken to achieve the prescribed signal (see below).

2.9. Doubling (or generation) time

In the time taken for cell numbers to double, N will increase to $2N$ and q_N will increase to $2q_N$. Thus, from Equation 3;

$$2N_0 = N_0 e^{kt_d}$$

Equation 21

Or;

$$2 = e^{kt_d}$$

Equation 22

$$\ln 2 = kt_d$$

Equation 23

$$t_d = \frac{0.693}{k} = 0.693g$$

Equation 24

Finally, the number of cells at time *t* is given by;

$$N_t = N_0 2^n$$

Equation 25

If we note that;

$$N_t - N_0 = \frac{q_{N,t}}{\Delta_N H} = N_0 2^n - N_0$$

Equation 26

Then it follows that;

$$\frac{q_{N,t}}{\Delta_N H} = N_0[2^n - 1]$$

Equation 27

Or;

$$\frac{q_{N,t}}{\Delta_N H . N_0} = 2^n - 1$$

Equation 28

3. Results and Discussion

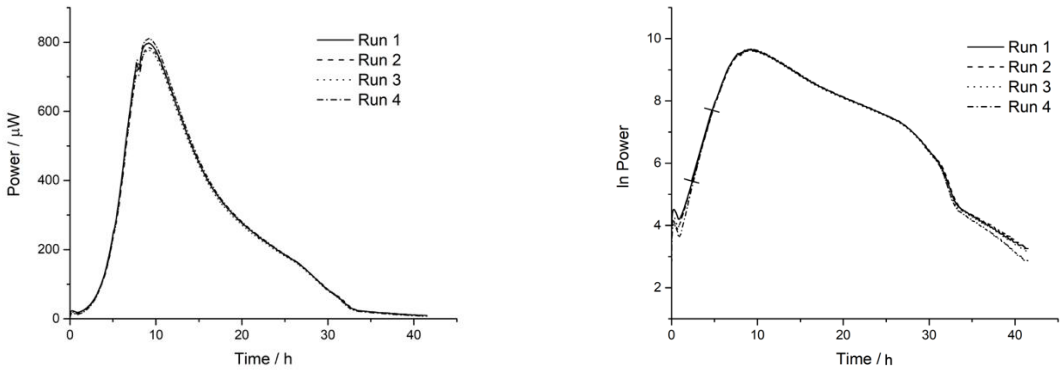

Figure 1: Typical power-time plots for growth of *L. rhamnosus* in MRS broth (left) and the transformation of these plots into the *ln* power versus time format (right). Note that, following transformation, it is possible to identify three linear periods – only the first, exponential period, is analysed here.

Figure 1 shows typical power-time data for the growth of *L. rhamnosus* in MRS and their corresponding *ln* plots. The first observation to be drawn is that the *ln* transformation yields a number of linear periods (4-6h, 20-24h and 34-40h), corresponding to exponential growth or decay events. In this treatment we only analyse the first linear portion, since this relates directly to the exponential growth phase. Table 1 shows the results of analysing the first linear region using (i) linear fitting to determine the gradient and intercept of the line and (ii) the coding tool. We note that the slopes derived from each method are of the same order of magnitude and have similar values for the percentage standard deviations. The actual value of the slope differs between methods, indicating that positioning of the line of best fit is critical. Obviously, the coding tool performs this task against a set of predetermined criteria, rather than using human judgement, which is why it was developed for automation of the analysis. Unfortunately, given the limitations of the laboratory facilities at Symprove (the industrial site) it wasn't possible to perform parallel cell counts on the study growth systems and thus to yield a more conventionally based value for *k*. However, the reproducibility of the analysis (via either method) of the IMC experiments is sufficiently robust in comparison with

conventional cell counting procedures involving complex media that we are satisfied that these data are very useful in application to the selected systems.

	Slope (s^{-1})	Intercept
Manual analysis	$1.89 \pm 0.06 \times 10^{-4}$	2.13 ± 0.07
Coding tool	$1.34 \pm 0.06 \times 10^{-4}$	3.00 ± 0.1

Table 1. Average values for the slope and intercept calculated for the first linear region of the ln (power) versus time plots derived by manual analysis and with the coding tool

In a recent paper, Fricke et al (2020) used IMC to detect growth of *Legionella pneumophila* and make oblique reference to the use of differential power data to determine *k*. As we noted explicitly above, a plot of differential power versus power should itself yield a straight line of slope *k* (Figure 2).

