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Simultaneous thermogravimetry and differential thermal analysis for comparing burning characteristics between oleaginous and non-oleaginous microorganisms

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ABSTRACT

Simultaneous thermogravimetry (TG) and differential thermal analysis (DTA) were applied to the studies of microorganisms, including *Escherichia coli* JM109, *Rhodococcus opacus* B-4, *Saccharomyces cerevisiae*, and *Mortierella alpina* IFO32281. *M. alpina* IFO32281 is known as an oleaginous fungus, while the other microorganisms examined are non-oleaginous species. The mass-difference baseline method, where the DTA curve for a small-mass sample was used as the baseline for a large-mass sample, was employed to quantify the endothermic and exothermic peaks on the DTA curves. A marked difference in heat energy between the oleaginous fungus and the non-oleaginous microorganisms was detected in the temperature range from 280 to 360 °C. The heat evolved from *M. alpina* IFO32281 in this temperature range was approximately 5.5 J/g, which was 3.3- to 11-fold greater than those detected from the non-oleaginous microorganisms.

1. Introduction

Microorganisms have long been recognized as an alternative to agricultural and animal sources for the production of oils and fats [1]. Microorganisms that accumulate more than 20–25% of their weight as lipid are referred to as oleaginous species [2]. Oleaginous yeasts, fungi, and microalgae are under active study as an alternative source for biodiesel production [2,3]. To improve their prospects of biofuel production, the biochemical and physiological characteristics of oleaginous microorganisms have been intensively investigated [1,2]. The evaluation of the burning characteristics of microorganisms is also essential for screening potential oleaginous microorganisms for biodiesel production. However, little is known about the physical and chemical changes occurring in microorganisms when they are heated in air.

Thermal analysis comprises a group of techniques in which the physical property of a substance is measured as a function of temperature, while the substance is subjected to a controlled temperature program [4,5]. Thermogravimetry (TG) is the study of the relationship between a sample's mass and temperature. Differential thermal analysis (DTA) measures the temperature difference that develops between a sample and an inert reference material, when both are subjected to identical heat treatments [6]. DTA has been used in the study of phase changes in polymer systems [7] and in the determination of the thermal stability of simple molecules [8]. Previous workers have used DTA for the study of the burning properties of heterogeneous organic systems such as peat and soil organic matter [9]. DTA has also been applied to the investigation of higher plants, including *Allium ursinum*, *Cheiranthus cheiri*, *Beta vulgaris*, and *Ranunculus ficaria* [4], and a marine phytoplankton, *Tetraselmis suecica* [10].

Considerable attention has been paid to simultaneous TG/DTA analysis, because it is useful for characterizing thermal behaviors of materials. Simultaneous TG/DTA has been applied in the thermal characterization of mumiyo [11] and reconstituted tobacco [12], in the investigation of thermal decomposition of natural and modified sepiolites [13], and in the property analysis of the volatile components of crude oil [14]. However, to our knowledge, no attempts have been made to apply simultaneous TG/DTA for comparing the burning characteristics between oleaginous and non-oleaginous microorganisms.

Theoretically, the baseline of DTA measurement is the DTA trace measured with an empty pan (blank measurement), and it should be a straight line parallel to the temperature axis. However, DTA curves are affected by factors associated with apparatus and sample properties [15]. Asymmetric heat transfer is caused by the asymmetric location of sample holders in the DTA furnace (instrument effect) and the differences in thermal properties between the sample and the reference, for example, emissivity and thermal conductivity (sample influence) [16]. To overcome these problems, the mass-difference baseline method has been proposed by Yang and

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Roy [15]. In this method, the DTA curve for a small-mass sample is used as the baseline for a large-mass sample instead of blank measurement.

In this study, we applied simultaneous TG/DTA to the studies of oleaginous and non- oleaginous microorganisms in order to assess their burning characteristics. The microorganisms examined in the present study were an oleaginous fungus, *Mortirella alpina* IFO32281 [17], and non-oleaginous species (e.g., *Escherichia coli* JM109; *Rhodococcus opacus* B-4 [18]; and *Saccharomyces cerevisiae* (Oriental Yeast Co., Ltd., Tokyo, Japan)). The mass-difference baseline method was employed to quantitatively determine the exothermic heat evolved from the microbial samples. The areas under exothermic peaks were calculated using the mass-difference DTA curves and converted to heat energy using indium as a standard material.

