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Calorimetry of microbial growth using a thermopile based microreactor

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Abstract

A miniaturized calorimeter, based on silicon integrated thermopile chips, has been developed for the determination of growth-related heat production in microbial cultures. The calorimetric vessels consists of two independent sensors located within a thermostated aluminum frame, the heat sink, and covered by a 0.6 ml reaction chamber made of PTFE for improved thermal insulation. The second sensor was used as a reference to minimize temperature perturbations on the output signal. Baseline stability was better than 0.08 $\mu W\,h^{-1}$. The Si thin-film membrane which supports the Al–Si thermopiles enabled an excellent dynamic response and a temperature resolution of 50 μK. The sensitivity for the heat power measurement was 0.39 $V\,W^{-1}$. Batch measurements of *Escherichia coli* activity under different conditions have been performed. The thermal profiles matched the exponential growth kinetics usually found in batch cultures of bacteria. A simplified model based in the Monod equation is used to analyze the influence of oxygen depletion on cell growth.
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Keywords: Thermopile silicon chip; Heat-flow microcalorimeter; Bacterial growth; Metabolic activity

1. Introduction

Control of biotechnological processes requires the use of reliable and robust sensors able to provide real-time information on the main variables of the process. Sensors for monitoring pH, redox, oxygen or carbon dioxide have long been available. On-line measurements of microbial biomass can also be carried out using several commercial devices. However, determination of microbial activity is usually accomplished after analyzing the kinetic data obtained from the reactor. Since microbial growth is accompanied by an enthalpy change, heat dissipation measured by calorimetry represents a suitable procedure to monitor metabolic activity. Moreover, although non-specific, the calorimetric signal is related to well defined thermodynamic quantities [1]. To be useful and widely applicable in the study of living systems the calorimetric technique must yield quantitative measurements of the small heat released by biological systems during the long periods of time involved in those processes. Several groups have measured microbial growth using commercial calorimeters focusing on aspects such as the thermodynamics of cell growth [2], the conversion of toxic compounds [3] or the metabolic rate in animal cell cultures [4,5]. Both, batch and heat flow calorimeters, have been used and their specificities have been already pointed out [6,7]. Batch calorimetry [6,8] allows to monitor in convenient time scales the influence of a number of effects, such as the role of inhibitors or depletion of nutrients on cell growth.

Integrated chips analogous to the ones used in this study have already been shown to work as batch type microcalorimeters for the detection of enzyme catalyzed

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reactions [9], or the determination of heats of absorption onto thin coatings [10]. Their use as flow-through systems has also been reported and demonstrated for reaction calorimetry with fluid reactants [11]. Other studies have even shown the ability of microsized calorimeters to measure the heat evolved during chemical stimulus on single cells [12]. Progress in modern calorimetry aims at increasing sensitivity and long-term stability together with the ability to perform multiple measurements in parallel [13]. Moreover, microfabrication technologies efficiently allows the production of a large number of identical microreactors. The integration of multiple calorimeters in a single instrument offers the unique advantage of allowing the simultaneous screening of a large number of factors affecting microbial activity, such as in substrate evaluation, toxicity testing or biodegradation studies. The simplicity of the overall system presented here, its reduced size and easy implementation into assemblies formed by arrays of microcalorimeters, and its low cost are important advantages of using miniaturized calorimeters based on microfabrication technologies.

This study shows that microcalorimeters based on thermopile silicon chips can be used successfully for the determination of the heat evolved during bacterial activity over long periods of time. The choice of *Escherichia coli* as a model organism relies on the large body of literature describing the physiology of this organism and on its ability to grow under different conditions.

2. Experimental

2.1. Construction of the calorimeter

The basic parts of this microcalorimeter are integrated silicon–aluminum thermopile chips NCM 9924 (Xensor Integration, Delft, NL). These chips were used as the sensor part of the calorimetric system (Fig. 1). The calorimetric setup consists of two independent sensors located within an aluminum frame, the heat sink, and covered by a 0.6 ml reaction chamber made of PTFE for improved thermal insulation from the surroundings, assuring that the heat flow takes place mainly through the silicon membrane. The second sensor was used as reference to minimize temperature perturbations on the output signal.

