Article

Calorimetric Analysis of the Growth of Anaerobic Microbes Cultured on Insoluble Carbon Sources

Naoko Aoki and Hideo Miyake

Graduate School of Bioresources, Mie University, Tsu, Mie 514-8507, Japan

(Received Jan. 24, 2018; Accepted Feb. 27, 2018)

Methods for the measurement of anaerobic microbial growth are generally complex. Furthermore, aggregation and precipitation make it difficult to estimate bacterial cell counts using turbidity. Recently, we demonstrated that heat evolution is a good indicator of the growth of anaerobic bacteria cultured on soluble carbon sources. In this study, we validated the use of calorimetry to measure growth on insoluble carbon sources using *Clostridium* spp. When *Clostridium cellulovorans* and *C. thermocellum* were cultured on crystalline cellulose, their optical density values over time were irregular. However, the thermograms indicating the heat evolution and the integrated ATP concentrations of the cultures showed highly correlated growth curves. When *C. acetobutylicum* and *C. beijerinckii* were cultured on corn starch, peaks were observed at the initial stage based on thermograms and ATP concentration measurements. Thin-layer chromatography revealed that these peaks corresponded to the rapid metabolism of monosaccharides and disaccharides in the culture broth. Thus, the growth of anaerobic microbes cultured on insoluble carbon sources can be measured conveniently and accurately by calorimetry, without confounding effects of aggregation or precipitation. Growth can be measured with high sensitivity, comparable to that of ATP measurements, indicating that calorimetry is a promising method for measuring microbial growth.

Keywords: calorimetry, cell growth processes, anaerobic microbes, *Clostridium*

1. Introduction

Some *Clostridium* spp. produce cellulosomes, which are enzyme complexes that efficiently degrade cellulose and hemicellulose. These cellulosome-producing Clostridia are promising for the microbial pretreatment and saccharification of lignocellulosic biomass (1-3). Solvent-producing *Clostridium* spp. have long been important industrial microbes (4-6). They produce biobutanol, which has many advantages over bioethanol, such as its higher combustion efficiency. Currently, starch and cellulosic biomass are used as raw materials for biofuels. As these materials are insoluble, it is difficult to accurately estimate the number of bacterial cells based on culture turbidity. In addition, some cellulosome-producing *Clostridium* spp. aggregate and bind to cellulose, resulting in a heterogeneous bacterial broth (1, 2, 7).

Optical density (OD) and ATP concentration are common measures of microbial growth (8, 9). Clostridia are anaerobic bacteria. It can be difficult to measure the growth of anaerobes; it is necessary to extract an aliquot of the culture broth, which must be maintained in strictly anaerobic conditions. Recently, calorimetry has been used to continuously and non-destructively measure the heat generated by the growth of anaerobic microbes as a function of time. We previously demonstrated that heat evolution is a good indicator of the growth of anaerobic bacteria cultured on soluble carbon sources (10). However, it is not clear if the growth of microbes cultured on insoluble carbon sources, such as crystalline cellulose and starch, which precipitate at the bottom of the culture vessel, can be measured by calorimetry. And furthermore, there is no way to continuously and non-destructively measure the growth of anaerobic microbes when cultured on insoluble carbon source. Therefore, in this study, we aimed to validate the use of calorimetry to measure the growth of anaerobic microbes cultured on insoluble carbon sources. We assessed the growth of the cellulosome-producing *Clostridium cellulovorans* and *C. thermocellum* and the solvent-producing *C. acetobutylicum* and *C. beijerinckii*, which are important for biofuel production. Our results have practical implications for the evaluation of the efficacy of the microbial pretreatment of various biomass materials.

2. Materials and Methods

2.1 Bacterial strains, culture media, and inoculation

The strains *C. cellulovorans* 743B (ATCC 35296), *C. thermocellum* (NBRC 103400), *C. acetobutylicum* (NBRC 13948), and *C. beijerinckii* (NCIMB 8052) were used in this study. Cultures were grown in 50-mL vials (Chemglass Life Sciences, Vineland, NJ, USA). Special-grade reagents from Nacalai Tesque (Kyoto, Japan) or Wako Pure Chemical Industries (Osaka, Japan) were used throughout the study.

