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Chapter 1

General introduction

Continued use of petroleum-sourced fuels is now widely recognized as unsustainable because of depleting supplies and the contribution of these fuels to the accumulation of carbon dioxide in the environment (Chisti, 2007). CO_2 emissions have risen over the last two decades, reaching an atomospheric content of 360 ppm, estimating the world CO_2 emission at about 26 billion metric t per year, 80% of which comes from the combustion of fossil conbustibles such as coal, petroleum and natural gas (J. A. López Sastre *et al.*, 2003). Increased attention has been paid to the use of renewable alternatives to fossil fuels. Among many energy alternatives, biofuels, hydrogen, natural gas, and syngas (synthesis gas) may likely emerge as the four strategically-important and sustainable fuel sources in the foreseeable future (Nigam and Singh, 2011). Oils produced by crops and microorganisms are potentially renewable and carbon-neutral alternatives to petroleum fuels. The use of vegetable oils as raw materials for fuel production would compete with edible oils, thus leading to the soar of food price. Oil-accumulating microorganisms can produce oils more efficiently than crop plants. Consequently, microbial oils, which have similar composition of fatty acids to that of vegetable oils, are now believed as a promising potential feedstock for fuel production.

1.1 Single cell oils

Microorganisms that accumulate more than 20-25% of their weight as lipid are referred to as oleaginous species (Ratledge, 1991; Chisti, 2007). The principal species which are regarded as oil-accumulating are listed in Tables 1 and 2. Oils from oleaginous microorganisms, otherwise referred to as Single Cell Oils (SCOs), are potentially usable for the production of biodiesel as alternative energy sources. Biodiesel fuel is fatty acid methyl or ethyl esters produced by transesterification of triglycerides with methanol or ethanol (Koutb and Morsy, 2011). Biodiesel is rather an attractive alternative to the conventional petroleum diesel fuels because of its biodegradable, nontoxic, and clean renewable characteristics.

Oleaginous microorganisms can accumulate triacylglycerols (TAGs) as cellular storage lipids, sometimes up to 70% of the biomass (Ratledge, 2002). The ability of an organism to accumulate large quantities of oils must lie outside the immediate area of fatty acid biosynthesis, as this biosynthetic machinery is common to all microorganisms (Ratledge, 2004). It has been demonstrated that SCOs can be transformed to fatty acid methyl esters by means of both enzymatic and inorganic catalyses (Hassan et al., 1996). SCOs are also produced by various microorganisms as commercial sources of arachidonic acid (AA) and docosa-hexaenoic acid (DHA) (Ratledge, 2004). These oils are now used extensaively as dietry supplements in infant formulas.

Microalgae can be the source of renewable biodiesel that is capable of meeting the global demand for transport fuels (Chisti, 2007). The colonial green alga, *Botryococcus braunii*, is able to produce large amounts of hydrocarbons which are similar to those found in petroleum (Hu *et al.*, 2008). The heterotrophic microalga, *Schizochytrium limacinum*, has been shown that the

growth, lipid content and fatty acid composition were sensitive to changes in salinity and temperature (Zhu et al., 2007) and different carbon and nitrogen sources (Yokochi et al., 1998). This alga appears to be a suitable feedstock for producing biodiesel via the direct transesterification method (Johnson and Wen, 2009). Oil productivity of many microalgae greatly exceeds that of the best oil-producing crops. Microalgae may allow biodiesel production to achieve the price and scale of production needed to compete with, or even replace, petroleum (Demirbas and Demirbas, 2011). The growth and lipid content of *Nannochhloropsis oculata* and *Chlorella vulgaris* were dependent on the culture conditions, including temperature and nitrogen concentration (Converti et al., 2009).

