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Growth measurement of *Escherichia coli* by differential scanning calorimetry

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Using differential scanning calorimetry (DSC) and plate count (PC), growth of *Escherichia coli* was measured in four initial *E. coli* cell concentrations cultured in tryptic soy broth (TSB) at 35°C. The calorimeter measured the energy released and/or absorbed by *E. coli* cells during growth and expressed it as thermograms. The resulting thermograms accurately reflected cell growth and activity, and exhibited a close correlation with growth values recorded by PC. Heat release decreased notably in the final stage of the logarithmic growth phase, and no signal was generated once the stationary phase had begun. DSC accurately quantified growth in *E. coli* at different initial cell concentrations grown in TSB at 35°C. Depending on initial *E. coli* concentration, DSC detected growth starting after just a few minutes and up to five hours. The detection limit of calorimeter was 4.3 ± 0.4 $\log_{10}$ CFU. DSC is an effective alternative method for measuring bacterial growth in TSB in real time. This is the first report of DSC use for measuring bacterial growth.

Key words: Differential scanning calorimetry (DSC), *Escherichia coli,* thermograms.

INTRODUCTION

Plate count and turbidimetric (e.g. Bioscreen C) are the most commonly used techniques to monitor microbial growth (Huchet et al., 1995). Plate count is widely used to estimate viable cell numbers in culture medium (Madrid and Felice, 2005), but has the disadvantage that it requires 24 to 48 h.

Techniques based on turbidity measurement are easy and fast, use small volumes and can be monitored in real time. However, they are useless for measuring microbial growth in opaque samples (McClure et al., 1993; McKellar and Knight, 2000). In addition, with turbidimetric is required a large numbers of cells (10^6-10^7 cells) for detect differences in optical density (Biesta-Peters et al., 2010).

Calorimetry involves use of a thermal sensor to directly measure the heat emitted or absorbed by a material.

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Microcalorimetry and differential scanning calorimetry (DSC) are based on material thermal response and have been used to study some aspects of microorganisms. They are generally distinguished by measurement cell size and calorimeter specifications (von Stockar and Marison, 1989). Microcalorimetry has been used to measure bacterial metabolism (Braissant et al., 2010a; 2010b) whereas DSC has been used to measure microbial thermal death (Lee and KaletunÇ, 2002; Honglin et al., 1993). Microcalorimetry can measure changes in heat released and/or absorbed during bacterial growth (Boe and Lovrien, 1990; Braissant et al., 2010a; 2010b; Von et al., 2009), and has been used to monitor growth of *E. coli* in broth under different culture conditions. Sugar degradation during *E. coli* growth generates heat that can be measured by a microca-lorimeter sensor, producing thermograms with exother-mal peaks (Belaich and Belaich, 1976a; 1976b; Dermoun and Belaich, 1979; 1980; Braissant et al., 2010a; 2010b). A distinct disadvantage with microca-lorimetry is the need for large sample volumes. Microcalorimetry for studying cellular metabolism has been limited to the use of batch and flow microca-lorimeter and has required large numbers of cells (10^5 - 10^6 cells) for reproducible measurements (Braissant et al., 2010a). In addition, most cases, microcalorimetry requires an initial equilibration time of 1 h, during which data cannot be collected (Braissant et al., 2010a). DSC offers a more sensitive method for studying cellular metabolism in living cell. In a Differential scanning calorimeter a sample (in crucible) and a suitable reference material (in crucible) are heated in separate chambers at a constant rate. A feedback system maintains an essentially zero temperature difference between the chambers and provide an output that measures the excess power requirement of one chamber relative to the other one. The differential scanning calorimeter requires only 1 minute for tempe-rature equilibration of crucibles. A limitation of DCS is the low throughput: just one sample crucible per DSC per day.

Differential scanning calorimeters function within a wide temperature range (-45 to 700°C), have a <0.04 µW signal resolution, require small sample volumes and can analyze liquid, solid, turbid and/or opaque samples. Bacterial growth has not been measured previously using DSC, although this technique is a promising alternative for real-time monitoring of bacterial growth in opaque, turbid and/or solid samples that cannot be monitored with turbidimetric methods. In food microbiology, DSC is potentially promising since it can directly measure microbial growth in food, in real time and continuously. It also makes it possible to use bacterial growth heat generation data in predictive equations with applications in predictive microbiology or in food biotechnology for monitoring microbial fermentations.

