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Calorimetric studies of the growth of anaerobic microbes

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This article aims to validate the use of calorimetry to measure the growth of anaerobic microbes. It has been difficult to monitor the growth of strict anaerobes while maintaining optimal growth conditions. Traditionally, optical density and ATP concentration are usually used as measures of the growth of anaerobic microbes. However, to take these measurements it is necessary to extract an aliquot of the culture, which can be difficult while maintaining anaerobic conditions. In this study, calorimetry was used to continuously and nondestructively measure the heat generated by the growth of anaerobic microbes as a function of time. *Clostridium acetobutylicum*, *Clostridium beijerinckii*, and *Clostridium cellulovorans* were used as representative anaerobic microbes. Using a multiplex isothermal calorimeter, we observed that peak time (t_p) of *C. acetobutylicum* heat evolution increased as the inoculation rate decreased. This strong correlation between the inoculation rate and t_p showed that it was possible to measure the growth rate of anaerobic microbes by calorimetry. Overall, our results showed that there is a very good correlation between heat evolution and optical density/ATP concentration, validating the use of the method.

[**Key words:** Calorimetry; *Clostridium acetobutylicum*; *Clostridium beijerinckii*; *Clostridium cellulovorans*; Anaerobic microbes; Growth processes]

It is necessary to understand the growth process of a microorganism to evaluate its activity. A growth curve is often generated from measurements of the density of microbial cells at arbitrary time points throughout growth, which are classified into lag phase, log phase, stationary phase, and death phase. The pattern of the growth process is basically conserved between different microbial species, but the time spent in lag phase and the slope of the curve during log phase growth differ depending on the type and number of microorganisms and the growth environment. This information is extremely important for the management of fermentation, sampling inspection of microbial contamination of food and the environment, and for screening for useful microorganisms. Measurement of the optical density (OD) of a culture is commonly used to assess the growth phase, and reflects the number of microorganisms in the culture at a given time (1). Measurement of integrated ATP concentration can also be used to assess the growth of some microorganisms (2). However, when a microorganism aggregates in a biofilm, it is difficult to measure growth. This is particularly true for OD measurement, as microbial cells must be uniformly dispersed in the culture medium to obtain an accurate OD reading. In contrast, ATP can be measured with good sensitivity from aggregated cells, but a sample of the culture medium must still be removed for each measurement. However, because ATP is present not only in the microbial cell but also in components of the culture medium, assessment of growth using ATP measurement is complicated and requires removal of the ATP from culture medium components. Both OD and ATP measurements are thus laborious work.

During the growth of microbial cells, heat is generated at around 1–100 pW per cell (3–6). Microcalorimetry can reliably measure heat evolution at values of approximately 1 μW. During microbial cell culture, it is possible to measure the growth process by detecting variations in heat evolution. A multiplex isothermal calorimeter (7) can detect heat evolution of samples from differences in temperature between each sample cell and a reference cell by monitoring the thermoelectromotive force of each thermopile (7) (Fig. 1). The thermograms generated by the calorimetric measurement indicate time-dependent changes in heat evolution of each sample, and are independently obtained by outputting the data from an acquisition unit. Using this method, it is not necessary to remove samples from the culture as calorimetry can continuously and nondestructively measure heat evolution of the culture. Previous studies have used a multiplex isothermal calorimeter to measure the growth of yeast under aerobic conditions and determine the effects of inhibitory substances on growth (8,9). The growth of *Escherichia coli* under anaerobic conditions has also been examined using this method (10). However, there is no research into whether this method can be used to measure the growth of obligate anaerobes quantitatively.