The principal attraction of using moulds as sources of oils lies in the occurrence of the nutritionally important polyunsaturated fatty acids (PUFAs) in their oils (Ratledge, 1991). TAGs in fungal oil are rich in PUFAs such as AA, γ- linolenic acid, and eicosapentaenoic acid (Berger et al., 2002). For example, the oleaginous fungi, *Mortirella alpina* and *Mortirella alliacea*, have been shown to produce high levels of AA (Aki et al., 2001; Higashiyama et al., 2002). Especially, a filamentous fungus, *Mortirella alliacea* YN-15, producing significant levels of AA (C20:4n-6) was isolated from a freshwater pond sample (Aki et al., 2001). The production of AA was also carried out by other oleaginous fungi, such as *Mortirella alpina* I_{49}-N_{18} (Yuan et al., 2002), and *Entomorphthora exialis* (Ratledge, 1991). Recently, some papers have introduced a filamentous fungus as a potential feedstock for biodiesel production (Vicente et al., 2009; Koutb and Morsy, 2011). However, a little information is available for the practical use of oleaginous fungi in biodiesel production.

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Oleaginous yeasts also accumulate TAGs rich in PUFAs including oleic and linoleic acids (Meng *et al.*, 2009). Oleaginous yeasts have many advantages due to their fast growth, high oil content and the resemble of their TAGs fraction to plant oil (Zhao *et al.*, 2010). The oil produced by the oleaginous yeast, *Cryptococcus curvatus*, resembles plant seed oils like palm oil (Meesters *et al.*, 1996). The PUFAs from oleaginous yeast are now used extensively as dietary supplements, and consequently a large market is available to ensure their worldwide sales (Ratledge, 2004). Yeast cells are large which makes the separation of cells from culture become easy to handle (Zhu *et al.*, 2008). On the basis of these advantages, the lipid obtained from yeast cells can be used for biodiesel production. The potential of yeast cells to accumulate oils was assessed when grown on sewage sludge as carbon and energy sources (Angerbauer *et al.*, 2008).

The accumulation of TAGs seems to be widespread among bacteria belonging to the actinomycetes group, such as species of *Mycobacterium, Streptomyces, Rhodococcus* and *Nocardia.*(Alvarez and Steinbüchel, 2002). Particularly, *Rhodococcus opacus* PD630 has been reported to accumulate up to 76% of TAGs when grown on different carbon sources under nitrogen-limiting conditions (Wältermann and Steinbüchel, 2000).

Oleaginous yeasts, fungi, and microalgae are under active study as an alternative source for biodiesel production (Ratledge *et al.*, 1991;Rubin, 2008). Screening oleaginous microorganisms and developing lipid-accumulating strains have drawn considerable attention for the improvement of microbial oil production (Meng *et al.*, 2009). The biochemical and physiological characteristics of oleaginous microorganisms have been intensively investigated to improve their ability to accumulate oils (Meng *et al.*, 2009). However, little is known about the

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thermal characteristics of microorganisms and the physical and chemical changes occurring in

microorganisms when they are heated in air.

Table 1 Total lipid content in oleaginous yeasts and mucorales (Ratledge, 1991)

Yeasts	Max lipid content (w/w %)	Mucorales	Max lipid contents (w/w %)
Candida curvata	58	*Blakeslea trispora*	37
Candida diddensiae	37	*Cunninghamella eshinulata*	45
Candida guilliermondi	22	*Cunninghamella elegans*	56
Candida tropicalis	23	*Cunninghamella homothallica*	38
Candida sp. 107 (NCYC 911)	42	*Cunninghamella japonica*	60
Cryptococcus albidus	65	*Mortierella isabellina*	86
Cryptococcus laurentii	32	*Mortierella pusilla*	59
Cryptococcus neoformis	22	*Mortierella vinacea*	66
Hansenula ciferri	22	*Mucor albo-ater*	42
Hansenula saturnus	22	*Mucor circinelloides*	65
Lipomyces lipofer	64	*Mucor mucedo*	51
Lipomyces starkeyi	63	*Mucor plumbeus*	63
Lipomyces tetrasporus	67	*Mucor ramanianus*	56
Rhodosporidium toruloides	66	*Mucor spinosus*	47
Rhodotorula glutinis	72	*Rhizopus arrhizus*	45
Rhodotorula graminis	36	*Rhizopus oryzae*	57
Rhodotorula mucilaginosa	28	*Zygorhynchus moelleri*	40