The present study objective was to measure *E. coli* growth in tryptic soy broth at 35°C by both differential scanning calorimetry (DSC) and plate count (PC), and compare the resulting data.

MATERIALS AND METHODS

Bacterial strain

The strain used was *E. coli* ATCC (American Type Culture Collection) 25922 preserved in inclined blood agar base under refrigeration. Before use, the strain was activated three times in trypticase soy broth (TSB) and incubated for 18 h at 37°C.

Inoculum preparation

E. coli cultured for 18 h at 37°C in TSB was washed twice with isotonic saline solution by centrifuging at 1507 *g* for 20 min each time and then resuspended in TSB at pH 7. Decimal dilutions were then done in TSB at pH 7 to produce flasks containing 100 ml TSB with final bacterial concentrations of 4 x 10^7, 4000, 40 or 4 colony-forming units (CFU)/ml.

Calorimetry and bacterial count

An 822e/400 (Mettler-Toledo) calorimeter was used, and calibrated with indium. Samples (40 µl) were taken of each bacterial suspension and placed in previously sterilized 40 µl aluminum crucibles for thermal analysis (ME-00026763, Mettler-Toledo). These were hermetically sealed with a press and placed in the calorimeter. A sample of sterile, uninoculated TSB was used to verify device baseline. Temperature was maintained at 35°C for 14 h. The resulting thermograms were analyzed to record initial signal (heat) generation (that is, onset), maximum signal generation (peak) and signal termination (final), as well as total heat generated based on the area below the curve (Toro-Vazquez et al., 2003). The onset, peak, and final temperature for the different transitions were determined using the first derivative of the heat capacity of the sample calculated with the DSC software library. Thus, onset and final temperature were established as the temperatures where the first derivative of the heat capacity of the sample initially departed from or returned to the baseline, respectively. In contrast, peak was established as the temperature where the first derivative of the heat capacity of the sample crossed the baseline (i.e. the inflexion point of the transition curve). The enthalpy (total heat generated) was calculated by integration of the corresponding exothermal peak. Integration was done between the respective onset and final temperature with the DSC software (Toro-Vazquez et al., 2003). Three replicates were done per experiment.

In a parallel procedure, different sealed crucibles (one for each time-point) containing inoculated TSB with the different initial inoculums level were incubated for 14 h at 35°C and the standard plate count technique applied (FDA, 2010), briefly, in the sampling time, a crucible was taken and punched. The culture was taken from crucible with a micropipette and serial dilutions (1:10, 1:100, 1:1000, etc) were made with peptone diluent. Dilutions were plated in trypticasein soy agar (TSA) to quantify *E. coli* growth. The CFU data were $\log_{10}$-transformed and bacterial growth results from the DSC and plate count techniques were compared. Three replicates were done per experiment.

Statistical analysis

A completely random design was used and significant differences ($p<0.05$) calculated with an analysis of variance (ANOVA) and a