Obligate anaerobes cannot grow in the presence of oxygen. When measuring the growth of these organisms, it is necessary to maintain strict anaerobic conditions even when collecting samples for measurement. This is often very difficult to achieve and requires an anaerobic chamber. Therefore, in the current study we investigated the use of calorimetry to measure the heat evolution of

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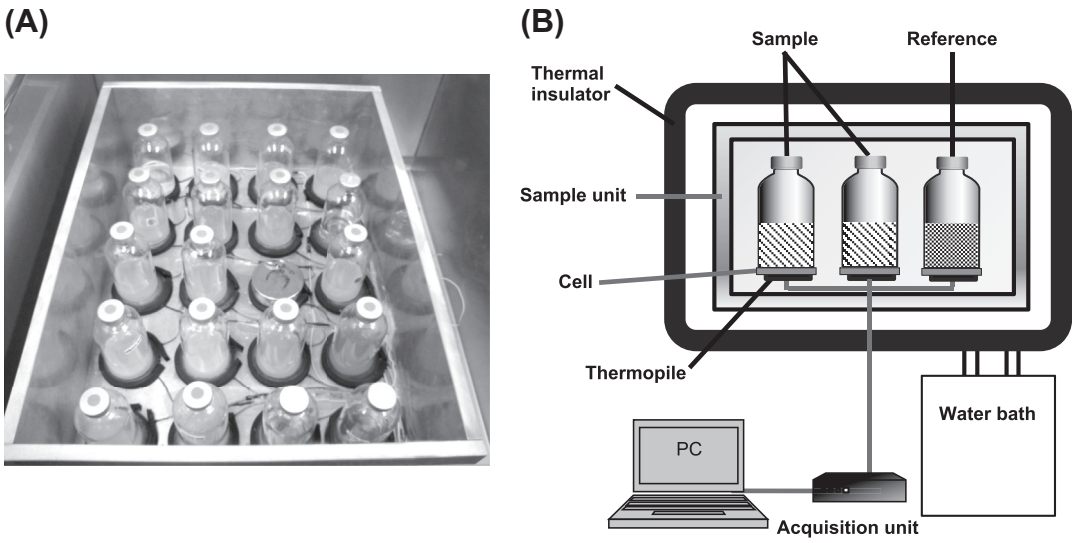

FIG. 1. Photograph of sample unit in the multiplex isothermal calorimeter (A) and the cross-sectional drawing of it (B). Each vial was placed on the thermopile and incubated in the sample unit. The temperature was maintained using a circulating water bath with a temperature control of ±0.1°C.

obligate anaerobes and butanol-producing *Clostridium* strains *Clostridium acetobutylicum* and *C. beijerinckii*, which are important industrial microbes (11–13). The aggregating and cellulosome-producing species *Clostridium cellulovorans* was also used (14–16). Using this method we attempted to determine the growth processes of each strain by measuring heat evolution by calorimetry.

MATERIALS AND METHODS

Determination of calorimetric parameters A multiplex isothermal calorimeter with 20 cells was used for calorimetry (7,17). A schematic diagram is shown in Fig. 1. Both sample cells and reference cells were included in the sampling unit of the device. The temperature was maintained using a circulating water bath with a temperature control of ±0.1°C. The change in voltage corresponds to the heat evolution. Eq. 1 was used to correct the heat loss to determine the heat evolution $q(t)$ assuming ideal adiabatic conditions (18,19),

$$q(t) = g(t) + K\int g(t)dt \quad (1)$$

where $g(t)$ is a raw data thermogram, which was obtained by subtracting the heat evolution value of the reference from that of the sample. The thermal conductivity constant (K) value of each cell was determined using the method described by Takahashi (7).

The calorimetric parameters of the growth process were calculated using the Richards model equation, which is a model of the growth curve (17,20) (Eq. 2).

$$q(t) = Q\left(1 + (d-1)\exp\left(-B(t-t_p)\right)\right)^{\frac{1}{1-d}} \quad (2)$$

where Q is the total amount of heat evolution, t_p is the time taken to reach peak heat evolution, d is a dimensionless adjustable parameter of the growth curve which determines the asymmetry of the differential form $q'(t)$ of Eq. 2 with respect to t_p (17), and B is a rate parameter (17). When t is infinite, q becomes equal to Q. The maximum heat evolution rate V_{max} can be described using Q, B, and d by the following equation:

$$V_{max} = QBd^{\frac{d}{1-d}} \quad (3)$$

The growth process of each anaerobic microbe was evaluated using these parameters on the basis of the differential form $q'(t)$ of Eq. 2 (17).

