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Microcalorimetric study of the growth of *Enterococcus faecalis* in an enriched culture medium

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Abstract *Enterococcus faecalis* is a Gram-positive bacteria, considered one of the most common causes of nosocomial infections. Bacterial cultures produce an exchange of energy as a result of the bacteria metabolisms. The rate of heat production is an adequate measure of the metabolic activity of the organisms and their constituent parts. Microorganisms produce small amounts of heat: 1–3 pW per cell. Although the heat produced by bacteria is very small, their exponential reproduction in a culture medium permits heat detection through microcalorimetry. In this study, we analyzed the microcalorimetric behavior of *Enterococcus faecalis*. A thermal Calvet microcalorimeter was used. The inside of the calorimeter contains two stainless steel cells (experimental and reference). Experiments were carried out at final concentrations of 10^6,10^5,10^3, and 10 CFU/mL and a constant temperature of 309.65 K was maintained within the microcalorimeter. Recording the difference in calorific potential over time we obtained *E. faecalis*'s growth curves. Thermograms were analyzed mathematically allowing us to calculate the constant growth, generation time and the amount of heat exchanged over the culture time.

Keywords *Enterococcus faecalis* · Microcalorimetry · Metabolism · Bacteria

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Introduction

Enterococcus faecalis

Enterococcus faecalis is a Gram-positive cocci, catalase-negative and it is one of the few pathogenic species to humans in the genus Enterococcus.

They are enteric bacteria usually isolated from the feces of humans and various animals. They are one of the most frequent causes of nosocomial infections, been the urinary tract, peritoneum, and heart tissue the most affected places. Enterococcus infections are particularly common in patients with intravascular catheters or urinary catheters and in patients hospitalized for a long time who have received broad spectrum antibiotics. A particularly serious complication is endocarditis by Enterococcus, a process that has high mortality [1, 2].

Microcalorimetry and its applications

Microcalorimetry is an experimental technique that allows us to precisely measure the energy released by a process or transformation [3].

This technique is interesting to biological science since heat flow is strongly related to the kinetics and thermodynamics of biological processes. Heat variations resulting from chemical reactions, which take place during metabolism, can be used to monitor bacterial growth, and monitor the influence of external agents [4, 5].

All living beings produce heat during metabolism. The rate of heat production is an adequate measure of the metabolic activity of the organisms and their constituent parts, cells and sub-cellular levels [6]. The heat generated by a single cell is in the range of 1–80 pW. Human connective tissue cells (fibroblasts, adipocytes…) have

metabolic rates of approximately 25–80 pW per cell. In contrast, micro-organisms produce small amounts of heat, in the order of 1–3 pW per cell. Despite the low levels of heat produced by the bacteria, their exponential growth in culture medium allows their detection using microcalorimetry within a few hours.

In this study, we analyzed the microcalorimeter behavior of *E. faecalis* at different concentrations in an enriched culture medium. As experimental equipment we used a Calvet microcalorimeter.

Materials and methods

Sample preparation

We have used *E. faecalis* from the American Type Culture Collection (ATCC 29212).

Samples were inoculated on blood agar plates and incubated at 309.65 K in an incubator for 24 h. The blood agar plates with multiple bacterial colonies were then used to prepare a bacterial suspension with 0.9% saline solution, whose concentration was adjusted to the corresponding 0.5 on the McFarland scale, using a Densichek® optical densitometer. This solution was then used to prepare further dilutions with 0.9% saline solution to get final concentrations of 10^6, 10^5, 10^3, and 10 CFU/mL [7, 8].

The culture medium consisted of a liquid enriched with digested soya-casein.

Experimental equipment

The measures were carried out in a Calvet microcalorimeter equipped with a device allowing operation in the absence of vapor phase, and having aluminum and Teflon cells of approximately 10 cm^3 [3]. A Philips PM2535 multimeter and a data collection system were coupled to the microcalorimeter. Calibration was performed electrically using the Setaram EJP30 stabilized current source. The precision in calorimetric signal was ± 1 μW. Further details about the experimental method have been previously published by Professor Paz Andrade [9].

Experimental setup and data collection

The external environment of the calorimeter was maintained at a constant temperature of 309.65 K. The reference cell was injected with 7 mL of culture medium + 1 mL of saline while the experimental cell was injected with just 7 mL of culture medium. Both cells were then introduced through two cylindrical holes aligned in parallel, which extended from the upper part of the microcalorimeter to the internal thermopile chamber. The large distance that separates the cells from the entrance permits minimization of heat flow to the exterior. The system is allowed to stabilize for about 2 h, after which 1 mL of the established concentration is introduced into the experimental cell [8].