2. Experimental

2.1. Preparation of microbial samples

E. coli JM109 and *R. opacus* B-4 were grown in LB (Lysogeny broth) medium with shaking at 170 rpm at 37 and 30 °C, respectively. *S. cerevisiae* was grown in YPD (Yeast Peptone Dextrose) medium containing 2.0% glucose, 1.0% yeast extract, and 2.0% tryptone with shaking at 170 rpm at 30 °C. Cells were harvested by centrifugation at 8000 × *g* (=9.80 m/s^2) for 10 min. The oleaginous fungus *M. alpina* IFO32281 was maintained on Czapek-Dox agar plates (Oxiod Co., Ltd., Cambridge, UK). Czapek-Dox agar plates were inoculated with fungus cells for spore generation and incubated at 28 °C for 14–17 days.

Spores were harvested by adding 2 mL of sterile Tween 80 (0.1%, v/v). The mycelium was scraped off the surface of Czapek-Dox agar plates. The spore suspension was filtered with sterile polyallomer wool (Iwaki Co., Ltd., Tokyo, Japan). Then the spores were collected from the filtrate by centrifugation at 140 × *g* for 10 min at 4 °C. The spores obtained from several agar plates were pooled together and resuspended in a small volume of Tween 80. The spores were counted using a hemacytometer (Erma Co., Ltd., Tokyo, Japan). The density of spores of this fungus was maintained at 1600 spores/mm^3. One hundred microliter of the spore suspension was inoculated into a 15-mL test tube containing 5 mL of YG medium with 1.6% yeast extract and 8.0% glucose and cultivated at 28 °C with shaking at 170 rpm. After 3 days of incubation, the preculture was inoculated into a 500-mL Erlenmeyer flask containing 100 mL of fresh YG medium. The culture was incubated at 28 °C with shaking at 180 rpm on an orbital shaker for 4 days. Cells were harvested by centrifugation, washed three times with distilled water and dried overnight in an incubator at 30, 40, or 70 °C. The dried cells were pulverized in a bowl prior to the analytical experiments. This gave satisfactory reproducibility of TG/DTA curves. To prepare wet samples, the washed cells were subjected to two times of centrifugation in order to remove extracellular water. All chemicals used were of analytical grade.

2.2. Instrumentation

A simultaneous TG/DTA instrument (model DTG 60/60H, Shimadzu, Kyoto, Japan) was used to measure the heat evolved from microbial cells. The Shimadzu DTG 60/60H was capable of ramping a sample from 0.1 to 99.9 °C/min. The DTG furnace assembly was continuously flushed with air (21% oxygen and 79% nitrogen) at a flow rate of 250 mL/min. For complete-combustion determination, sample powders of approximately 5.01(±0.04) and 10.02(±0.04) mg were placed in platinum pans of 5 mm in diameter. Unless otherwise noted, 20 mg of α-alumina (α-Al_2O_3) powder (Shimadzu Co., Ltd., Kyoto, Japan) was used as an inert reference. The linear heating program was conducted with a heating rate of 18 °C/min from 30 to 900 °C. At least three measurements were performed for each sample.

The areas of the endothermic and exothermic peaks in the DTA curves were obtained using TA-60 software (Shimadzu Co., Ltd., Kyoto, Japan). In the mass-difference baseline method [15], the heat evolved in the temperature range from T_1 to T_2 (°C), Q (J/g), was calculated using Eq. (1) (see Appendix A).

$$\begin{aligned} Q &= \left[\left\{M_L(T_1)H_L(T_1) - M_S(T_1)H_S(T_1)\right\} - \left\{M_L(T_2)H_L(T_2) - M_S(T_2)H_S(T_2)\right\}\right] \\ &\quad / \left\{M_L(T_1) - M_S(T_1)\right\} \\ &= \alpha \int_{T_1}^{T_2} (\mathrm{DTA_L} - \mathrm{DTA_S})\, dT / \left\{M_L(T_1) - M_S(T_1)\right\} \\ &= \alpha \Sigma / \left\{M_L(T_1) - M_S(T_1)\right\} \end{aligned} \quad (1)$$

where Σ (μV °C) is the area between two DTA curves in the temperature range from T_1 to T_2; α (J/μV °C) is the overall conversion coefficient from the DTA signal to the heat evolved; $H(T)$ (J/g) is the enthalpy at the temperature T °C; $M(T)$ (g) is the TG signal at the temperature T °C; the subscripts L and S indicate large- and small-mass samples, respectively. α was determined using the melting heat of indium (28.4 J/g at 157 °C). The standard material, indium, was purchased from the National Institute of Standards and Technology (Gaithersburg, USA).