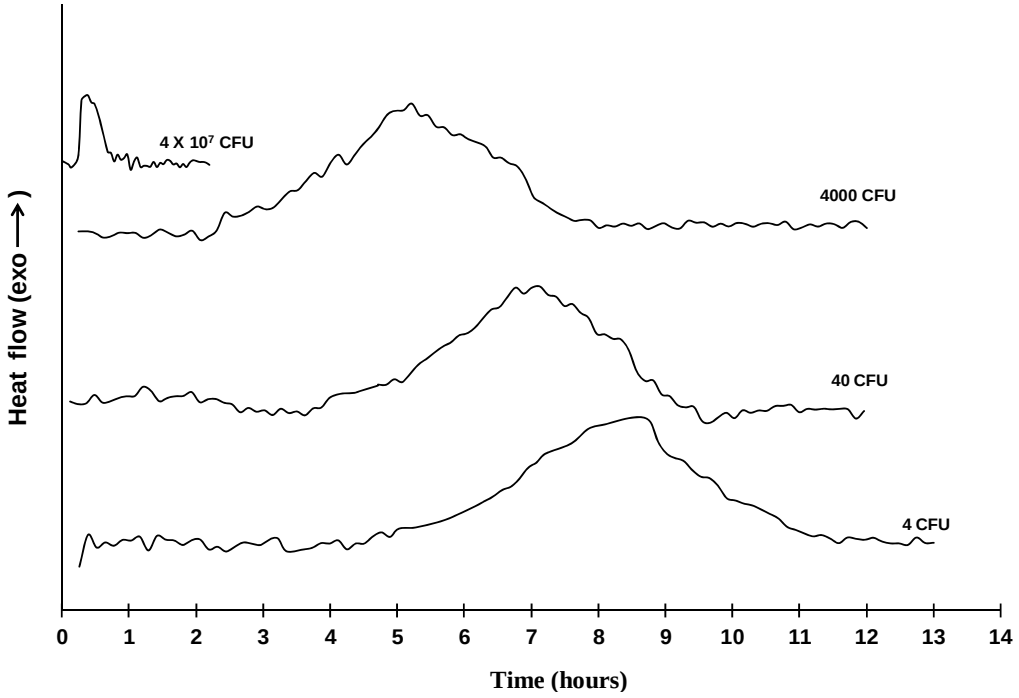

Figure 1. DSC thermograms of *E. coli* growth for four different initial inoculum concentrations in TSB at 35°C and pH 7.

Table 1. Time in minutes at signal detection onset, peak heat generation and final signal detection, and total heat generated during growth of four *E. coli* initial inoculum concentrations.

Initial concentration (CFU)	Onset	Peak	Final	Heat released (Integral [mJ])
4	*290 ± 20[a1]	522 ± 28[a]	693 ± 31[a]	163 ± 13[a]
40	233 ± 72[b]	437 ± 26[b]	581 ± 11[b]	150 ± 71[a]
4000	136 ± 24[c]	297 ± 14[c]	450 ± 12[c]	113 ± 29[a]
4×10^7	14 ± 0.3[d]	28 ± 6[d]	43 ± 1[d]	8 ± 2[b]

*Mean ± SD of three replicates, [1] Different letters in the same column indicate statistically significant difference ($p < 0.05$).

Duncan test (Statistica 8, StatSoft, Inc., 2007). Simple correlation coefficient (r) between initial inoculum concentration and total heat output for *E. coli* growth in TSB were determined with a statistical analysis system (Statistica 8, StatSoft, Inc., 2007).

RESULTS AND DISCUSSION

Thermograms illustrate microorganism growth by generating a curve with three main points: onset, peak and final signal detection (Figure 1). In thermograms, signal onset is related to heat generation, which can be associated with microbial activity. Samples with different initial cell concentrations produced different responses, with higher concentrations producing more rapid heat generation (Figure 1). *E. coli* grew well in TSB at low oxygen levels and under these conditions the thermograms showed it to exhibit a hyperbolic growth pattern (Figure 1). This coincides with a previous report of hyperbolic growth in *E. coli* when metabolizing glucose and with use of a calorimetric pump (Boe and Lovrien, 1990). This can also be observed in the onset, peak and final time results (Table 1). At an initial concentration of 4 CFU, onset was at 4.83 ± 0.33 h, while at 40 CFU it was 3.88 ± 1.19 h, at 4000 CFU it was 2.27 ± 0.4 h and at 4 $x10^7$ CFU it was 0.23 ± 0.005 h. As mentioned above, a higher initial concentration produced a more rapid thermal response, probably due to greater metabolic activity (Table 1). In addition, final time occurred more

rapidly (0.71 ± 0.02 h) at the highest initial concentration ($4x10^7$) than at the lowest (4 CFU; 11.55 ± 0.51 h). There are not previous reports about DSC used for growth measurement of bacteria to compare our data. However, there are data obtained with microcalorimetry. Although a direct comparison between DCS and microcalorimetry is not possible, it is useful to have results from the previous study with microcalorimetry as a reference.

