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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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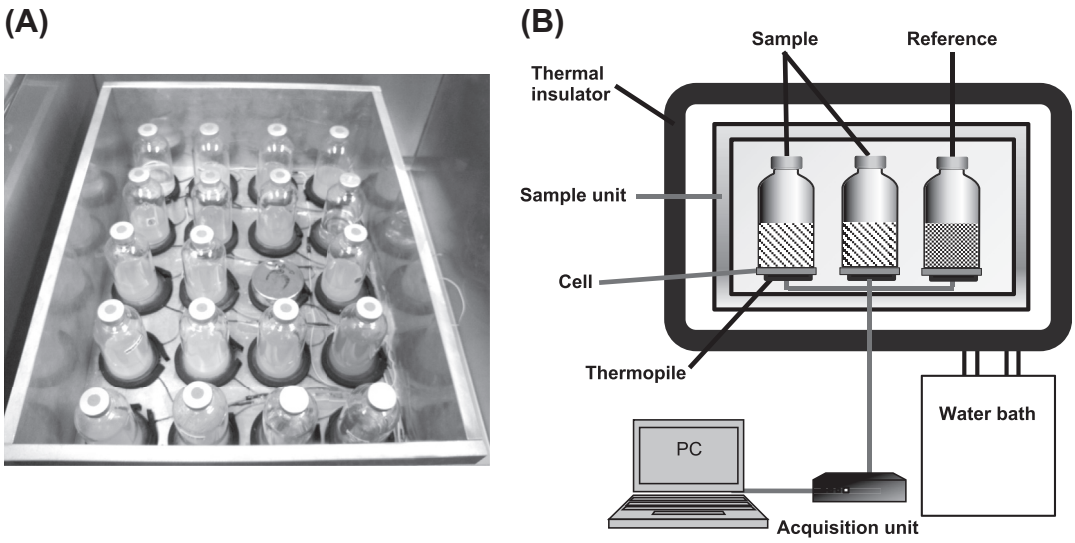

FIG. 1. Photograph of sample unit in the multiplex isothermal calorimeter (A) and the cross-sectional drawing of it (B). Each vial was placed on the thermopile and incubated in the sample unit. The temperature was maintained using a circulating water bath with a temperature control of ±0.1°C.