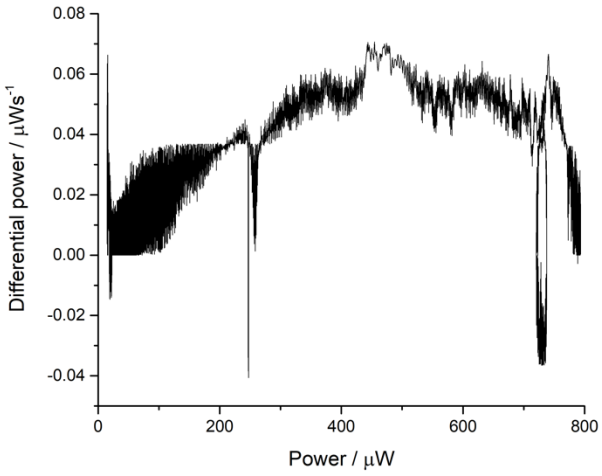

Figure 2: Representative example of power-time data for growth of *L. rhamnosus* in MRS broth transformed to $\frac{d^2q}{dt^2}$ versus power.

It is apparent that there is considerable noise in these data (the power data themselves are smoothed, as a result of the data collection method used). However, it was still possible to use the linear regression and coding methods to analyse these data also (Table 2). Here, greater divergence between the methods were seen; the coding method in particular returned *k* values substantially different from the other analyses, and for both methods the correlation coefficients of the fitting were poor. This is a result of (i) noise in the signal following differentiation and (ii) the gradient being very shallow.

	Slope (s^{-1})
Manual analysis	$1.71 \pm 0.07 \times 10^{-4}$
Coding tool	$5.13 \pm 0.22 \times 10^{-5}$

Table 2. Average values for the slope calculated for the first linear region of the derivative (power) versus power plots derived by manual analysis and with the coding tool.

Recognising that taking the slope of the differential power versus power data was difficult, we note that it is possible to effect an alternative calculation to determine *k* by dividing each differential power data point with its corresponding power value and averaging the values returned (effectively the same outcome as smoothing the data shown in Figure 2). One potential benefit of this approach would be to get indicative *k* values from the early

exponential growth phase, potentially reducing the time needed to record calorimetric data. Table 3 shows the results for this approach. While the average *k* values agree well with those determined form the *ln* plots, the standard deviations are high, rendering this approach unsuitable.

	Slope (s^{-1})
Manual analysis	$1.87 \pm 1.71 \times 10^{-4}$
Coding tool	$1.37 \pm 0.834 \times 10^{-4}$

Table 3. Average values for the slope calculated by dividing each derivative power value by its corresponding power value.

The intercept of the *ln* power versus time plots is determined as $lnk\Delta_N HN_0$. The assignment of N_0 thus requires an assignment of the value for t_0 to which it refers. This is problematic as the identification of the "best" linear portion of the plot is found by successive iteration and is not based on theoretical evaluation of when the exponential period starts. The slope values reported in Table 1 are independent of the choice of when $t_0 = 0$ but, of course, that of the intercept is not. In practice we find that for a set of experiments conducted via a constant procedure, such as those reported here, there is consistency in the intercept values (see Table 1). These may not, however, be directly useful in deriving values of *h* or N_0. Their consistency is, nonetheless, useful in the application of IMC data to the determination of a successful production process and product as a statistically sound value for a repeatable process is what is required.

The data derived from the $\frac{dq}{dt}$ v *q* plot produce *k* values (Table 4) which are consistent with those found from the $ln\frac{dq}{dt}$ *v t* plots. However, they also reveal the dependence on the selected time at which time is set to zero, because our model does not account for the initial lag phase in the way that other models do (see for instance, Braissant et al, 2010). The equations refer only to an exponential period of growth and this definition appears to be problematic with both equations appearing to have a different dependence on *t*. However, as noted above, this does not affect the values of *k*. The differences in the values of *k* seen between the manual and coding outcomes results from the iterative strategies employed to determine a "best" straight line plot.

	Slope (s^{-1})	Intercept
Manual analysis	$1.88 \pm 0.01 \times 10^{-4}$	31.05 ± 10.05
Coding tool	$1.57 \pm 0.03 \times 10^{-4}$	17.27 ± 0.46

Table 4. Average values for the slope and intercept calculated from power versus heat plots derived by manual analysis and with the coding tool.

Equation 17 indicates that *if* exponential growth persists from inoculation to the observation of a specified signal then it should be possible to determine relative cell numbers from a given reference experiment. Table 5 describes data from a series of experiments in which the inoculum volumes were varied. The data analysed showed that the values of *k* are consistent with those shown in Table 1 (the values for the intercepts are less well evaluated for reasons outlined above).