Our results agrees with microcalorimetry results in which both onset and final signals occurred more rapidly at higher initial concentrations of *Lactococcus lactis* (Kobanova et al., 2012). In addition, our results are in agreement with the studies made with microcalorimetry and reported by Maskow et al. (2012), who observed that initial rapid heat generation depends of the initial inoculum concentration.

In the other hand, in a microcalorimeter, the heat released by the microbial cultures during growth has been found to correlate quantitatively with biomass generation, uptake of substrates or oxygen (Birou et al., 1987). Metabolic events, such as shifts from one substrate to another, change of limitations, inhibitions and overflow metabolism would cause characteristic changes in the heat evolution curves (Braissant et al., 2010b; von Stockar and van der Wieler, 1997; Yi et al., 2000). It is possible that during the growth of *E. coli* in crucibles of DCS occur to changes in oxygen concentration and affect the behavior of *E. coli* and biomass generation and the heat released. However, in the present study we did not evaluate the effect of changes in oxygen concentration in *E. coli* behavior.

Initial *E. coli* concentration significantly ($p < 0.05$) affected detection of exothermal signal onset, peak and final (Figure 1) generated by *E. coli* metabolic activity. However, except for the $4x10^7$ CFU initial concentrations, no difference ($p > 0.05$) was detected in total heat detected for the 4, 40 and 4000 CFU initial concentrations (Table 1). This coincides with a report of lack of difference ($p > 0.05$) in total heat detected by microcalorimetry between initial *L. lactis* concentrations ranging from 10^2 - 10^4 CFU (Kobanova et al., 2012). However, total heat values were significantly lower ($p <0.05$) at very low (10^0-10^1) and very high (10^5-10^6) concentrations compared to those of the 10^2 - 10^4 CFU concentrations (Kobanova et al., 2012).

The low total detected heat value in the present study at the $4x10^7$ CFU concentrations versus the other initial concentrations suggests that cellular multiplication was minimum in the TSB contained in the crucibles during incubation at 35°C. In addition, the rapid heat release observed in this broth (0.23 h) is probably related to high enzymatic activity (e.g. sugar degradation) rather than cellular multiplication. Indeed, a series of biochemical reactions known to occur before the lag phase can lead to heat generation (Zhang et al., 1993). In the present study, it is therefore probable that the very high initial *E. coli* concentration ($4x10^7$) allowed heat generated by lag phase reactions to be detected by the calorimeter. This hypothesis would agree with the rapid heat generation over a short time reported at a high initial cell concentration for *E. coli* grown in culture broth at 25°C and monitored by calorimetric pump (Boe and Lovrien, 1990). These authors state that caloric yield is a measure of metabolic capacity which in turn is dependent on and proportional to cell number and mass.

To estimate cell concentration during thermal monitoring in the calorimeter, *E. coli* growth in TSB inoculated with the same initial cell concentrations was measured simultaneously by the plate count (PC) technique. Final cell concentrations measured in the PC trial did not differ, independent of initial inoculum concentration (Figure 2). At higher initial cell concentrations, maximum cell concentration from microbial growth was reached more rapidly (Figure 2). This indicates that growth rate was lower at higher initial concentration. The 4×10^7 CFU initial inoculum concentration exhibited the lowest multiplication rate, supporting the hypothesis proposed above to explain this inoculum's thermal behavior. Indeed, heat production in bacterial cultures is directly linked to microorganism multiplication (Marison and Stockar, 1985).

To estimate cell concentrations at onset, peak and final signal detection, as well as total heat generated, plate count values were related to the thermograms based on initial inoculum concentration (Table 2). For the 4, 40 and 4000 CFU initial concentrations, onset was detected when the *E. coli* had begun to grow and had reached a concentration of 4 $\log_{10}$ to 4.5 $\log_{10}$ CFU, but this occurs in different times. In other words, the calorimeter detection limit is 4.3 ± 0.4 $\log_{10}$ CFU for *E. coli* at 4 to 4000 CFU initial concentrations (Table 2).