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

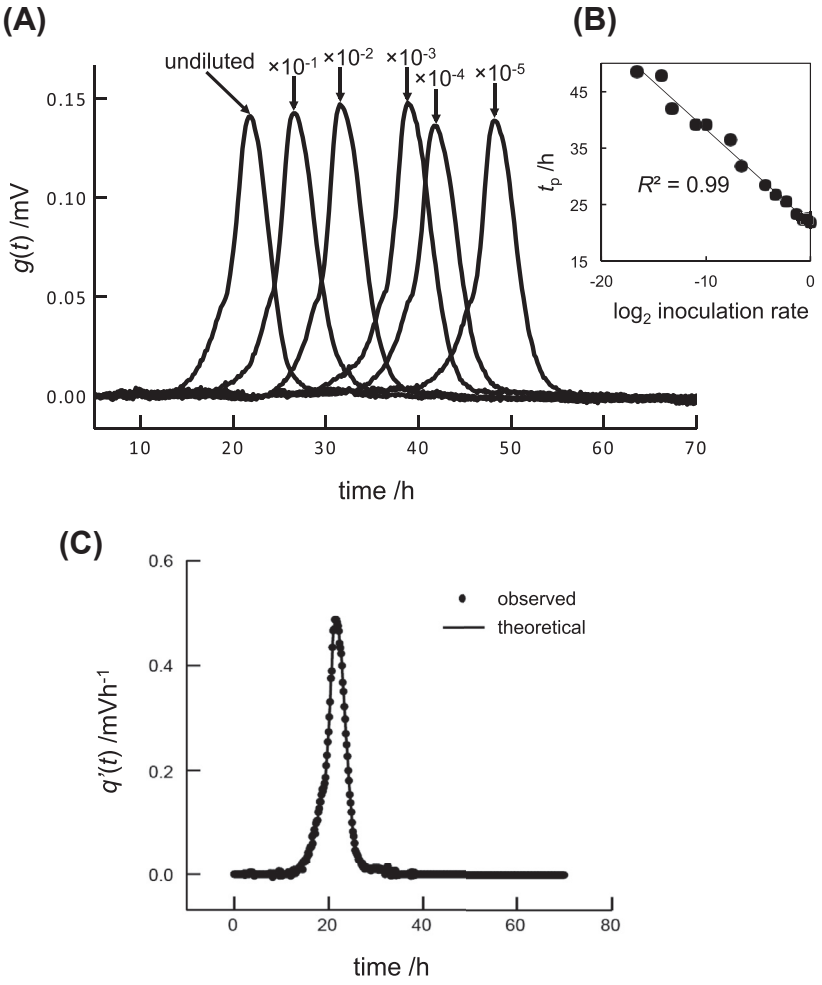

FIG. 2. The growth thermograms generated for cultures with various inoculation rates. Each sample was used the medium containing 0.5% glucose. The inoculation rate indicates dilution rate of pre-culture (diluted 10–100,000,000-fold and undiluted). (A) Thermogram for *C. acetobutylicum*. (B) Plot of t_p versus the converted $\log_2$ value from the inoculation rate. (C) Comparison of the observed (closed circles) and the theoretical (solid line) values of $q'(t)$ of the undiluted experiment in Fig. 2A. The former values were obtained from $g(t)$ using Eq. 1, and by numerical differentiation with respect to the time. The solid line is drawn using the values listed in Table 1 and the differential form of Eq. 2 (17).

values listed in Table 1. Good agreement between these values indicates that the evaluated values of the parameters are reliable. The t_p (time taken to reach peak heat evolution) for this bacterium changed between 22 and 49 h. When the inoculation rate was small, the value of t_p increased, indicating a highly negative correlation between the inoculation rate and t_p (Fig. 2B). Conversely, no significant change in the V_{max} and Q values were observed when the inoculation rate was altered (Table 1). No heat evolution was observed at dilutions of the inoculum of greater than 10^6-fold.

The heat evolution recorded by the multiplex isothermal calorimeter was then analyzed to determine whether it reflected the growth processes of *C. acetobutylicum* and *C. beijerinckii*. The thermograms generated for each of the strains were compared with the growth curves obtained by OD measurements under the same conditions (Fig. 3A and B, respectively). *C. acetobutylicum* entered logarithmic phase after 18 h, and reached stationary phase 40 h later. *C. beijerinckii* reached logarithmic phase at 10 h, and reached stationary phase 30 h later. The thermograms of both strains were highly reproducible, with the time taken to shift from lag phase to log phase and the growth rate in log phase being almost identical across the replicates. It is thus evident that the curves generated by the thermograms corresponded with OD measurements.

When *C. cellulovorans* is cultured in medium containing a soluble carbon source, the cells aggregate (14). Therefore, the thermogram generated for *C. cellulovorans* was compared with the growth curve generated from integrated ATP concentration measurements instead of OD. Heat evolution by this strain was observed after 10 h, and lasted for 40 h (Fig. 3C). Again, the $q(t)$ thermograms and the integrated ATP concentration growth curves corresponded based on time.

Butanol-producing *Clostridium* strains grow by metabolizing a carbon source such as glucose (13). By varying the glucose concentration in the culture medium, the cell concentration at stationary phase also changes. Therefore, the growth of *C. beijerinckii* was analyzed under different glucose concentrations by calorimetry to determine whether there were observable changes in the thermograms and the calorimetric parameters (Fig. 4A and B).

TABLE 1. Summary of calorimetric parameters of the growth of *C. acetobutylicum* when cultured using varying inoculation rates.