Inoculation volume (μL)	Time to reach signal of 100 μW (s)
100	9430
30	15130
3	24331

Table 5. **The time (s) from inoculation to achieve a signal of 100μW**.

Manipulation of these limited data has been made through application of Equation 17 and the use of the mean value of *k* shown in Table 1. The nominal ratios are, taking the 3 μL value as the reference: 1:10: 33.3. The derived values from Equation 17 are 1: 17: 50. The agreement is rather modest, underlining the crucial dependence on the assumption that exponential growth commences at inoculation. From this very limited data set this assumption is only approximately held to. The more substantial, empirically-based, data sets (i.e. not based on any theoretical basis) present in the literature that relate known inoculum numbers to the time to achieve a defined signal suggest an extension of Equation 17 to the estimation of *k.*

Here we show the result of this calculation for growth of *Pseodomonas brassicacearum* pure culture and growth of this organism coated on to seeds as described by Nykyri et al (2019).

P. brassicacearum (pure culture) $k = 0.73\ h^{-1}$

P. brassicacearum (on seeds) $k = 0.92\ h^{-1}$

These data yield doubling times (from Equation 20) of 57 min and 45 min respectively. They also reinforce that that observation of the whole calorimetric growth record is not necessary

for the establishment of the doubling time if relative or individual cell numbers are available. A further demonstration of this application can be taken from the work of Agyeman and Gaisford (2019) on the growth of *Pseudomonas aeruginosa*. Despite the lack of numerical data on the time taken to yield an IMC signal it is possible to extract from the graphs presented a value for k of ca. 1 h^{-1} making t_d equal to ca. 42 min.

4. Conclusions

The significance of these results is that they clearly indicate that the application of IMC to a real industrial situation is plausible. We are confident that this analysis (particularly of the values of k) will be sufficiently sensitive to allow exploration of production variables, which in the context of Symprove include the following; (i) As the production process involves fermenting pairs of organisms in separate fermenters then it is plausible that the study and analysis of the consequent power time curves will yield information on the interaction of the organisms and yield relative numbers. It can be imagined that, by extension, symbiosis or antagonism between organisms may be quantitatively examined (ii) the approach should allow direct comparison of “synthetic” (i.e. made by combining pure samples of two organisms in MRS medium) and actual production samples (i.e. samples taken from the fermenters and inoculated into MRS). This approach naturally implies that, as the production process attempts to control the growth medium (barley wort) by use of consistent procedures, then the outcomes of the analysis should reveal the impact of the variation in this “biologically derived” wort medium. The larger implication of such studies will be the possibility to investigate an organism’s history by establishing a standard calorimetric investigation system (here it is the use of MRS medium). Further, the product in use will, on swallowing by a consumer, arrive in the stomach where the pH is between 1 and 3. It will be important to know whether the organisms are affected by this and the capacity to determine k will contribute to this understanding, If k remains the same after exposure to acid for varying periods of time then it is likely that the metabolism of the organisms is unchanged. We also anticipate that this approach to the study of organism metabolism in complex media

may have wider application in other areas of current interest (e.g. drug/cell interactions, synergy and antagonism in drug/cell interactions, identification of MRSA, neonatal septicaemia and many other cell-based systems).

The coding procedure can be automatically entrained after production (i.e. a “black box” can be incorporated directly in to the process). The only requirement is that a suitably trained operative can load the IMC with a product sample correctly. Thus, the IMC data analysis outcome, which is available some 15 h after the product is sampled, is a remarkable improvement on the current 7 – 10 days required for conventional microbiological assessment (to allow release of product for bottling and marketing) via plating methods. If it can be shown that (i) these results hold in the examination of the pairs of organisms used in the two fermenters, and (ii) the mixture of these (i.e. the final product), and that (iii) contaminants can be detected at appropriate levels via IMC then the product can be safely packaged and be available to customers within a working day (the examination of the pairs, product and IMC consequences of the presence of contaminants will be the subject of further papers).

The product-manufacturing procedure can also be imagined as being controlled via IMC and feed-back information. A continuous flow IMC could be placed in loop with the fermenter vessels with the output data continuously monitored. Conformity with established outcomes could then be used to, in principle, allow a product to be processed for customers immediately following production. This more elaborate application awaits study.

The data reported here in Tables 1, 2 and 4 are, for microbiological systems, of excellent reproducibility. This encourages the exploration of the applications identified as plausible in the introduction. The identification of t_0 is a challenge as is the detailed exploration of the capacity of the IMC to allow dependable enumeration of the organisms present in a study system. However, as discussed above, whilst a more dependable value for the intercept (i.e. for t_0) is desirable, for an industrial control system, reproducibility is more important.

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