Microcalorimeters have a higher detection limit. For example, in a study of *L. lactis* growth in culture broth the microcalo-rimeter detection limit was 10^5 cells (Kobanova et al., 2012). In the present study, by contrast, the calorimeter detected a heat signal within the first few minutes for the 4×10^7 initial concentration, which was associated with enzymatic activity rather than cell multiplication.

Cell concentrations at the peak signal detection had reached an advanced logarithmic phase: 7.2 $\log_{10}$ CFU for the 4 CFU initial concentrations, 6.82 $\log_{10}$ CFU for the 40 CFU, and 6.92 $\log_{10}$ CFU for the 4000 CFU (Table 2). Heat release was no longer detected when growth had concluded the logarithmic phase, at 8.5 to 8.8 $\log_{10}$ CFU. The drastic drop in heat release observed as the *E. coli* entered the final stage of the logarithmic phase can be attributed to a decrease in growth rate as the cultures neared stationary phase (Ramírez et al., 2005), and a consequent reduction in the amount of heat generated. In bacteria, a lack of nutrients, among other reasons, causes cellular multiplication rates to decrease notably and almost stop as they enter the stationary phase (Ramirez et al., 2005). In addition, the amount of heat

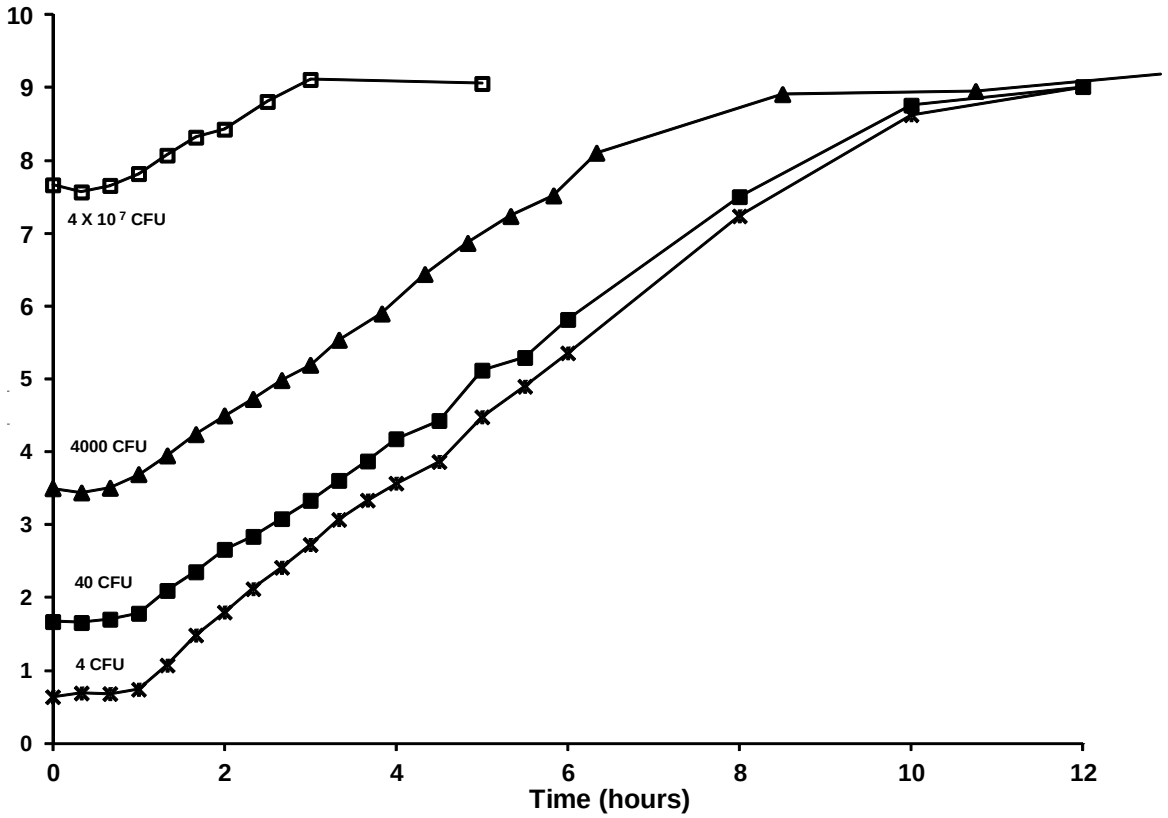

Figure 2. *E. coli* growth curves from plate counts for four different initial inoculum concentrations in TSB at 35°C and pH 7.