The inoculation rate[a]	t_p/h	V_{max}/mV h^{-1}	Q/mV	B/h^{-1}	d
1.0 (undiluted)	21.9	0.480	2.43	0.98	2.80
1×10^{-1}	26.7	0.486	2.55	0.80	2.17
1×10^{-2}	31.8	0.510	2.64	0.82	2.21
1×10^{-3}	39.2	0.505	2.79	0.85	2.60
1×10^{-4}	42.0	0.468	2.60	0.82	2.48
1×10^{-5}	48.5	0.525	2.75	0.90	2.61

[a] Inoculation rate indicates dilution of pre-culture.

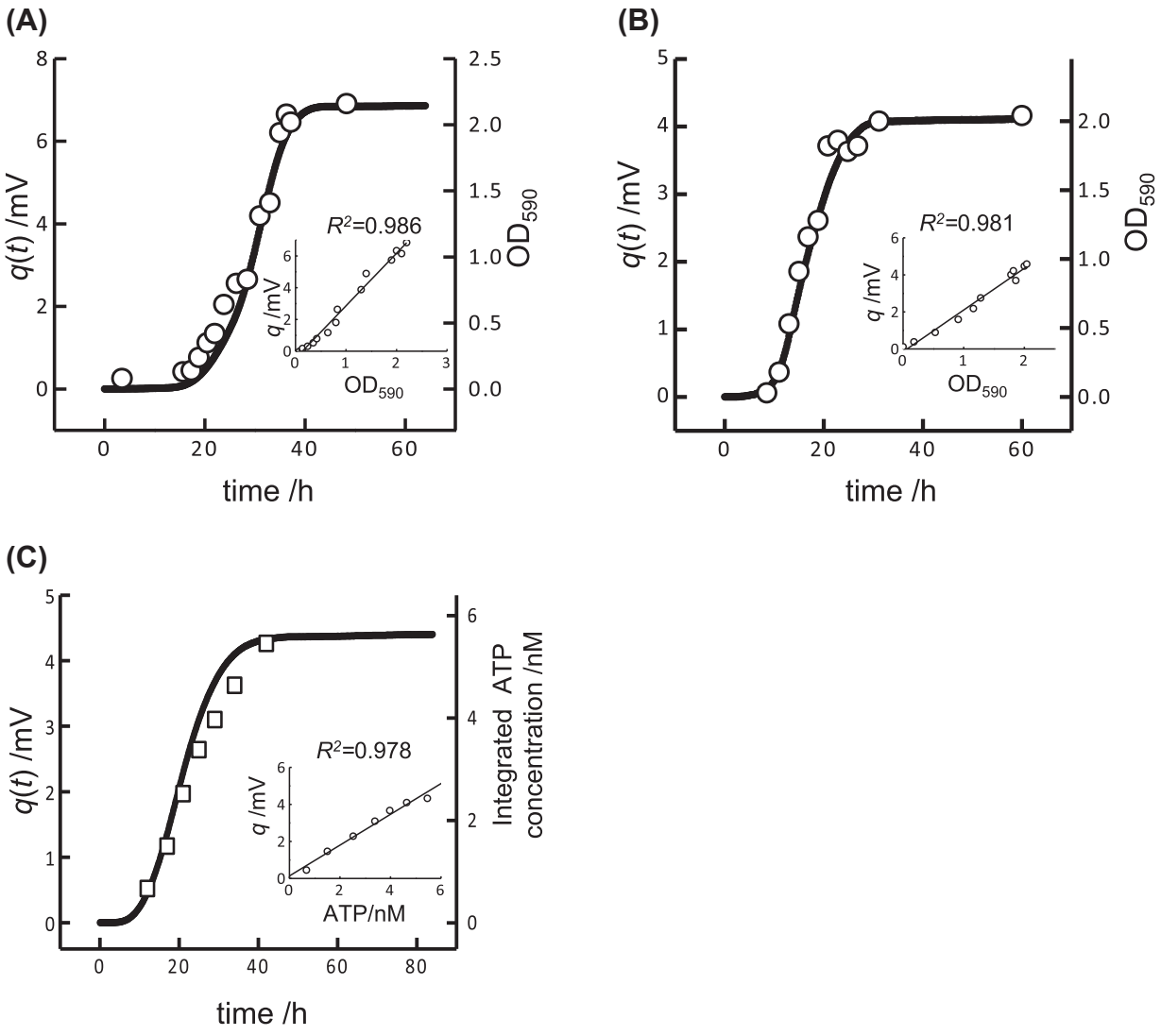

FIG. 3. Time courses of heat evolution by *C. acetobutylicum* (A), *C. beijerinckii* (B), and *C. cellulovorans* (C). *C. acetobutylicum* (A) and *C. beijerinckii* (B) were grown with 5% glucose. *C. cellulovorans* (C) was grown with 0.5% cellobiose. The $q(t)$ of each strain was calculated from $g(t)$ obtained using the multiplex isothermal calorimeter (line). Circles and squares indicate optical density and integrated ATP concentration, respectively.

When the glucose concentration in the culture medium was increased, heat evolution was detected for a longer period. No significant differences in the peak heat evolution were observed for glucose concentrations of >0.1%. The calorimetric parameters and OD measurements for the glucose concentration assays are shown in Fig. 5. Q, t_p, and OD all increased when the glucose concentration was increased. V_{max} remained similar at glucose concentrations of 0.1% or greater.

DISCUSSION

Calorimetric analysis of the growth of the obligate anaerobe *C. acetobutylicum* showed that the t_p increased as the inoculation rate decreased (Fig. 2A, Table 1), indicating a strong correlation between the inoculation rate and t_p (Fig. 2B). Similar results have been observed for yeast under aerobic conditions (8). Therefore, the t_p value accurately reflects the characteristics of microbial growth for both