The culture medium without cellobiose for *C. cellulovorans* was prepared as described by Sleat *et al.* (11). The culture medium for *C. thermocellum* contained (per liter): 0.65 g of $K_2HPO_4 \cdot 3H_2O$, 0.5 g of KH_2PO_4, 1.3 g of $(NH_4)_2SO_4$, 42 g of 3-(*N*-morpholino)propanesulfonic acid, 5 g of yeast extract, 1 g of L-cysteine・HCl・H_2O, 0.5 g of $MgCl_2$, and 2 mg of resazurin as an oxygen indicator. Additionally, 0.1 g of Avicel® (microcrystalline cellulose; Sigma–Aldrich, St. Louis, MO, USA) was added as the insoluble carbon source. The appropriate medium (19.9 g), which had been flushed with CO_2 gas, for *C. cellulovorans* or *C. thermocellum* was placed into 50-mL vials in the anaerobic chamber, and the media was adjusted to 0.5 % (w/w) Avicel® prior to autoclaving (40 min at 121°C). When the OD and ATP concentration were assessed, resazurin-free culture medium was used.

C. acetobutylicum and *C. beijerinckii* were cultured in thioglycollate medium without glucose, as previously described (10). Corn starch (0.1 g; Nihon Shokuhin Kako, Tokyo, Japan) was added as the insoluble carbon source. The medium (19.9 g), which had been flushed with N_2 gas, was placed into 50-mL vials in the anaerobic chamber, and was adjusted to 0.5% (w/w) corn starch prior to autoclaving (40 min at 121°C).

Pre-culture aliquots were inoculated into vials containing the appropriate culture media with Avicel® or corn starch for use in calorimetry, OD, and ATP assays.

2.2 Measurement of heat evolution

Calorimetry was performed with a multiplex isothermal calorimeter with 25 cells (10, 12, 13). The inoculated culture

media were immediately placed within the sample unit of the multiplex isothermal calorimeter. The heat evolution of each culture sample was measured (at 35°C for *C. cellulovorans*, *C. acetobutylicum*, and *C. beijerinckii*, or at 55°C for *C. thermocellum*) until no further evolution was observed. An aliquot of non-inoculated culture medium was used as a reference. The change in voltage indicated by the calorimeter corresponded to the heat evolution of the culture medium. The calorimetric parameters of the growth process were calculated using Richards equation, a model of the growth curve, as follows (10, 13, 14) (Eq. 1):

$$q(t) = Q(1 + (d-1)\exp(-B(t - t_{\mathrm{p}})))^{\frac{1}{1-d}} \qquad (1)$$

where Q is the total 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. 1 with respect to t_{p}; and B is a rate parameter (10, 13). The growth of each anaerobic microbe was evaluated using these parameters on the basis of the differential form $q'(t)$ of Eq. 1, as previously described (10, 13).

2.3 Measurement of OD and ATP concentration

As it is not possible to remove samples of culture medium during calorimetry, duplicate cultures were prepared in an incubator maintained at the same temperature as that used in the calorimetry assay. The OD values and ATP concentrations were determined by sampling the cultures over the growth period, as described previously (10). The integrated ATP concentration was determined by approximating the integral of each sequential ATP concentration using the integration function in Origin (OriginLab, Northampton, MA, USA).

2.4 Identification of sugars in the culture broth by thin-layer chromatography

Culture supernatants harvested at various times were spotted on a TLC Silica Gel 60 plate (Merck, Darmstadt, Germany). Sample separation on the thin-layer chromatography (TLC) plate was performed using a mixture of butanol, acetic acid, and water at a 2:1:1 ratio by volume. After development, the spots of the sugars were visualized by heating the plate after spraying with 80 % (v/v) H_2SO_4.