Table 2 Total lipid content in oleaginous ascomycetes and algae ([a]Ratledge, 1991; [b]Chisti, 2007)

Ascomycetes[a]	Max lipid contents (w/w %)	Algae[b]	Max lipid contents (w/w %)
Aspergillus fischeri	53	*Botryococcus braunii*	75
Aspergillus nidulans	51	*Chlorella* sp.	32
Aspergillus ochraceus	48	*Crypthecodinium cohni*	20
Aspergillus oryzae	57	*Cylindrothea* sp.	37
Aspergillus terreus	57	*Dunaliella primolecta*	23
Chaetomium globosum	54	*Isochrysis* sp.	33
Fusarium bulbigenum	50	*Monallanthus salina*	20
Fusarium equiseti	48	*Nannochloris* sp.	35
Geotrichum candidum	50	*Nannochloropsis* sp.	68
Gibberella fujikoroi	48	*Neochloris oleoabundans*	54
Humicola lanuginosa	75	*Nitschia* sp.	47
Penicillium javanicum	39	*Phaeodatylum tricormutum*	30
Penicillium lilacinum	56	*Schizochytrium* sp.	77
Penicillium soppi	40	*Tetraselmis sueica*	23

1.2 Thermal analysis

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 (Mitchell and Knight, 1965; Price *et al.*, 2000). Thermogravimetric analysis (TGA) is an analytical technique used to determine a material's thermal stability and its fraction of volatile components by monitoring the change in mass reduction that occurs as a specimen is heated (Fig. 1A) (Stephen *et al.*, 2008). 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 (Fig. 1B and C) (Arseneau, 1961). In DTA analysis, the temperature difference between reference and sample materials, is recored as an electric potential as shown by the equation (1.1) (see 2.1.1 later) :

$$- \, \mathrm{d}\Delta q/\mathrm{d}t = - \, 1/(R \, S_{AB}) \, \Delta E \tag{1.1}$$

where $\mathrm{d}\Delta q/\mathrm{d}t$ is the difference in velocity of heat transfer rate between sample and reference materials (J/s); R is the heat resistance ($^{\circ}$Cs/J) ; ΔE is the thermoelectromotive force caused by the temperature difference between sample and reference materials (V). S_{AB} is the conversion coefficient from ΔT to ΔE ($\Delta E = S_{AB} \, \Delta T$). The positive electrical potential means exothermic heat evolved from the sample, while the negative one indicates endothermic heat absorbed by the sample.

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Fig. 2 shows a schematic diagram of the Shimadzu DTG-60/60H analyzer used in the present study. After putting sample and reference materials in the two pans, the furnace is moved down to the joint section to start heating. Thermocouples are connected to the sample and reference pans for the DTA measurement. The change in sample weight for TG analysis is measured by a beam balance system. According to the change in weight, the shutter rotates to change the light intensity detected by the photoelectric conversion element.

Recently, considerable attention has been paid to simultaneous TG/DTA analysis, because it is useful for characterizing thermal behaviors of materials. TGA and DTA measurments have been applied to the investigation of the thermal behaviours in the inorganic materials including metals, alloys, carbonates, salts and minerals (Mackenzie, 1970). Simultaneous TG/DTA analysis has a long use history to determine the transition temperature and enthalpy changes in bulk inorganic materials such as polymers, minerals, and ceramics (Ochiai and Ozao, 1992). These thermal analyses have also been used for the studies of organic materials such as polymeric and biological materials (Mackenzie, 1970). Simultaneous TG/DTA has also been applied in the thermal characterization of mumiyo (Garedew *et al.*, 2004) and reconstituted tobacco (Wang *et al.*, 2005), in the investigation of thermal decomposition of natural and modified sepiolites (Turhan *et al.*, 2008), and in the property analysis of the volatile components of crude oil (Ali *et al.*, 1998). Additionally, TG/DTA has been applied to the investigation of higher plants, including *Allium ursinum, Cheiranthus cheiri, Beta vulgaris*, and *Ranunculus ficaria* (Mitchell and Knight, 1963), and a marine phytoplankton, *Tetraselmis suecica* (Pane *et*

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al., 2002). Previous workers have used DTA for the study of the burning properties of heterogeneous organic systems such as peat and soil organic matter (Mitchell and Birnie, 1970).