aerobic and anaerobic microbes. Additionally, the slope of the relationship between the $\log_2$ of the inoculation rate and t_p is a measure of the doubling time, which in this case was 1.67 h (Fig. 2B). When q_1 at time t_1 reaches q_2 at t_2, the heat evolution rate (v) is expressed by the equation $v = \ln(q_2/q_1)/(t_2 - t_1)$. Here, when the heat evolution is assumed to correspond to the concentration of microbial cells, the heat evolution should double when the number of microbial cells doubles; that is $q_2/q_1 = 2$. Doubling time (t_d) of microbial cells at the maximum growth rate (V_{max}) is represented by $t_d = \ln 2/V_{max}$. The average doubling time calculated from V_{max} in Table 1 was 1.4 h, indicating that V_{max} determined by calorimetry is a reliable parameter. These results also confirm that it is possible to determine the growth rate of obligate anaerobes using calorimetry in the same way as aerobic bacteria, and that t_p and V_{max} are useful parameters for characterizing the growth rate.

The current study also showed that there is a good correlation between the $q(t)$ thermograms and growth curves generated from OD measurements in *C. acetobutylicum* and *C. beijerinckii* (Fig. 3A and B). Additionally, there was good correlation between the $q(t)$ thermogram of *C. cellulovorans* and the growth curve generated from integrated ATP concentration analysis (Fig. 3C). Therefore, we concluded that the heat evolution of these strains obtained by calorimetry reflects the number of anaerobic microbial cells present in a culture at a given time point. Using a multiplex isothermal calorimeter, it is possible to continuously measure heat evolution while maintaining anaerobic conditions. Furthermore,