3. Results and Discussion

Previous calorimetric studies of *C. acetobutylicum*, *C. beijerinckii*, and *C. cellulovorans* cultured in media containing soluble carbon sources under aerobic conditions have shown a strong correlation between the heat generated by bacterial growth and the OD and ATP concentration (10). We previously found that the heat evolution, as determined by calorimetry, reflects the number of anaerobic microbial cells present in a culture (10). However, the thermopiles in the calorimeter are in contact with the bottom of the culture medium vials; if precipitates form in the vials, they may interfere with the detection of the heat that accompanies the growth of anaerobic bacteria. Thus, we examined the correlation between the heat evolution and the OD and the ATP concentration in cultures of *C. cellulovorans*, *C. thermocellum*, *C. acetobutylicum*, and *C. beijerinckii* in the presence of insoluble carbon sources, such as microcrystalline cellulose (Avicel®) or starch, under anaerobic conditions. We compared the thermograms generated for each of the strains with the growth curves obtained by OD measurements and integrated ATP concentrations (**Fig.1**). For all strains, the $q(t)$ thermograms were strongly correlated with the integrated ATP concentrations. As expected, the OD values over the time course were high variance and did not correlate with the thermograms. In the media containing Avicel® and corn starch, the OD values could not be measured accurately owing to the non-uniformity of the culture broths. The $q(t)$ thermograms representing the heat evolution process and the integrated ATP concentrations both formed a general growth curve. Thus, the $q(t)$ thermograms obtained by calorimetry appear to reflect microbial growth, even in cultures with an insoluble carbon source.

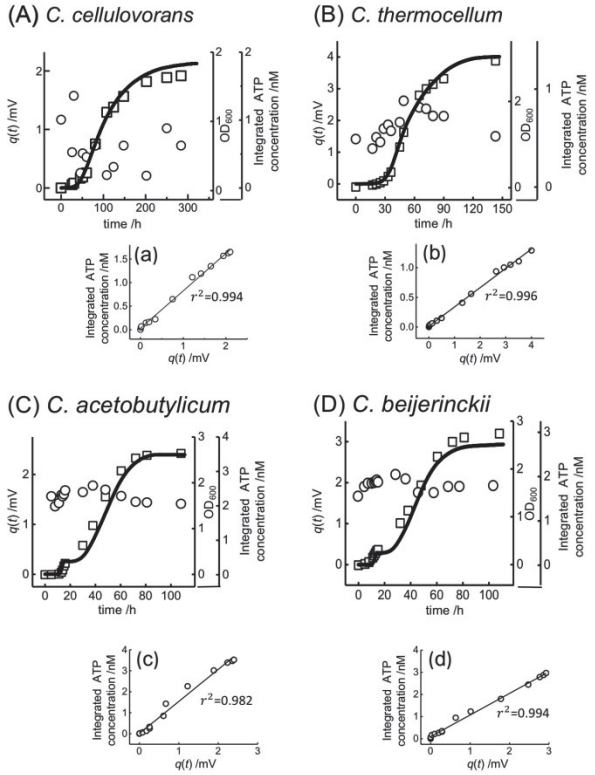


Fig.1 The heat evolution time courses for *C. cellulovorans* (A), *C. thermocellum* (B), *C. acetobutylicum* (C), and *C. beijerinckii* (D). *C. cellulovorans* and *C. thermocellum* were grown with 0.5 % (w/w) Avicel®. *C. acetobutylicum* and *C. beijerinckii* were grown with 0.5 % (w/w) corn starch. The $q(t)$ of each strain was calculated from raw data obtained with a multiplex isothermal calorimeter (line). The circles and squares indicate the ODs and integrated ATP concentrations, respectively. (a)–(d) indicate the correlations between the $q(t)$ thermogram and the integrated ATP concentrations of each strain.