The performance of the chips has been improved with the aid of a polymer box photolithographically patterned on the Si membrane. The polymer thermally insulates the liquid sample from the frame and enhances the thermal conductivity between the hot and cold zone of the thermopiles. The nominal physical volume of the measuring vessel was 0.6 cm^3 with a calorimetric sensitive area of 25 mm^2 on the center of the chip. The enclosed reaction chamber eliminated evaporation of the liquid and assures a good reproducibility of the measurements. To minimize perturbations on the baseline caused by thermal variations on the surroundings of the calorimeter, the aluminum frame was placed on top of a 25 cm × 25 cm × 8 cm copper block acting as a thermal reservoir and all this assembly was introduced into a custom-made porex box. To increase thermal stability and reduce thermal drift during the experiments the whole setup was placed inside a hot air incubator with temperature control of ±1 °C (J.P. Selecta).

When operated in differential mode the twin system showed a baseline drift lower than 0.08 $\mu W\,h^{-1}$. The reduced long-term drift of the system is the key point that enables the study of processes in which weak heat effects are extended over long periods of time. In addition, the small time constant of the calorimeters, $\tau \sim 20$ ms, allows for multiple averaging of the experimental data, significantly improving the noise, without severely affecting kinetic measurements. The peak-to-peak electrical noise associated to the measurements was less than 0.2 $\mu W\,min^{-1}$.

2.2. Calibration and data acquisition

The calibration of the sensors was initially carried out introducing a controlled amount of power through a resistance heater integrated in the chip, and comparing its electrical response. The measurements were performed under steady state conditions with the chamber full of distilled water

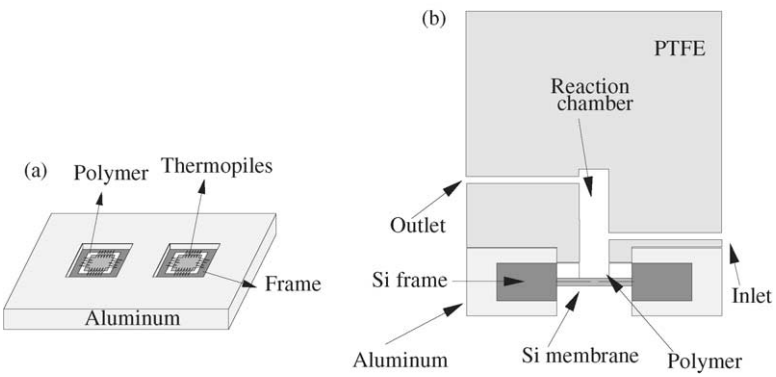

Fig. 1. Schematics of the microcalorimetric vessels. (a) Upper view of the silicon thermopile chips located within the aluminum frame, (b) cross-sectional view showing the different elements. The reaction chamber was made of PTFE for improved thermal insulation.

or microbial culture medium. A sensitivity of $1.24\,V\,W^{-1}$ was obtained. However, due to the specific location of the heater, electrical calibration may give rise to inhomogeneous temperature distributions in the reaction vessel resulting in a different heat flow pattern compared to the heat evolved during bacterial growth. Consequently, a chemical calibration, based on the imidazole catalyzed hydrolisis of triacetin as test reaction was performed following the procedure described in Beezer et al. [14] and Beezer and co-wokers [15]. The sensitivity obtained was $0.39\,V\,W^{-1}$. This value has been used throughout this work to obtain the thermal power generated by the cells. The signal recorded by the calorimeter is the differential voltage of the sample and reference vessels. The voltage output of the thermopile was digitized by a two channel Keithley nanovoltmeter, model 2182. A PC using a LABVIEW program controls the acquisition through a GPIB bus. Averaging over multiple points was used to improve the signal-to-noise ratio of the measured data.

2.3. Materials and procedure

E. coli K12 (CGSC 5073) was used throughout the experiments. Growth was carried out either in AB or in LB culture medium. AB medium [16] contains a mixture of inorganic salts which provide the nutrients required for growth, and 0.02% glucose as the only carbon and energy source. LB medium [17] contains 10 g of tryptone, 5 g of yeast extract and 15 g of NaCl per liter of medium. The medium was heat sterilized at 121 °C during 15 min before inoculation and insertion into the microcalorimeters.