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 (Yang and Roy, 1996). 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) (Yang and Roy, 1999). To overcome these problems, the mass-difference baseline method has been proposed by Yang and Roy (Yang and Roy, 1996). 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. The mass difference baseline method allow more stable baseline and relatively close to zero baseline by using two measurements having different masses.
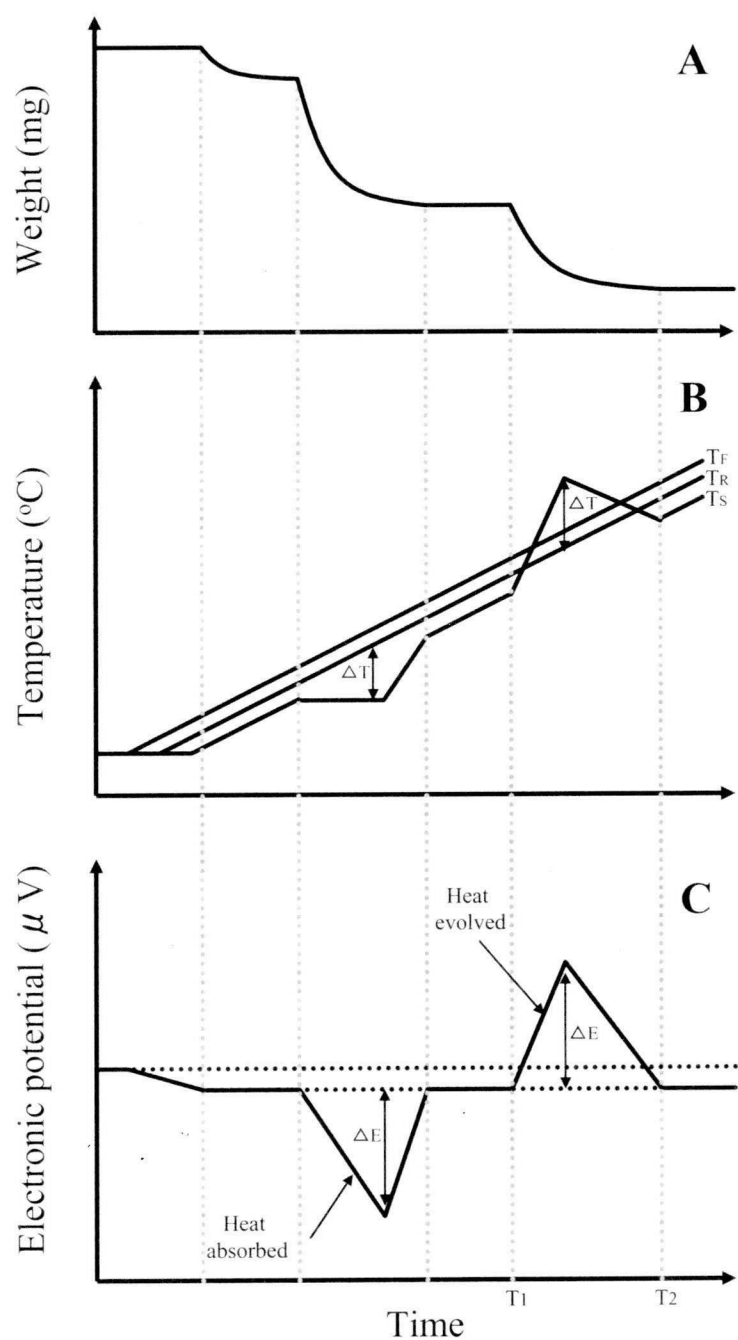
Fig. 1 Principle of TGA and DTA measurements

A: TG curve, B: temperature change of reference (T_R), sample (T_S), and furnace (T_F), C:

DTA curve. $\Delta T = T_R - T_S$, ΔE is thermoelectromotive force detected by thermocouples.