3. Results

3.1. Effect of pretreatment temperature on TG/DTA curves

Typical TG/DTA curves of *E. coli* JM109, *R. opacus* B-4, and *S. cerevisiae* are shown in Fig. 1. Previous workers suggested that the moisture content of biological samples could significantly affect the results of TG/DTA measurements [19–21]. To determine the optimal pretreatment temperature for removing the moisture of microbial cells, TG/DTA curves were obtained with the samples dried overnight at 30, 40, or 70 °C in an incubator and compared with those of the wet ones. All the measurements were performed with samples of approximately 5.0 mg, regardless of their moisture content. The wet samples showed an approximately 50% reduction in their weight below 100 °C (Fig. 1A). The DTA curve showed a large endothermic peak at approximately 75 °C because of water evaporation (Fig. 1E). For the pretreated samples, the weight loss of *R. opacus* B-4 was larger than those of *E. coli* and *S. cerevisiae* in the temperature range from 30 to 900 °C. No significant endothermic peak was detected with the DTA curves at approximately 75 °C. However, a small mass loss could be detected at the beginning of the TG curves. This suggested that small amounts of moisture were likely to remain in the samples, though they had been pretreated at temperatures of 30–70 °C.

There were no significant differences in TG curves among the samples pretreated at 30, 40, or 70 °C. The DTA curves of the samples pretreated at 30 °C (Fig. 1F) were similar to those of the samples pretreated at 40 °C (Fig. 1G). The DTA curves exhibited pronounced exothermic peaks at approximately 350 and 550 °C. The two-exothermic peak system was also observed with the DTA curves of the wet samples (Fig. 1E). However, the peaks were weaker than those detected with the pretreated samples, because the wet samples lost approximately 50% of their weight below 100 °C. When the samples were pretreated at 70 °C, the DTA curve of *S. cerevisiae* exhibited a considerable difference from those prepared at 30 or 40 °C. A new peak appeared at approximately 370 °C on the DTA curve of the *S. cerevisiae* sample prepared at 70 °C (Fig. 1H). Although the reason for this is unclear, it is obvious that drying the microbial samples at 70 °C affected the burning characteristics of microbial cells. Thus,