Prior to the experiment, a three step cleaning and sterilization procedure of the flow tubing and measuring cell was carried out. Sterilized distilled water was pumped through the system for 15 min at a rate of $40\,ml\,h^{-1}$; afterwards an alcohol solution at 96%, and finally distilled water again were pumped at the same rate and amount of time. After an initial transient period, stabilization of the baseline followed and the bacteria were pumped into the sample cell. The culture medium was inoculated with an overnight culture of *E. coli* K12 at a final concentration around 10^3 cells ml^{-1}. Once inoculated, the medium was pumped into the reaction chamber of the calorimeter using a peristaltic pump (Watson–Marlow 505U) operated at $40\,ml\,h^{-1}$. Simultaneously, the reference cell was filled with fresh culture medium. Recording of the thermal profiles was performed using the flow-step method, i.e., when the microcalorimeter cell of 0.6 ml was full, the pump was stopped and recording of the power versus time data started. Measurements at different temperatures 18 °C, 32 °C and 37 °C, in LB and/or AB medium were conducted to assess whether the calorimeter was able to discriminate changes of microbial activity due to variations of temperature or composition of the culture medium.

The concentration of viable cells was determined at given intervals from samples taken directly from a parallel reactor incubated under exactly the same conditions. The samples were diluted in 0.9% NaCl and plated in LB agar plates. The plates were incubated 24 h at 37 °C and counted.

3. Modeling of cell growth

A simplified model has been developed to describe cell growth in AB medium. The effect of nutrient depletion on the specific growth rate (μ) of the organism has been modeled using the Monod equation [18]. The biomass production rate, X, can be simply related to the biomass at time t, $X(t)$, through the specific growth rate, μ, as $(dX/dt) = \mu X$. Considering oxygen concentration (S) as the primary growth limiting factor, oxygen uptake by the cells can be written as $(dX/dt) = -(\mu X/Y)$, where S is the concentration of oxygen dissolved in the medium, i.e. initially $S_0 = 5.4 \times 10^{-6}$ g, and Y_{X/O_2} is the aerobic growth yield of *E. coli* on oxygen, i.e.: $Y_{X/O_2} = 1.47\,g\,DW\,g^{-1}\,O_2$; [19]. The relationship between the specific growth rate and the concentration of limiting substrate, i.e.: oxygen, is given by the Monod equation, $\mu = \mu_m S/(K_s + S)$, where μ_m corresponds to the maximum specific growth rate and K_s is the half-saturation coefficient. To obtain the thermal power generated by cell growth, a linear relationship between power and mass at time t is assumed $(d\dot{Q}/dt) = \beta X$, being β the specific heat production rate which accounts for the heat production rate per unit of biomass. Combining the above mentioned equations a second order differential equation is obtained, i.e.

$$\frac{d\dot{Q}}{dt} = \beta\mu_m \frac{-\frac{1}{Y}\left(\frac{\dot{Q}}{\beta}\right)^2 + \left(\frac{\dot{Q}}{\beta}\right)\left(S_0 + \frac{X_0}{Y}\right)}{K_S + S_0 - \frac{1}{Y}\left(\frac{\dot{Q}}{\beta} + X_0\right)} \quad (1)$$

This equation is solved for $\dot{Q}$ using Mathematica. The main parameters of the model, assuming oxygen depletion is the limiting factor, are summarized in Table 1. S_0, Y_{X/O_2}, K_s are set to well defined literature values [19,20] and remain constant in all cases.