When we introduce the sample to be tested in the experimental cell, a small voltage variation of $\pm$ 10 μV is recorded in the thermogram, returning to stabilize within a few minutes.

All experiments were realized in triplicate.

The experiment was also carried out with a sample not containing any bacteria (control).

Data were collected using the earlier mentioned data collection and processing system, at intervals of 15 s, throughout the duration of the experiment.

Bacterial metabolism produces residues which modify the pH of the medium. Samples were subject to pH control both before and after each experiment using a basic 20+ pH meter.

Results

By plotting heat voltage difference versus time between the experimental cell and the reference cell, we were able to obtain the characteristic bacterial growth curves of *E. faecalis* at different concentrations, where we can distinguish the different phases of the growth curve: latency, exponential or logarithmic, stationary, and cell death.

The curves presented a characteristic shape which was repeated at all concentrations studied (Fig. 1). As we can see, *E. faecalis* has unique energetic phase in which stand out four peaks of electromotive force, and the signal is recorded for about 6 h, returning to baseline levels after this time. The shape of the curve of heat flow is repeated for the four concentrations studied, behaving like a "thermal footprint."

Furthermore, we observed an inversely proportional relationship between the inoculum size and the time until signal detection and the record of electromotive force peaks, been these times smaller as we increase the concentration of the culture. In any case, even at low concentrations (10 CFU/mL), we can identify bacterial growth in the sample within 5 h (Table 1).

In the earliest records of the thermograms of *E. faecalis* 10 and 10^6 CFU/mL, we observed a slight modification of the voltage returning to baseline levels within a few minutes. This voltage variation is due to the variation that results from the injection of the sample in the experimental cell. In the thermograms 10^3 and 10^5 CFU/mL of *E. faecalis*, this voltage variation is also produced, but it is not contained in Fig. 1 where it is only represented a voltage range of 0–80 μV.

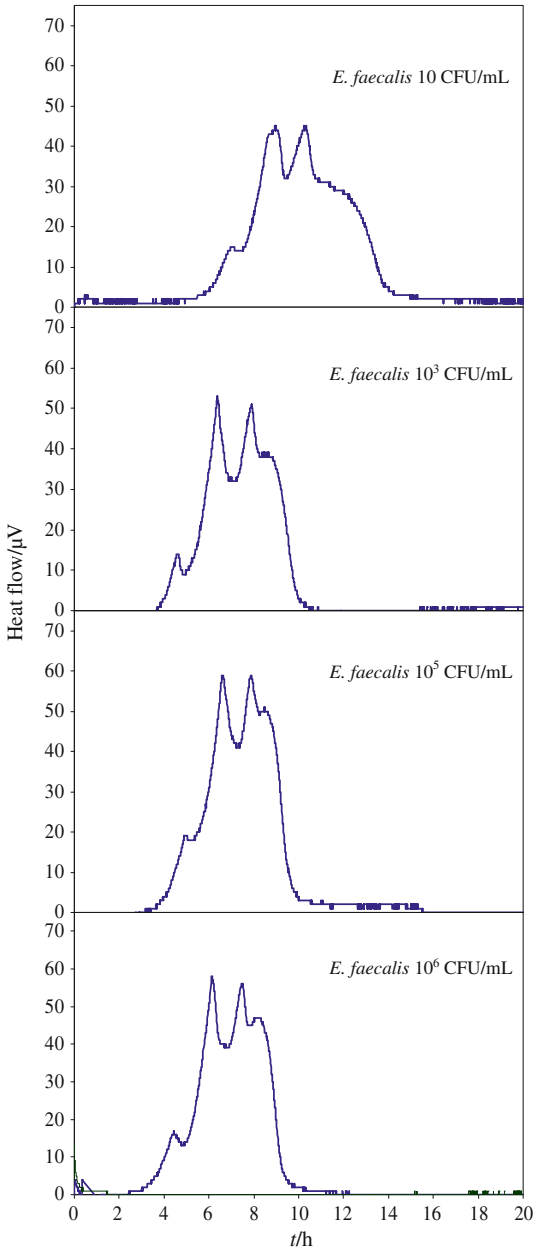

Fig. 1 Calorimetric signal versus time for the *E. faecalis* at the different studied concentrations: 10^6, 10^5, 10^3, and 10 CFU/mL

We have treated the data mathematically, fitting them to exponential equations in ascending and descending phases of each peak of electromotive force and to polynomial-type equations in the areas of higher activity of the thermogram.

From the polynomial equations, by the first derivative we determinate the value of the electromotive force maximum peaks and the time of it registration in the thermogram (Table 1).