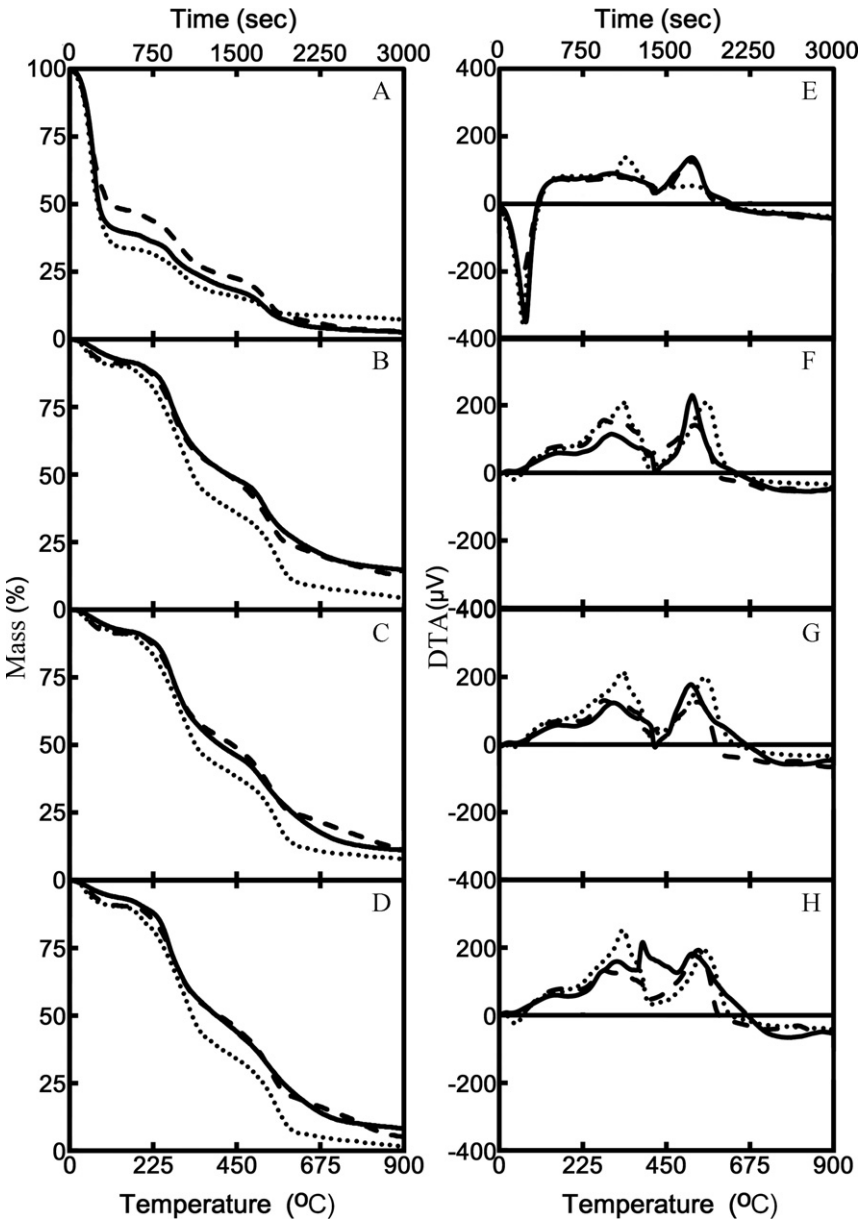

Fig. 1. TG and DTA curves of wet and pretreated samples. Except wet samples (A and E), microbial samples were pretreated at 30 (B and F), 40 (C and G), or 70 °C (D and H). The microorganisms examined were *E. coli* JM109 (broken line), *S. cerevisiae* (closed line), and *R. opacus* B-4 (dotted line). Ten milligram of α-alumina was used as reference. TG values are given by the percentage of the sample mass relative to the initial one.

microbial samples were pretreated overnight at 30 °C for further study.

3.2. Mass-difference TG and DTA curves

Previous workers have reported that the DTA baseline obtained by blank measurement is inadequate for quantifying a peak area under a DTA curve because of instrumental and sample effects [15]. The instrumental effect is caused by an asymmetrical heat transfer towards the reference and sample pans and also by the inconsistency of heat transfer between the blank and sample measurements [15]. For example, if the sample has a heat capacity significantly smaller than the reference, the sample temperature can be increased at a rate greater than the reference. This temperature difference leads to a positive DTA signal through the thermocouple detection. Since the rate of heat transfer is dependent on the temperature difference between the furnace and the pan, the temperature difference between the two pans will be readily cancelled if neither exothermic nor endothermic reactions occur. This time course of the temperature difference results in an apparent peak signal in the DTA curve.

To solve this problem, the mass-difference baseline method, which has been proposed by Yang and Roy [15], was employed for quantifying the peak area under DTA curves in the present study. Fig. 2A and B shows typical TG and DTA curves of *M. alpina* IFO32281 for approximately 5 and 10 mg of the sample. Despite the difference in the sample mass, the DTA curves with a function of temperature showed similar patterns. Both the DTA curves exhibited two exothermic peaks at approximately 350 and 550 °C and one endothermic peak at approximately 70 °C. Below 200 °C, the DTA signal for the 10-mg sample was more distinct than that for the 5-mg sample. This was due to water evaporation. The size and sharpness of the first exothermic peak at approximately 280–360 °C significantly differed between *M. aplina* IFO32281 (Fig. 2B) and the non-oleaginous microorganisms (Fig. 2E, H, and K). Although the non-oleaginous species showed the two-peak system, their second