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Fig. 2 A schematic diagram of the Shimadzu DTG-60/60H TG/DTA analyzer
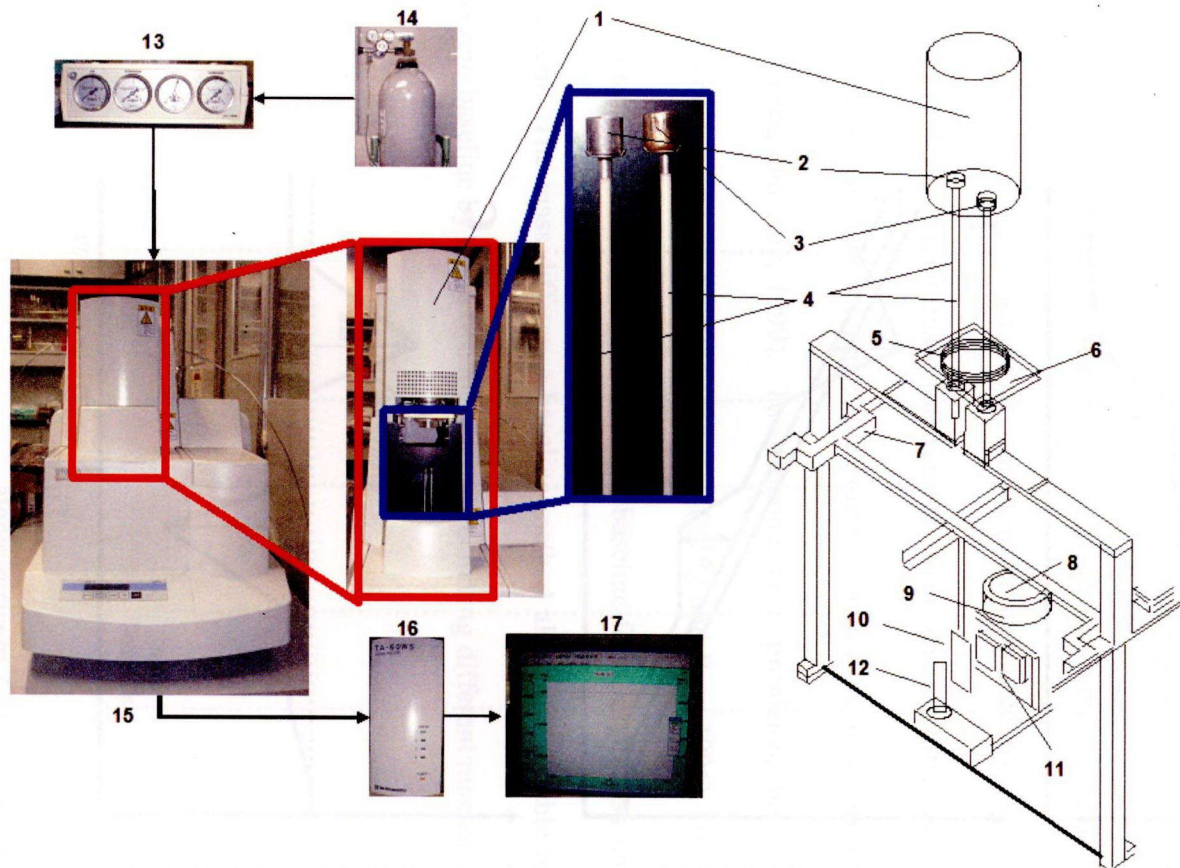
The analysis system (15) consists of furnace (1), reference pan (2), sample pan (3), thermocouples (4), furnace base (5), Joint section (6), beam (7), coil (8), magnet (9), shutter (10), photoelectric conversion element (11), lamp (12), carrier gas controller (13), carrier gas slinder (14), computer controller (16), monitor display (17).