In the logarithmic phase of the growth curve, we adjusted the data to an exponential equation. At this stage of the growth curve, microbial proliferation can be expressed as:

$$n_t = n_0 \cdot e^{k \cdot t} \quad (1)$$

where n_0 is the number of cells at onset (time 0), n_t is the number of cells at time t, and k is the growth constant [4, 8].

If we consider P_w as energy released by each cell, then it leads to the following equation:

$$n_t \cdot P_w = n_0 \cdot P_w \cdot e^{k \cdot t} \quad (2)$$

Taking into account that P_0 is the energy released at onset and P_t is the energy released at time t:

$$P_t = P_0 \cdot e^{k \cdot t} \quad (3)$$

$$\ln P_t = \ln P_0 + kt \quad (4)$$

The generation time (G) is defined as the time taken by a population to double in size, and is expressed as:

$$G = \frac{(\ln 2)}{k} \quad (5)$$

Therefore, derived from the mathematical adjustment of the powers obtained from the microcalorimetric study of bacterial cultures we can quickly and easily extrapolate the value of the constant growth and the generation time of the bacteria under study (Table 2). In the thermograms obtained from all samples analyzed, the fitting equation in the exponential phase presents an R^2 higher than 0.99, therefore we can say that the parameters obtained show a good correlation. As we can observe in Table 2, the

Table 1 Acquisition time and signal values and detection time for the first, second, third, and fourth peaks in the growth curves of *E. faecalis* at the different studied concentrations

Conc./CFU/mL	Detec. *t*/h	First peak		Second peak		Third peak		Fourth peak	
		t/h	Signal/μV	*t*/h	Signal/μV	*t*/h	Signal/μV	*t*/h	Signal/μV
10^6	2.67	4.43	16	6.13	58	7.46	56	8.14	47
10^5	3.32	4.99	19	6.61	59	7.87	59	8.49	51
10^3	3.63	4..55	13	6.37	53	7.88	51	8.59	39
10	4.72	6.97	15	8.98	45	10.27	45	11.36	31

Table 2 Growth constant (k) and generation time (G) of the *E. faecalis*

Conc./CFU/mL	k/h^{-1}	G/h	R^2
10^6	1.35	0.51	0.996
10^5	1.11	0.63	0.996
10^3	1.41	0.49	0.991
10	1.01	0.68	0.991

Table 3 Heat produced during culture of *E .faecalis*

Conc./CFU/mL	AUC/μV h^{-1}	Q/J g^{-1} K^{-1}
10^6	188.63	4.414
10^5	203.81	4.769
10^3	175.48	4.106
10	219.48	5.136

Table 4 pH values before and after each experiment

Conc./CFU/mL	Initial pH	Final pH
10^6	7.08	6.01
10^5	7.08	6.03
10^3	7.06	6.04
10	7.03	6.04

constant growth and the generation time are parameters dependent on the starting concentration, although there is not a relationship of proportionality.

From the thermogram, we can also calculate the amount of heat (Q) exchanged over the culture time.

$$Q = K \cdot A \tag{6}$$

where A (μV h) represents the area, calculated by the trapezoidal method, and K is a constant whose value, 24.8 J/μV h, was calculated from the electric calibration performed by the Joule effect on the equipment.

In Table 3, we compare the heat exchanged in different cultures. As can be seen, although there is no relationship of proportionality between exposed energy and starting concentration, the heat is located between 4 and 5 kJ by experience.

As a result of metabolic wastes are generated, usually acidic, producing a decrease in pH in the culture medium after the experience. In Table 4, we compare the values of pH of samples before and after each experiment, confirming the acidification of the medium.

Discussion

Microcalorimetry is an analytical technique to measure the produced or absorbed heat by chemical reactions or physical changes of state. It can also be used to track heat generated during complex biological processes. The technique has been used in biology, pharmacology, biotechnology, and ecology because of its high sensitivity, precision, and simplicity [10–16]; however, its clinical use so far has been limited [6, 17].

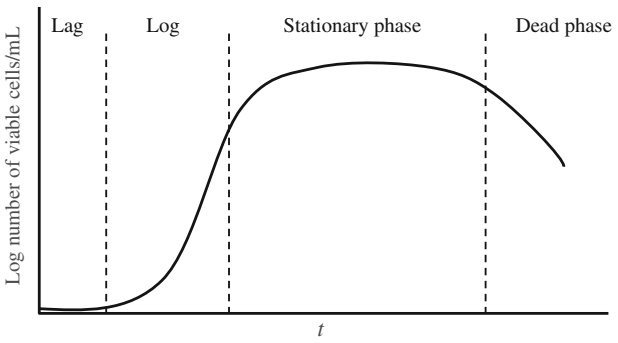

Fig. 2 Growth phases of bacteria: latent phase (lag), exponential or logarithmic phase (log), stationary phase, and dead phase

Microcalorimetry can determine the presence of microorganisms in a sample within a few hours. Even though the initial inoculum contains a low concentration of bacteria, their exponential replication permits measuring the metabolic heat generated. This information is very important from a clinical point of view. As we can see in the thermograms obtained in this study, it is possible to identify the presence of bacteria in a sample in less than 5 h, even in low concentrated samples (10 CFU/mL).