Table 2. Bacterial concentration (log_{10} CFU) at signal detection onset, peak heat release and final signal detection during growth of four initial inoculum concentrations of *E. coli*.

Initial inoculum concentration (CFU)	Cell concentration (CFU)		
	Onset	Peak	Final
4	4.3 ± 0.3[a1]	7.2 ± 0.4[a]	8.8 ± 0.2[a]
40	3.9 ± 0.5[a]	6.9 ± 0.2[ab]	8.5 ± 0.5[a]
4000	4.7 ± 0.2[ab]	6.9 ± 0.6[ab]	8.6 ± 0.3[a]
4×10^7	7.0 ± 0.07[c]	7.6 ± 0.1[ac]	7.5 ± 0.4[b]

*Mean ± SD of three replicates, [1]Different letters in the same column indicate statistically significant difference ($p < 0.05$).

released by a bacterial cell depends on its cellular activity, the types of energy and carbon sources it has access to (especially their degree of reduction), the biomass production coefficient, physical conditions such as temperature and pH, microorganism respiration and culture medium chemical composition (Chang-Li et al., 1988). The reduced growth rate of *E. coli* as it entered the stationary phase in the present study manifested as a progressive decrease in heat generated until the calorimeter ceased to detect a change in heat flow. This is more visible when the thermograms for a low initial concentration (4 CFU) are compared point-by-point with the corresponding PC values (Figure 3). In the PC, growth was detected at 4.83 h and 4.3 log_{10} CFU. In the DSC, the highest heat flow was detected during the logarithmic growth phase, the peak occurred at the end of this phase and the stationary phase is clearly visible as the point at which heat flow is stable. Finally, although no difference ($p>0.05$) was detected in total heat output during the growth of *E. coli* for 4, 40 and 4000 CFU of initial inoculum concentrations (Table 1), a negative correlation ($r=0.845$) between initial inoculum (including