1.3 The aim of present study

The biochemical and physiological characteristics of oleaginous microorganisms have been intensively investigated to improve their prospects of oil production (Meng *et al.*, 2009). Knowledge about the thermal characteristics of microorganisms is fundamentally important for screening oleaginous microorganisms and developing lipid-accumulating strains as a source of biodiesel. For a recent new decades, we have observed remarkable development in thermal analysis, especially in new techniques by which chemical and structural changes in the sample can be directly observed (Ozawa, 2000). However, little is known about the physical and chemical changes occurring in microorganisms when they are heated in air.

Arseneau have described biological materials is "multi-component, molecularly non-homogeneous material in which part of the components exist [*sic*] as continous, separate and intermingling structures" (Arseneau, 1961). The complexity of biological systems even in the static state and the difficulty to be expected in obtaining meaningful results and of establishing correlations between thermal characteristics and other physical and chemical properties presumably accounts for the paucity of DTA studies on these organic systems (Mitchell and Birnie, 1970). For this reason, it has been appreciated that reproducibility and resolution in thermal analysis may be poor.

This study employed simultaneous TG and DTA measurements to microorganisms to examine the potential of a thermal technique for screening oleaginous microorganisms. To do this, thermal behaviours of oleaginous and non-oleaginous microorganisms were investigated by

using the mass-difference baseline method (Yang and Roy, 1999) for obtaining stable baseline significantly affected in reproductivity of DTA results. To resolve overlapping DTA peaks, a two-step linear temperature program was examined for discriminating oleaginous and non-oleaginous microorganism.

The chapter 2 will describe the theory of TG/DTA for the measurement of heat evolved from a sample material and also for the mass-difference-baseline method. Experimental procedures for preparing of microbial samples and lipid extraction will also be described.

The chapter 3 will demonstrate the application of simultaneous TG/DTA to 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, will be employed to quantify the endothermic and exothermic peaks on the DTA curves.

The chapter 4 will examine the potential of thermal analysis for differentiating between oleaginous and non-oleaginous microorganisms. The model oleaginous microorganisms used will be fungi, *Mortierella alpina* IFO32281 and *Mortierella alliacea* YN-15, a unicellular alga, *Aurantiochytrium* sp. CB 15-5, and a yeast, *Rhodosporidium toruloides* DMKU3-TK 16. *Escherichia coli* JM109, *Rhodococcus opacus* B-4, and *Saccharomyces cerevisiae* will be employed as control non-oleaginous microorganisms. In this simultaneous TG/DTA analysis, the furnace temperature will be linearly increased from 30 to 280 °C, decreased down to 30 °C, linearly increased from 30 to 360 °C, and isothermally held at 360 °C for 30 min. This two-step

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linear temperature program will be effective in resolving overlapped exothermic peaks in the DTA curves in the temperature range from 280 to 360 $^{\circ}$C. Heat evolved from a microbial sample will be estimated from the area under the exothermic peak between 280 and 360 $^{\circ}$C using indium as a standard material.

Chapter 2

Theory and experimental procedures

2.1 Theory

2.1.1 Calorification determination

When sample and reference pans are located symmetrically, the temperatures of furnace (T_P), reference (T_R) and sample (T_S) are changed with the same rate as the furnace temperature ($d(T_F)$ /dt) after a relaxtion time:

$$d(T_F) / dt = d(T_R) / dt = d(T_S) / dt \qquad (2.1)$$

When the furnace temperature is linearly increased ($d(T_F)$ /dt = const), T_P - T_S is constant with time. The heat transfer rate from the furnace to the sample pan ($d(q_s)$ /dt (J/g·s)) is given by (2.2):

$$d(q_S) / dt = (1/R)(T_F - T_S) \qquad (2.2)$$

Eq.(2.2) describes that the heat transfer rate is proportional to the temperature difference between the furnace and the sample pan. The heat conductivity, ($1/R$) (J/g·°C·S) is expressed by the

reciprocal of heat resistance (R). Heat resistance is determined as an instrumental constant which is changed by the property of furnace and pan.