Traditionally, bacterial growth is studied by analyzing the growth curve obtained when microorganisms are grown in a closed system or discontinuous culture. That is, microorganisms are incubated in a closed container in which no more amount of medium is added to the initial, therefore, nutrient concentrations decrease as the concentration os the waste increase.

By a sampling method, that analyzes the number of cells in the culture medium at different times, we get the traditional bacterial growth curve (Fig. 2) in which we observe four different phases: latency (lag), logarithmic or exponential (log), stationary, and cell death.

When microorganisms are introduced into a fresh culture medium, generally it is not produced an immediate increase in the number of cells, this initial period is called the lag phase (lag). During the exponential or logarithmic (log) phase, microorganisms grow and divide at the highest possible rate that allows their genetic potential, the nature of the medium, and culture conditions. In a closed system, there comes a time when population growth stops and the growth curve becomes horizontal, we are in the stationary phase when the number of viable cells is constant. And finally, the privation of nutrients and the accumulation of toxic waste, characterize the cell death phase.

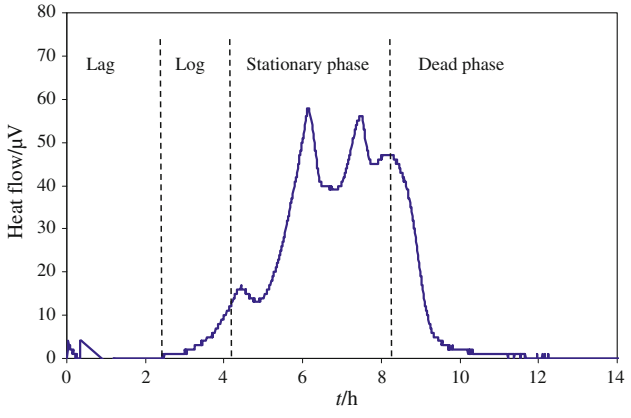

Fig. 3 Growth phases of *E. faecalis* 10^6 CFU/mL: latent phase (lag), exponential or logarithmic phase (log), stationary phase, and dead phase

In the case of the thermograms obtained by microcalorimetry, the electromotive force recorded versus time represents the metabolic activity in the culture medium, which is conditioned by the type of bacteria and its ability to assimilate the different components of culture medium. In these thermograms, the different phases of bacterial growth curve can also distinguished (Fig. 3).

Comparing Figs. 2 and 3, we observe that the main differences between both curves are observed in the stationary phase of the curve obtained by microcalorimetry, where far from being a horizontal line is a phase characterized by variations in the electromotive force, therefore, metabolically active, and whose form and duration will be characteristic of each bacterium and the starting concentration.

Despite the similarities reached between both growth curves, the results obtained by microcalorimetry can not adapt to the mathematical models that traditionally describe the bacterial growth. In the thermogram, it is represented the energy exchange that occurs in a culture as a result of the metabolic processes that accompany the growth of the microbial population, and it will be proportional to the number of viable cells, but its shape and intensity depend on other factors. However, traditional models describe the evolution of the number of viable bacteria during the study period.

Calorimetric curve is defined by a series of electromotive force (voltage) points recorded by the data collection system that corresponds to heat exchange produced during the culture period. This microcalorimeter behavior is kept at different concentrations of study. If we compare the growth curve obtained in the case of *E. faecalis* with *Pseudomonas aeruginosa* thermogram [8] studied at the same conditions, we can say that in a defined culture medium each species analyzed presents a growth profile that is repeated at all the concentrations studied, obtaining a "thermal footprint" for each bacteria species.

This heat exchange, as explained above, depends on the initial inoculant and the bacterial species. A set of peaks are furthermore observed during culture and such peaks are probably associated with the capacity of each bacterial species to assimilate the nutrient culture medium components. Therefore, there arises a need to study microcalorimetric behavior of such bacteria in simpler media, in order to be able to identify the origin of each peak recorded on the thermograph.

The microcalorimeter study was performed in triplicate for each concentration, reproducing the same results in the three experiments. Based on samples of *E. faecalis* to the same concentration, we obtain thermograms that retain the same shape and reproduce the electromotive force peaks, and the time to its appearance.