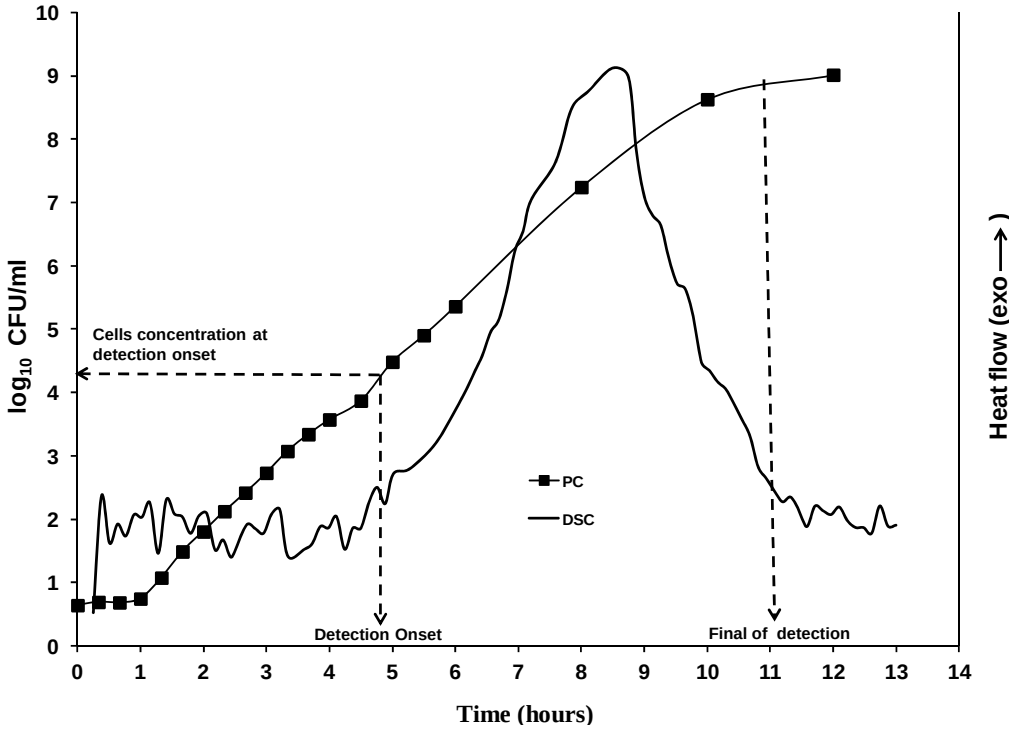

Figure 3. Comparison of DSC thermogram and plate count (PC) growth curve for *E.coli* growth at a 4 CFU initial concentration in TSB, at 35 °C and pH 7.

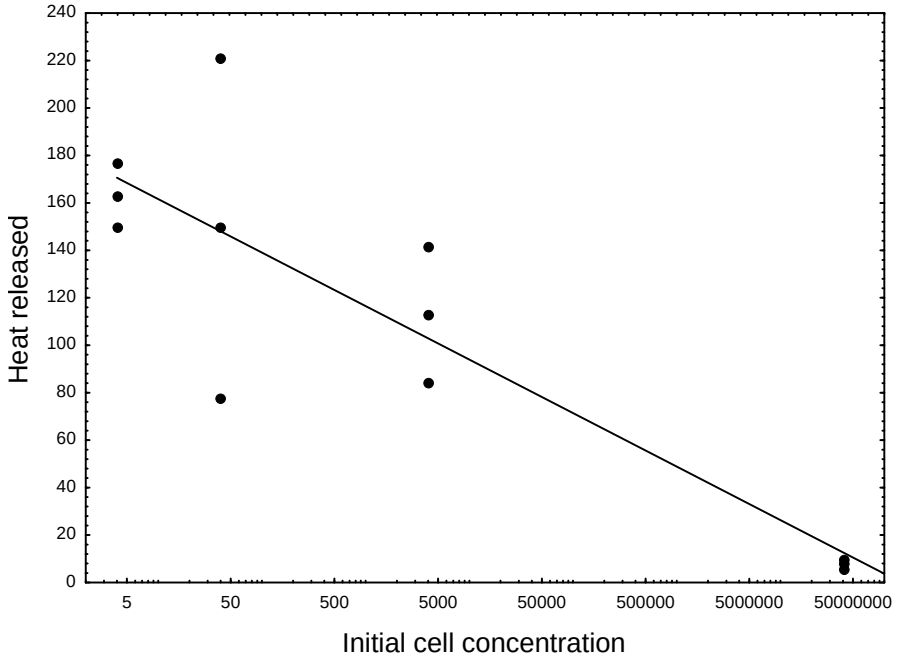

Figure 4. Correlation between initial inoculum concentration and total heat output for *E.coli* growth in TSB at 35°C and pH 7 (r=0.845).

4 x10^7 CFU) and total heat output was observed (Figure 4).

DSC effectively monitored growth in *E. coli* at different initial inoculum concentrations. It is a promising alterna-

tive technique for real-time monitoring of bacterial growth and has the potential to record different growth-related parameters. The calorimeter is a powerful tool for studying metabolic processes in living bacteria because it provides data on metabolism. Thermograms readily lend themselves to monitoring general microbial activity, and observing and pinpointing metabolic events such as product formation with subsequent oxidation, medium limitations and inhibition. The fast response of the differential scanning calorimeter and its direct correlation to plate count results suggests its use for generating convenient and rapid quantification of microbial growth kinetics. This is the first report of DSC use to measure real-time bacterial growth. It effectively measured growth of *E. coli*. Measurement of cellular growth by DSC can help in modeling cell behavior, and may contribute to creating mathematical models that could predict the behavior of microorganisms on various substrates and under different growth conditions.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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