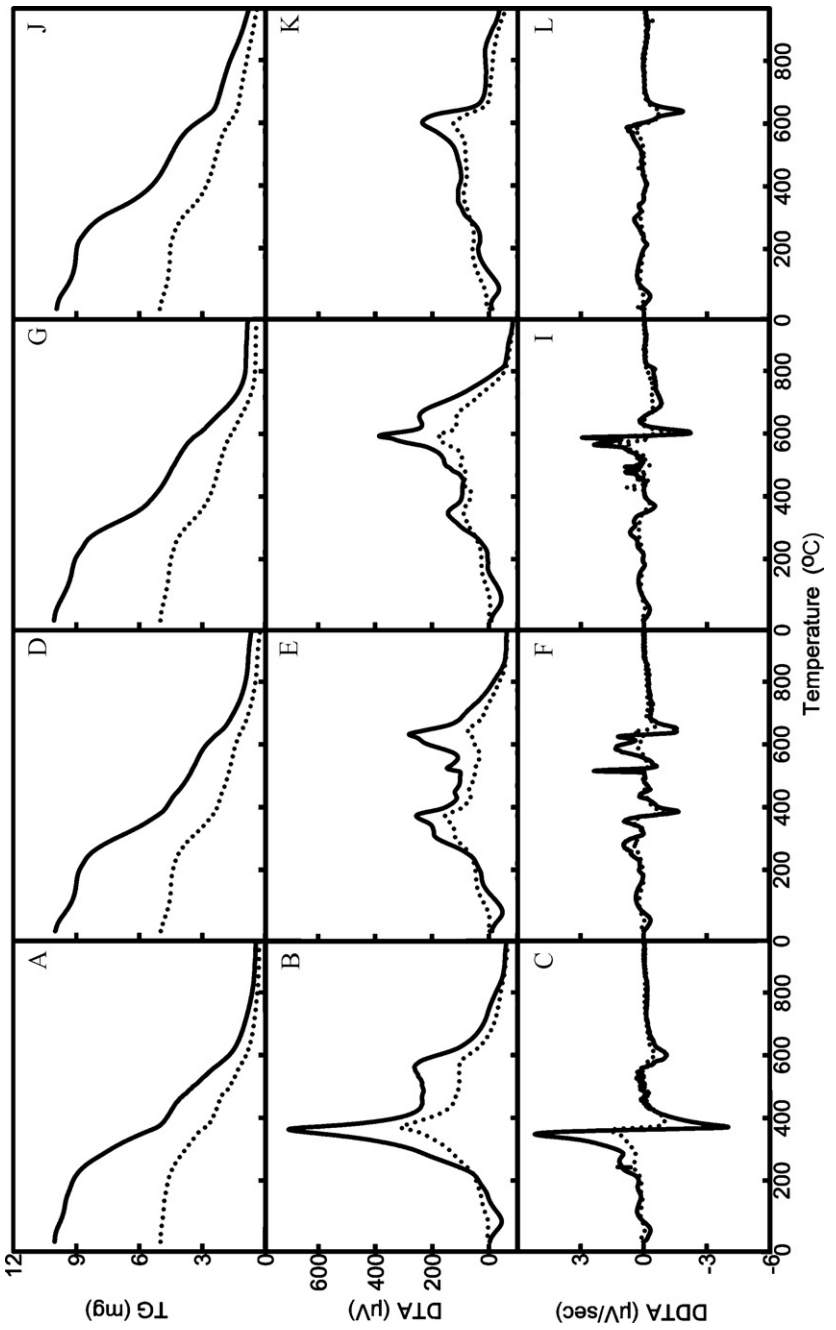

Fig. 2. TG, DTA, and differential DTA (DDTA) curves for microbial samples of 5 mg (broken line) and 10 mg (closed line). The microorganisms examined were *M. alpina* IFO32281 (A, B, and C), *R. opacus* B-4 (D, E, and F), *S. cerevisiae* (G, H, and I), and *E. coli* JM109 (J, K, and L).

exothermic peaks were larger than their first peaks. No sharp peak was detected with the non-oleaginous microorganisms at approximately 280–360 °C. The differential DTA (DDTA = dDTA/dT) showed that the remarkable change in the DTA signals of *M. alpina* IFO32281 occurred in the temperature range from 280 to 360 °C (Fig. 2C). It seemed likely that the exothermic peak at 280–360 °C in the DTA curve of *M. alpina* IFO32281 was a reflection of the combustion of oil accumulated in the oleaginous fungus.