Table 1
Main parameters of the cell growth model

	Y_{X/O_2} (g DW g^{-1} O_2)	K_s (g)	S_0 (g)	μ_m (s^{-1})	m_0 (g)	β (W g^{-1} DW)
AB at 37 °C	1.47	1.3×10^{-6}	5.4×10^{-6}	0.41×10^{-3}	0.26×10^{-9}	3.28
LB at 37 °C	1.47	1.3×10^{-6}	5.4×10^{-6}	0.82×10^{-3}	1.1×10^{-9}	4.1
LB at 32 °C	1.47	1.3×10^{-6}	5.4×10^{-6}	0.57×10^{-3}	0.80×10^{-9}	2.9

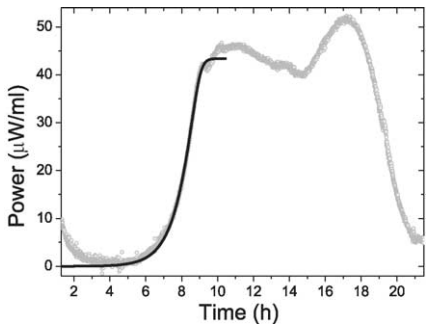

Fig. 2. Power–time of growth of *E. coli* in AB medium at 37 °C. Circles correspond to the experimental data. Continuous line is the best fit using Eq. (1).

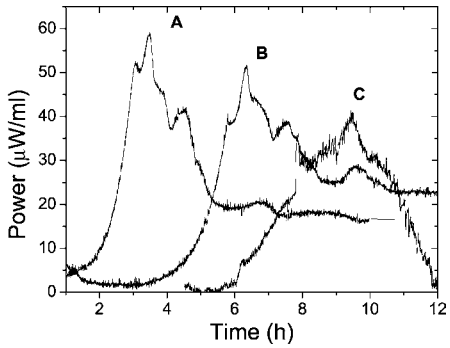

Fig. 3. Power–time curves of growth of *E. coli* in LB medium recorded at different temperatures. (A) 37 °C, (B) 32 °C and (C) 18 °C.

4. Results and discussion

A typical thermal profile of *E. coli* activity in AB medium at 37 °C is represented in Fig. 2. After an initial lag-phase in which the cells adapt to the culture medium, the thermal power increases exponentially with a doubling time of 40 min, which corresponds to the doubling time usually observed for *E. coli* growing in minimal medium at this temperature. A stabilization of the heat production rate observed after 9 h is presumably due to oxygen depletion. Since the culture was not aerated and the amount of oxygen initially present in saturated AB medium is about 9 mg l^{-1}, the maximum biomass which can be produced through aerobic growth can be calculated.

To carry out the calculation a previously reported yield for *E. coli* grown under aerobic conditions was used (Y_{X/O_2} = 1.47 g DW g^{-1} O_2; [19]). The results of the calculation indicate that the amount of biomass which can be formed, assuming an average cell weight of 0.433 pg DW $cell^{-1}$, is in the vicinity of 10^7 cells ml^{-1}, enough to detect exponential growth but about 10 times lower than the maximum numbers that could be obtained, with the amount of glucose available, if oxygen were continuously supplied during the length of the experiment. To test whether oxygen depletion might be responsible for the early stationary phase we have modeled the thermal power using Eq. (1) and the parameters given in Table 1. The excellent agreement between experimental and calculated data both in the exponential phase and in the onset of the stationary phase corroborates our initial assumption that oxygen depletion causes the end of exponential growth at population densities of ca. 10^7 cfu ml^{-1}. Because our model assumes a linear relationship between the heat production rate and the concentration of bacteria, the parameter β is related to the power output generated by a single bacteria. A value of 1.4 pW $cell^{-1}$ is found in AB at 37 °C. After oxygen depletion, a transient decrease is observed between 10 and 15 h followed by a final rise of activity. These changes can be attributed to fermentative growth once the culture has adapted to anoxic conditions. Towards the end of the experiment, thermal power drastically decreases due to glucose depletion as already evidenced by other authors [6]. In the final stage the cells are still active since the baseline is slightly higher than in the initial period.

Fig. 3 shows the effect of temperature on microbial activity for cultures with an initial concentration close to 10^3 cfu ml^{-1} in LB medium. As expected, the log phase starts later and the duplication time is longer in the thermal profile recorded at lower temperatures. The duplication time during the exponential growth is calculated assuming that the power generated by the population of *E. coli* is proportional to its concentration.