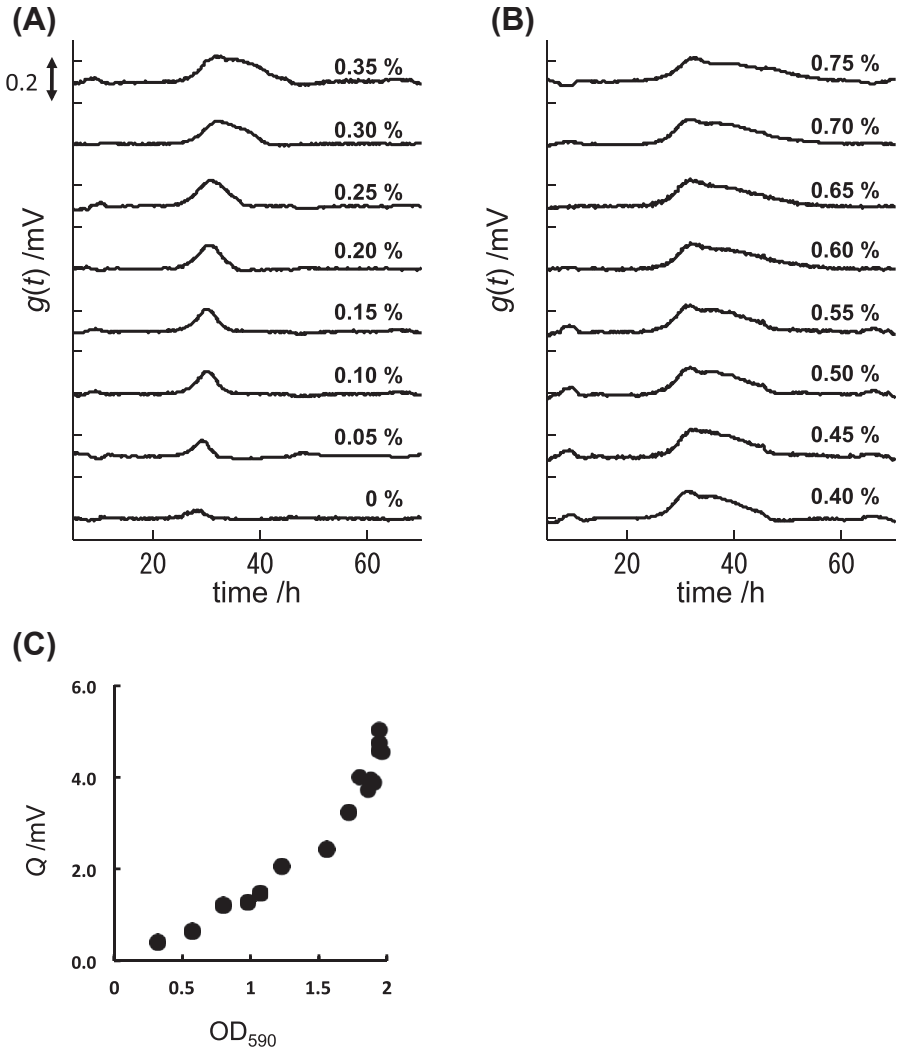

FIG. 4. Growth thermograms (A, B) of *C. beijerinckii* generated for cultures with various glucose concentrations. *C. beijerinckii* was grown with between 0% and 0.75% glucose. (A) From the bottom, 0%, 0.05%, 0.10%, 0.15%, 0.20%, 0.25%, 0.3% and 0.35% glucose. (B) From the bottom, 0.40%, 0.45%, 0.50%, 0.55%, 0.60%, 0.65%, 0.70% and 0.75% glucose. (C) Plot of *Q* versus OD.

the growth process of anaerobic microbes can still be measured using this method even if the cells are aggregated. Additionally, this method is effective in the presence of insoluble sugars and biomass given that calorimetry is not affected by the OD of the sample. This technique may therefore be particularly useful in industrial applications.

When *C. beijerinckii* was cultured with various glucose concentrations, OD was measured at the stationary phase, which was then compared with total heat evolution (*Q*) (Fig. 4C). A relatively high correlation was observed between OD and *Q*, suggesting that the value of *Q* obtained by calorimetry reflects the cell density in the culture. However, when the OD increased, that is, when the glucose concentration was high, the correlation weakened. It is likely that heat evolution from the metabolic products contributed to an overestimation of the actual value of *Q*. In future studies, the heat evolution from the metabolic products should be considered so as to improve the sensitivity of the thermopile and to accurately perform calorimetry under high glucose concentrations.