To estimate the heat evolved from the microbial samples, the TG and DTA signals for a small-mass sample (approximately 5 mg) were subtracted from those obtained with a large-mass sample (approximately 10 mg) (Fig. 3). Obviously, the peak area in the mass-difference DTA curve in the temperature range from 280 to 360 °C was greater in *M. alpina* IFO32281 than in the non-oleaginous microorganisms (Fig. 3B). Large peaks were also detected in the mass-difference DTA curves in the temperature range from 480 to 650 °C for all the microorganisms examined. The size and sharpness of the second peak were more distinct for the non-oleaginous species than for the oleaginous fungus. The effect of water evaporation on the mass-difference DTA curves was detected below 200 °C for all the microorganisms examined. There was no significant difference in the endothermic peak between the oleaginous and non-oleaginous microorganisms. The decrease in the mass-difference TG in the temperature range from 280 to 360 °C was largest in *M. alpina* IFO32281 (Fig. 3A). However, the difference in the mass-difference TG curve among the microorganisms examined was smaller than in the mass-difference DTA curve.

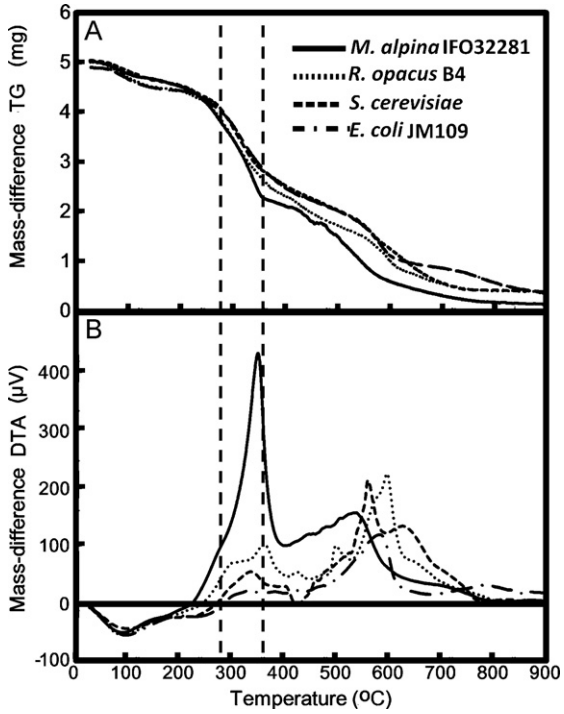

Fig. 3. Mass-difference TG (A) and DTA (B) curves of *E. coli* JM109, *S. cerevisiae*, *R. opacus* B-4, and *M. alpina* IFO32281. The mass-difference curves were obtained by subtracting the TG and DTA curves for the small-mass sample (approximately 5 mg) from those for large-mass sample (approximately 10 mg). Vertical broken lines indicate the temperature range from 280 to 360 °C. A horizontal line shows the zero baseline for the mass-difference DTA (0 μV).

The heat evolved from the microbial samples was estimated from the area under the mass-difference DTA curve using Eq. (1). To convert a DTA peak to exothermic heat, indium was employed as a standard material. The DTA analysis of indium showed an endothermic peak at the melting temperature of indium of 157 °C (data not shown). From the area above the DTA curve, the overall conversion coefficient α from the DTA signal to heat was estimated to be 8.10×10^{-7} (J/μV °C). Table 1 summarized the heat evolved from the microbial samples. The exothermic heat was calculated in the temperature range from 280 to 360 °C. The heat evolved from *M. alpina* IFO32281 was 5.54 ± 1.07 J/g which was 3.3- to 11-fold greater than those from the non-oleaginous microorganisms. The heat evolved from *R. opacus* B-4 in this temperature range was 1.82 ± 0.25 J/g which was relatively large among the non-oleaginous species.

4. Discussion

Knowledge of the burning characteristics of microorganisms is fundamentally important for screening potential oleaginous microorganisms for biodiesel production. To our knowledge, this is the first report on the application of simultaneous TG/DTA to the assessment of burning characteristics of oleaginous and non-oleaginous microorganisms. Quantitative thermal analysis has been generally conducted using differential scanning calorimetry (DSC) [22]. However, DSC requires a constant sample mass during the enthalpy change measurement. The burning of microbial cells is a complex process resulting in intensive decomposition and mass loss (Fig. 1). In contrast to DSC, simultaneous TG/DTA enables the measurement of the enthalpy change in an open system, thus allowing the simultaneous recording of changes in sample mass and temperature [15]. In simultaneous TG/DTA, DTA measures the temperature difference between the reference and sample pans, which is then converted to enthalpy change using an overall conversion factor (α in Eq. (1)). One major difficulty in DTA measurement is that the overall conversion factor α is rarely constant in a wide temperature range [15]. To overcome this problem, the mass-difference baseline method has been proposed by Yang and Roy [15].