Simultaneous measurements in a twin culture system of the heat production rate and *E. coli* concentration show that under the conditions used, the log phase finishes when the concentration has reached a value of 5×10^6 cfu ml^{-1}. The inset of Fig. 4 shows the relationship between bacteria concentration and heat production rate during the exponential growth. A duplication time of 96 min, 29 min and 21 min at 18 °C, 32 °C and 37 °C, respectively, has been measured. These results are in agreement with previous literature data [8].

The fit to the exponential growth in LB using Eq. (1) is also shown as a continuous line in Fig. 4. Table 1 summarizes the parameters used to fit the different growth conditions. The

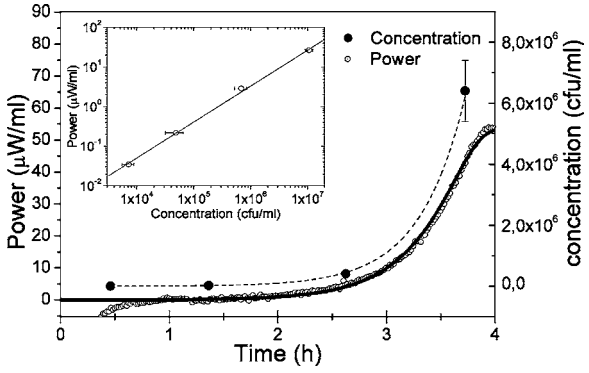

Fig. 4. Relationship between bacteria concentration and heat production rate during the exponential growth in LB at 37 °C. The dashed line is plotted as a guide to the eye. The continuous line is the best fit using Eq. (1) and the parameters given in Table 1. The inset shows the linear relationship between power and concentration.

parameters Y_{X/O_2}, K_s and S_0 are constant, μ_m is set to previously obtained values, m_0 changes slightly due to small variations in the initial conditions of the particular experimental run and β varies according to the different heat production rates, depending on both medium type and temperature. At 37 °C the power generated by a single bacteria ranges from 1.4 pW cell^{-1} in AB to 3.5 pW cell^{-1} in LB. This fitting parameter can be compared to the theoretical heat production rate for aerobic growth of *E. coli* on glucose. This value can be estimated from the oxygen consumption rate (q_{O_2}) using a well established conversion factor of 124 kcal mol^{-1} O_2 [21]. The rate of oxygen consumption can be calculated from the specific growth rate of the organism (μ) using the yield Y_{X/O_2} of 1.47 g DW g^{-1} O_2. In all cases, the heat production per cell has been determined using a mass of 0.865 pg DW for a single cell of *E. coli* growing with a doubling time of 20 min, and 0.433 pg DW cell^{-1} when growth occurs with a doubling time of 40 min [22]. The values of power output obtained in each case are 4.6 pW cell^{-1} for the fast growing cells and 1.2 pW cell^{-1} for slow growing cells. Those values agree well with those obtained from the fit to the experimental data using Eq. (1).

Another estimation of the quantitative validity of the calorimetric measurements relies on the integration of the Power–time curve in the interval where aerobic growth occurs. The total amount of heat evolved during oxidative growth on oxygen in LB medium is 124 J l^{-1}. This result is in excellent agreement with the enthalpy release of 130 J l^{-1} predicted using previously published heat yields (519 kJ mol^{-1} O_2) [21] and using the amount of oxygen initially present in the culture (9 mg l^{-1}). A similar calculation made using the growth stoichiometry and heat yield published by Winkelmann et al. [6] for the aerobic growth of *P. denitrificans* growing on glucose gives a estimate of 176 J l^{-1}, slightly higher that the value we have measured.

5. Conclusions

It has been shown that thermopile based microreactors are suitable to measure metabolic activity of living cells over extended periods of time. A temperature resolution of 50 μK, together with a baseline drift lower than 0.08 μW h^{-1} are the key points that enable the study of low heat production rate processes. The thermal profiles in AB or LB medium showed the main characteristics of the metabolic activity of *E. coli* during the different stages. Doubling times measured at different temperatures agreed with previously reported values. Modeling of the cell growth in AB or LB medium using standard parameters showed that oxygen depletion is the main responsible for the premature occurrence of the stationary phase. The heat production rate of individual cells of *E. coli* growing at 37 °C in AB and LB medium has been estimated as 1.4 and 3.5 pW cell^{-1}, respectively.

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