Interestingly, in the *C. acetobutylicum* and *C. beijerinckii* cultures, the thermograms showed small peaks with rapid increases in heat evolution and integrated ATP concentrations at approximately 15 hours of culture (**Fig.1(C)** and **(D)**). In our previous study, the theoretical $q'(t)$ thermogram values obtained using calorimetric parameters on the basis of the differential form $q'(t)$ of $q(t)$ were in agreement with the values obtained by calorimetry (10). Accordingly, we investigated the correlation between the theoretical $q'(t)$ thermogram values and the measured sequential ATP concentrations. Surprisingly, we observed strong correlations between the values of the spike-like peaks of the heat evolution of the $q'(t)$ thermograms, which corresponded to the small peaks of the $q(t)$ thermograms observed at the beginning of cultures (**Fig.1(C)** and **(D)**) and the values of the measured sequential ATP concentrations (**Fig.2**). We speculated that these peaks resulted from the metabolism of components of the culture broth, and performed TLC to identify them.

In the *C. acetobutylicum* cultures, spots corresponding to glucose and maltose were observed in the early stage of cultivation, but disappeared after 15 hours (**Fig.3(A)**). After 30

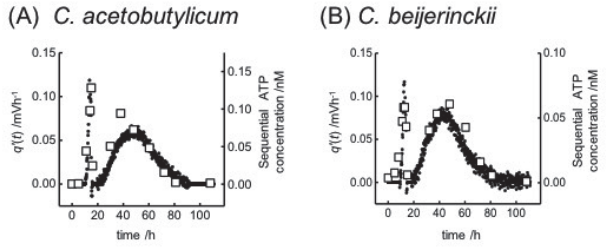


Fig.2 Comparison of the theoretical $q'(t)$ thermogram values and the values of the measured sequential ATP concentrations for *C. acetobutylicum* (A) and *C. beijerinckii* (B). The dots and open squares represent the theoretical $q'(t)$ thermogram values and the sequential ATP concentrations, respectively.

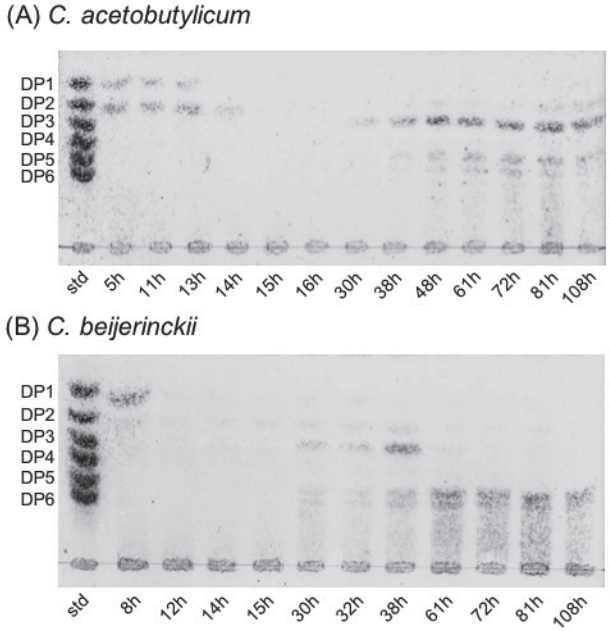


Fig.3 TLC analysis of maltooligosaccharides in the *C. acetobutylicum* (A) and *C. beijerinckii* (B) culture broths. Aliquots of culture media were taken at an arbitrary time point and spotted on the TLC plates. std: maltooligosaccharide standards. DP1-DP6 (in ascending numerical order): glucose, maltose, maltotriose, maltotetraose, maltopentaose, and maltohexaose.