The results were mathematically treated to allow us to quickly and easily calculate the value of the growth constant (k), and the generation time (G). These parameters are characteristic of each bacterial species. Calculating these parameters by the usual methods required very laborious techniques. In addition, microcalorimetry allows us to quantify the amount of heat exchanged (Q) in a bacterial culture over a defined period. Knowledge of these parameters opens up the possibilities of new studies that reveal the behavior of a bacterial species in the presence of different substances.

Conclusions

Microcalorimetry allow us to detect and quantify very small amounts of energy exchanged and thus detect the growth of *E. faecalis*, even with very small inoculation, 10 CFU/mL, in less than 5 h.

Microcalorimetry allows us to know the amount of heat and, therefore, the amount of energy exchanged in a bacterial culture for a determined period of time.

The calorimetric curves obtained allow us to characterize the bacterial species studied, both qualitatively as through the parameters obtained from mathematical analysis of curves: constant growth and generation time.

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References

1. Murray P, Rosenthal K, Pfaller M. Microbiología médica. España: Elsevier Mosby; 2009.
2. Kenneth J, Ryan C, George R. Microbiología médica. Una introducción a las enfermedades infecciosas. Madrid: McGraw Hill; 2004.
3. Calvet E, Prat H. Microcalorimétrie: aplications physico-chimiques et biologiques. Paris: Masson el Cie Editeurs; 1956.

4. Ma J, Qi WT, Yang LN, Yu WT, Xie YB, Wang W. Microcalorimetric study on the growth and metabolism of microencapsulated microbial cell culture. J Microbiol Methods. 2007;68:172–7.
5. James AM. Calorimetry. Past, present and future. In: Thermal and energetic studies of cellular biological systems. Bristol: IOP Publishing Ltd; 1987
6. Trampuz A, Salzmann S, Antheaume J, Daniela AU. Microcalorimetry: a novel method for detection of microbial contamination in platelet products. Transfusion. 2007;47:1643–50.
7. Lago N, Legido JL, Arias I, García F. Aplicaciones de la microcalorimetría como método de identificación precoz del crecimiento bacteriano. Invest Cult Cienc Tecnol. 2010;2(3):6–9.
8. Lago N, Legido JL, Paz-Andrade MI, Arias I, Casás LM. Microcalorimetric study on the growth and metabolism of *Pseudomonas aeruginosa*. J Therm Anal Calorim. 2011;105(2):651–5.
9. Paz Andrade MI. Les Dévelopements Récents de la Microcalorimétrie et de la Thermogenese. 1st ed. Paris: CRNS; 1967.
10. O'Neill M, Vine G, Beezer A, Bishop A, Hadgraft J, Labetoulle Ch. Antimicrobial properties of silver-containing wound dressings: a microcalorimetric study. Int J Pharm. 2003;263:61–8.
11. Wang X, Liu Y, Xie B, Shi X, Zhou J, Zhang H. Effects of nisin on the growth of *Staphylococcus aureus* determined by a microcalorimetric method. Mol Nutr Food Res. 2005;49:350–4.
12. Liang H, Wu J, Liu Y, Yang L, Hu L, Qu S. Kinetics of the action of three selenides on *Staphylococcus aureus* growth as studied by microcalorimetry. Biol Trace Elem Res. 2003;92:181–7.
13. Yang LN, Xian L, Xu F, Zhang J, Zhao JN, Zhao ZB. Inhibitory study of two cephalosporins on *E. coli* by microcalorimetry. J Therm Anal Calorim. 2010;100:589–92.
14. Xu XJ, Chen C, Wang Z, Zhang Y, Hou A, Li C. Antibacterial activities of novel diselenide-bridged bis(Porphyrin)s on *Staphylococcus aureus* investigated by microcalorimetry. Biol Trace Elem Res. 2008;125:185–92.
15. Yan D, Hna YM, Wei L, Xiao XH. Effect of berberine alkaloids on *Bifidobacterium adolescentis* growth by microcalorimetry. J Therm Anal Calorim. 2009;95(2):495–9.
16. Kong W, Li Z, Xiao X, Zhao Y, Zhang P. Activity of barberine on *Shigella dysenteriae* investigated by microcalorimetry and multivariate analysis. J Therm Anal Calorim. 2010;102:331–6.
17. Baldoni D, Hermann H, Frei R, Trampuz A, Steinhuber A. Performance of microcalorimetry for early detection of methicillin resistance in clinical isolates of *Staphylococcus aureus*. J Clin Microbiol. 2009;47(3):774–6.