Bacterial strains and culture media *C. acetobutylicum* (NBRC 13948) and *C. beijerinckii* (NCIMB 8052), representative butanol-producing strains, and *C. cellulovorans* 743B (ATCC 35296), a cellulosome-producing strain, were used. Cultures were grown in 50-mL vials (Maruemu Co., Osaka, Japan), while pre-cultures and dilutions were carried out in 16 × 125-mm Hungate tubes (Chemglass Life Sciences LLC, Vineland, NJ, USA). Thioglycollate medium was used to culture *C. acetobutylicum* and *C. beijerinckii*. The medium contained per liter: 15 g hipolypeptone (Wako Pure Chemical Industries Ltd., Osaka, Japan), 5 g dried yeast extract, 2.5 g NaCl, 0.5 g sodium thioglycolate, 0.5 g L-cysteine·HCl·H_2O, and 1 mg resazurin as an oxygen indicator. The culture medium was adjusted to pH 7.0. A 20% (w/v) glucose solution was prepared separately and added to the medium after autoclaving. Both the culture medium and the glucose solution were flushed with CO_2 gas for 20 min prior to autoclaving (40 min at 121°C). The culture medium for *C. cellulovorans* was prepared as described by Sleat *et al.* (14). Cellobiose (5 g/L) was added to the culture medium. When OD or ATP measurements were performed, a resazurin-minus culture medium was used.

Inoculation of culture media A 1-mL aliquot of pre-culture was inoculated into vials containing 20 mL of culture medium for use in calorimetry and OD and ATP assays. To vary the inoculation rate, pre-culture was diluted 10–10^8-fold in sterile culture medium in an anaerobic chamber. A 1-mL aliquot of each dilution was then individually inoculated into a vial containing 20 mL of culture medium containing 0.5% (w/v) glucose and used as a sample for calorimetry. The samples were immediately placed within the sample unit of the multiplex isothermal calorimeter, and the heat evolution of each sample was measured until no evolution was observed at 35°C. Uninoculated culture medium was used as a reference.

When carrying out calorimetry and OD measurements of samples with varying glucose concentrations, a one-way valve was mounted in the butyl rubber stopper of the vial. Thus, gas generated by microbial metabolism was released from the culture medium.

Measurement of optical density and ATP concentration As it is not possible to remove samples from the culture medium during calorimetry measurements, duplicate cultures were prepared in an incubator maintained at the same temperature as the calorimetry assay. ODs and ATP concentrations were determined by sampling these cultures across the growth period. The OD of each sample was measured at 590 nm using a WPA CO7500 colorimeter (Biochrom Ltd., Cambridge, UK). The ODs of samples with varying glucose concentrations were measured at the completion of the calorimetry. For ATP measurement, 10 μL of ATP eliminating reagent (Kikkoman Biochemifa Co., Tokyo, Japan) were added to 100 μL of culture solution and incubated for 30 min to eliminate extracellular ATP. The intracellular ATP concentration was then determined using a LuciPac Pen (Kikkoman Biochemifa Co.) and Lumitester PD-20 (Kikkoman Biochemifa Co.). The integrated ATP concentration was determined by approximating the integral value using the integration function of Origin software (OriginLab Co., Northampton, MA, USA).

RESULTS

Previous calorimetric studies using yeast under aerobic conditions have shown that the time taken to reach logarithmic growth phase and the time until peak growth rate change depends on the inoculation rate (8). Therefore, we first examined the effects of the inoculation rate on the growth rate of the anaerobic microbes using a multiplex isothermal calorimeter. The thermograms and calorimetric parameters for various inoculation rates of *C. acetobutylicum* are shown in Fig. 2 and Table 1, respectively. Fig. 2C shows an example of comparison of the observed $q'(t)$ values obtained on the basis of Eq. 1 with the theoretical $q'(t)$ values obtained using the