hours, spots corresponding to maltotriose and higher maltosaccharides were detected. Similarly, in the *C. beijerinckii* cultures, the spot corresponding to glucose was observed in the early stage of cultivation, but disappeared after 12 hours (**Fig.3(B)**). After 30 hours, spots corresponding to maltotriose and higher maltosaccharides were observed; after 61 hours, the maltotriose spot disappeared, and spots corresponding to maltopentaose, maltoheptaose, and higher maltosaccharides were observed. The disappearance of the glucose and maltose spots corresponded with the times that the spike-like heat evolutions ended, suggesting that the heat evolution was caused by the metabolism of small amounts of pre-existing monosaccharides and disaccharides in the media, which were probably generated from starch when the culture media were autoclaved. By calorimetry, we detected this small degree of sugar metabolism with high sensitivity, equivalent to that of the ATP concentration measurements. In addition, the $q'(t)$ thermograms appeared to correspond to the time course of the amounts of maltooligosaccharides measured by TLC. The thermograms of *C. acetobutylicum* and *C. beijerinckii* indicated that the cells cultured on glucose in the previous study and those cultured with insoluble carbon in this study took similar lengths of time to enter the logarithmic phase, but the cultures in this study took twice as long to enter the stationary phase (10). This may be due to the presence of small amounts of glucose and maltose in the culture broths; as these sugars were metabolized first, the cultures entered the logarithmic phase after nearly the same length of time. The time lag in the stationary phase likely reflects the requirement for cells to express amylase in order to hydrolyze starch into small sugars, which they then metabolized.

Our results demonstrated that, even if anaerobic microbes are cultured on insoluble carbon sources, their growth can be measured conveniently and accurately using calorimetry, without interference due to aggregation or precipitation. Calorimetry could be applied for the quantitative analysis of microorganisms that grow on surfaces, like in biofilms, and in solid cultures that contain little water, which is difficult to analyze by OD and ATP measurements. In addition, calorimetry can be used to measure microbial growth with high sensitivity, comparable to ATP measurements. The heat evolution, 1 μVh^{-1} obtained from calorimetry corresponds to 5×10^{-13} M ATP, which is close to the detection limit of ATP concentration by ATP measurements (15). Thus, calorimetric analysis of microbial growth may contribute to the evaluation of microbial pretreatments in industrial processes.

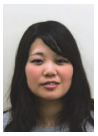
4. Acknowledgments

The authors would like to thank Professor Akiyoshi Tanaka of Mie University for technical assistance with experiments and useful discussions.

References

1) E. A. Bayer, J. P. Belaich, Y. Shoham, and R. Lamed, *Annu. Rev. Microbiol.* **58**, 521-554 (2004).
2) R. H. Doi, M. Goldstein, S. Hashida, J. S. Park, and M. Takagi, *Crit. Rev. Microbiol.* **20**, 87-93 (1994).
3) R. H. Doi, and Y. Tamaru, *Chem. Rec.* **1**, 24-32 (2001).
4) P. W. Wilson, W. H. Peterson, and E. B. Fred, *J. Bacteriol.* **19**, 231-260 (1930).
5) M. A. Cynkin, and M. Gibbs, *J. Bacteriol.* **75**, 335-338 (1958).
6) D. T. Jones, and D. R. Woods, *Microbiol. Rev.* **50**, 484-524 (1986).
7) R. H. Doi, and Y. Tamaru, *Chem. rec.* **1**, 24-32 (2001).
8) A. L. Koch, *Anal. Biochem.* **38**, 252-259 (1970).
9) J. M. Oades, and D. S. Jenkinson, *Soil Biol. Biochem.* **11**, 201-204 (1979).
10) H. Miyake, Y. Maeda, T. Ishikawa, and A. Tanaka, *J. Biosci. Bioeng.* **122**, 364-369 (2016).
11) R. Sleat, R. A. Mah, and R. Robinson, *Appl. Environ. Microbiol.* **48**, 88-93 (1984).
12) K. Takahashi, *J. Antibact. Antifung. Agents* **24**, 313-320 (1996).
13) A. K. Sakamiya, and A. Tanaka, *Netsu Sokutei* **37**, 58-63 (2010).
14) F. J. Richards, *J. Exp. Bot.* **10**, 290-301 (1959).
15) T. Sakakibara, S. Murakami, N. Hattori, M. Nakajima, and K. Imai, *Anal. Biochem.* **250**, 157-161 (1997)

Hideo Miyake
E-mail: miyake@bio.mie-u.ac.jp



Naoko Aoki
E-mail: 516m301@m.mie-u.ac.jp