The complete-combustion curves of four microorganisms examined showed two exothermic peaks at approximately 350 and 550 °C (Fig. 1). In addition to variations in peak temperatures, there were differences in the relative size and sharpness of the first and second peaks among the microorganisms examined. Since this two peak system reflects a series of discrete chemical reactions, it is difficult to understand the chemical and physical mechanisms behind the difference in the two peak system between the DTA curves. Both chemical and physical structures of a material affect its burning characteristics [9]. In addition, combustion curves do not necessarily reflect the ultimate thermal decomposition of a sample. The change in the molecular structure and the rearrangement of functional groups must also be involved [4]. DTA curves have been obtained for simple organic materials, including hydrocarbons, carbohydrates, and proteins [4]. However, very often, the information concerning the study of simple molecules is not applicable to heterogeneous, complex biological systems.

The strong first peak in the DTA curves of *M. alpina* IFO32281 is likely reflection of the high lipid content of this microorganism. Although no data was shown, *M. alpina* IFO32281 accumulated more than 50% of its dry weight as lipid in our laboratory. The lipid that accumulates in oleaginous microorganisms is mainly triacylglycerol [2]. To confirm this, the simultaneous TG/DTA and chemical composition analysis of *M. alpina* IFO32281 and other oleaginous microorganisms are now being undertaken in our laboratory. *R. opacus* B-4 exhibited a relatively large first peak in the mass-difference DTA curves among the non-oleaginous microorganisms (Fig. 3). Consequently, a relatively large exothermic heat was evolved from this organism in the temperature range of 280–360 °C among the non-oleaginous species (Table 1). The first peak in the DTA curves of *R. opacus* B-4 may be attributable to the unique cell envelope, because *R. opacus* B-4 is known to have long-chain-length fatty acids (typically mycolic acids) on the cell wall [23].

As can be seen in Fig. 3, the first peak sizes and sharpnesses in the mass-difference DTA curves were clearly different between the oleaginous and non-oleaginous microorganisms. In the DTA curve of *M. alpina* IFO32281, the first exothermic peak was more distinct than the second one. On the other hand, for the non-oleaginous microorganisms examined, the second peak was larger than the first peak in the DTA curves. This finding is likely important for discriminating oleaginous microorganisms from non-oleaginous species on the basis of their burning characteristics. The heat evolved from *M. alpina* IFO32281 in the temperature range from 280 to 360 °C was approximately 3.3- to 11-fold greater than those from the non-oleaginous microorganisms (Table 1). The data presented in this study suggest that the measurement of the heat evolved in the temperature range from 280 to 360 °C is a promis-

Table 1
The heat evolved from microbial samples in the temperature range from 280 to 360 °C.

Microorganisms	Temperature range (°C)	Peak temperature[a] (°C)	Heat evolved[a] (J/g)
E. coli JM109	281–363	333 ± 2	0.54 ± 0.11
S. cerevisiae	271–389	336 ± 3	1.08 ± 0.07
R. opacus B4	245–406	359 ± 2	1.82 ± 0.25
M. alpina IFO32281	226–400	339 ± 2	5.54 ± 1.07

[a] The data are given by the means ± the standard deviations for at least three different experiments.

ing means of screening oleaginous microorganisms for biodiesel production. Although no data was shown, *M. alpina* 8568 and *M. alliacea* YN-15 also showed the heat evolved in the temperature ranges from 316 to 364 °C and 322 to 367 °C, respectively. Microbial samples of 5–10 mg may be held isothermally in the temperature range from 280 to 360 °C to detect the exothermic heat for discriminating oleaginous microorganisms from non-oleaginous ones. Future study is directed toward the development of an inexpensive instrument for detecting oleaginous microorganisms by measuring the heat evolved in temperature range from 280 to 360 °C.

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Appendix A.