The value of t_p increased in accordance with increasing glucose concentrations (Fig. 5A). Although the initiation time of the heat evolution was approximately the same based on the shape of the thermograms (Fig. 4A and B), we inferred that this was because the heat evolution time became longer in accordance with increasing glucose concentration. There was no significant difference in V_{max} under any of the conditions except at glucose concentrations of 0% and 0.05% (Fig. 5B). As described above, V_{max} corresponds to the doubling time as $t_d = \ln 2/V_{max}$. When there is no carbon source, or a very low concentration of the carbon source in the culture medium, microbial growth is minimal. However, when there are sufficient amounts of carbon in the culture medium, regardless of the microbial cell density, the doubling time is approximately constant.

Calorimetry using the multiplex isothermal calorimeter can automatically collect data without the need to remove samples from the culture medium. Therefore, it is possible to accurately and simply observe the growth of anaerobic microbes while maintaining anaerobic conditions. While the OD cannot be accurately measured when cells are aggregated, calorimetry is a promising method of measuring microbial growth even when cells are aggregated. This study also showed that t_p, V_{max}, and *Q* are useful parameters for characterizing the growth rate and growth processes of microbes, even under anaerobic growth conditions. This method may be useful for high-throughput measurements of the growth of novel biotechnological strains, and to screen and breed mutant strains for tolerance to inhibitors such as alcohol and solvents.

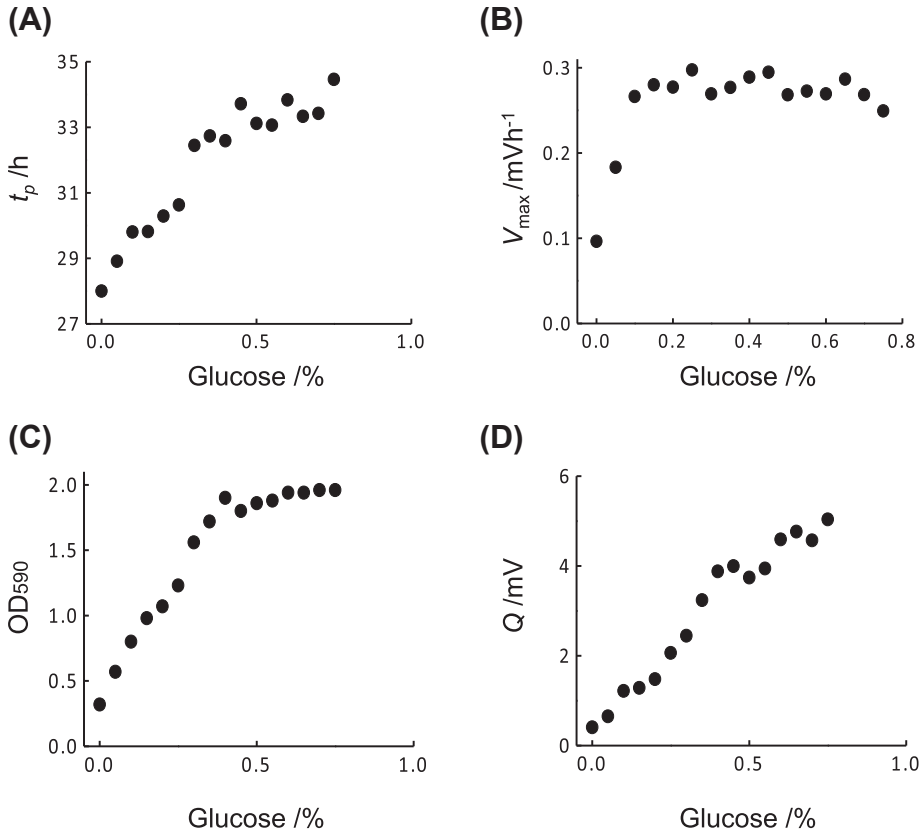

FIG. 5. Plot of calorimetric parameters and OD of *C. beijerinckii* versus glucose concentration.

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