Energy balance equations for the sample and reference holders are [15]:

On the sample side:

$$\frac{d(M_P C_{P,P} T_S)}{dt} + \frac{d\left(MC_{P,S}T_S\right)}{dt} + \frac{d(Mq)}{dt} = \alpha_S A(T_\infty - T_S) \tag{A.1}$$

and on the reference side:

$$\frac{d(M_P C_{P,P} T_R)}{dt} + \frac{d\left(M_R C_{P,R} T_R\right)}{dt} = \alpha_R A(T_\infty - T_R) \tag{A.2}$$

where M_P (g) and $C_{P,P}$ (J/g °C) are the mass and heat capacity of the pan; M (g) and $C_{P,S}$ (J/g °C) are the mass and heat capacity of the sample; M_R (g) and $C_{P,R}$ (J/g °C) are the mass and heat capacity of the reference; q (J/g) is the transition heat produced by phase changes or chemical reactions; α_S (J/m^2 s °C) and α_R (J/m^2 s °C) are the overall heat transfer coefficients from the furnace to the sample and the reference side, respectively; A (m^2) is the heat transfer area of the pan; T_∞ (°C), T_S (°C), and T_R (°C) are the temperatures of the furnace, the sample, and the reference, respectively; t (s) is the time.

Subtracting Eq. (A.2) from Eq. (A.1) yields:

$$\frac{d(M_P C_{P,P}\ \Delta T)}{dt} + \frac{d(MH)}{dt} - \frac{d(M_R C_{P,R} T_R)}{dt} = \alpha_S A(T_\infty - T_S) - \alpha_R A(T_\infty - T_R) \tag{A.3}$$

where $\Delta T = T_S - T_R$ and $H = C_{P,S}T_S + q$.

The first term on the left-hand side can be ignored as it is much smaller than the other terms. Rearranging Eq. (A.3),

$$\alpha_S A\ \Delta T = A(\alpha_S - \alpha_R)(T_\infty - T_R) + \frac{d\left(M_R C_{P,R} T_R\right)}{dt} - \frac{d(MH)}{dt} \tag{A.4}$$

In the mass-difference baseline method [15], ΔT derived from a small-mass sample is employed as the baseline for a large-mass sample. By subtracting ΔT of a small-mass sample from ΔT of a large-mass sample, the first and second terms on the right-hand side are eliminated:

$$\alpha_S A(\Delta T_L - \Delta T_S) = \frac{d(M_S H_S - M_L H_L)}{dt} \tag{A.5}$$

where the subscripts L and S indicate large- and small-mass samples, respectively.

In the linear temperature program, $dt = \beta dT$ where β (s/°C) is the conversion constant. The DTA signal (μV) is converted to ΔT (°C) using the conversion constant θ (°C/μV). Substituting α (the overall conversion coefficient from the DTA signal to the heat evolved (J/μV °C)) = $\alpha_S A\beta\theta$ into Eq. (A.5),

$$d(M_S H_S - M_L H_L) = \alpha(\mathrm{DTA_L} - \mathrm{DTA_S})\, dT \tag{A.6}$$

Integrating Eq. (A.6) from T_1 to T_2 yields:

$$\begin{aligned}&\left\{M_S(T_2)H_S(T_2) - M_L(T_2)H_L(T_2)\right\} - \left\{M_S(T_1)H_S(T_1) - M_L(T_1)H_L(T_1)\right\}\\ &= \alpha \int_{T_1}^{T_2} (\mathrm{DTA_L} - \mathrm{DTA_S})dT\\ &= \alpha\Sigma\end{aligned} \tag{A.7}$$

where Σ (μV °C) is the area between two DTA curves in the temperature range from T_1 to T_2.

The heat evolved from the sample in the temperature range from T_1 to T_2, Q (J/g), is given by:

$$\begin{aligned}Q &= \left[\left\{M_L(T_1)H_L(T_1) - M_S(T_1)H_S(T_1)\right\} - \left\{M_L(T_2)H_L(T_2) - M_S(T_2)H_S(T_2)\right\}\right] /\\ &\quad \left\{M_L(T_1) - M_S(T_1)\right\}\\ &= \alpha\Sigma / \left\{M_L(T_1) - M_S(T_1)\right\}\end{aligned} \tag{A.8}$$

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