In definition, heat capacity (C_P) is:

$$C_P = \Delta q / \Delta T \ (\text{J/g} \cdot {}^{\circ}\text{C}) \qquad (2.3)$$

where $\Delta q / \Delta T$ is the specific enthalpy change with temperature.

Using Eq. (2.3),

$$d(q_S)/dt = (C_{P(S)} + C_{P,S})dT_F/dt \qquad (2.4)$$

where $C_{P(S)}$ and $C_{P,S}$ are the heat capacities of the sample pan and sample, respectively.

From Eqs.(2.3) and (2.4),

$$d(q_S)/dt = (1/R)(T_F - T_S) = (C_{P(S)} + C_{P,S})dT_F/dt \qquad (2.5)$$

Similarly, the heat transfer rate from the furnace to the reference, $d(q_R)/dt$ (J/g$\cdot{}^{\circ}$C), is given by:

$$d(q_R)/dt = (1/R)(T_F - T_R) = (C_{P(R)} + C_{P,R})dT_F/dt \qquad (2.6)$$

where $C_{P(R)}$ and $C_{P,R}$ are the heat capacities of the reference pan and reference material, respectively.

Subtracting (2.5) from Eq. (2.6) yields

$$d(q_S)/dt - d(q_R)/dt = d\Delta q/dt = -(1/R)(T_S - T_R) = -(1/R)\,\Delta T$$

$$= \{(C_{P(S)} - C_{P,(R)}) + (C_{P,S} - C_{P,R})\}dT_F/dt \qquad (2.7)$$

If the heat capacity of the sample pans is same as reference pan ($C_{P(S)} = C_{P(R)}$), Eq. (2.7) is rewritten as:

$$d\Delta q/dt = -(1/R)\,\Delta T = (C_{P,S} - C_{P,R})\,dT_F/dt \qquad (2.8)$$

The temperature difference, ΔT, can be measured using thermocouples.

$$\Delta E = S_{AB}\,\Delta T \qquad (2.9)$$

where S_{AB} is the proportional constant between electromotive force (ΔE) and temperature difference (ΔT) (V·°C).

Using Eq.(2.9), Eq.(2.8) is rewrriten as

$$d\Delta q/dt = -1/(R\,S_{AB})\,\Delta E \tag{2.10}$$

Therefore, $\Delta q=(q_S- q_S)=\int_{T_1}^{T_2}(d\Delta q) =\int_{T_1}^{T_2}(-1/(R\,S_{AB}))\Delta E\;dt$

$= (-1/(R\,S_{AB}))\int_{T_1}^{T_2}(\Delta E)dt$

The difference in the heat transfer rate between the sample and reference materials is determined by the measurment of ΔE and Eq. (2.10).

2.1.2 Mass-difference-baseline method

Energy balance equations for the sample and reference pans are (Yang and Roy, 1996) :

On the sample side:

$$d(M_P C_{P,P} T_S)/dt + d(M C_{P,S} T_S)/dt + d(Mq)\,dt = \alpha_S A(T_\infty - T_S) \tag{2.11}$$

and on the reference side:

$$d(M_P C_{P,P} T_R)/dt + d(M_R C_{P,R} T_R)/dt = \alpha_R A(T_\infty - T_R) \tag{2.12}$$

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

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capacity of the reference; q (J/g) is the transition heat produced by phase changes or chemical reactions; α_S (J/m^{2}s^{o}C) and α_R (J/m^{2}s^{o}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_∞ (^{o}C), T_S (^{o}C), and T_R (^{o}C) are the temperatures of the furnace, the sample, and the reference, respectively; t (s) is the time.

Subtracting Eq. (2.12) from Eq. (2.11) yields:

$$\mathrm{d}(M_P C_{P,P} \Delta T)/\mathrm{d}t + \mathrm{d}(MH)/\mathrm{d}t - \mathrm{d}(M_R C_{P,R} T_R)/\mathrm{d}t = \alpha_S A(T_\infty - T_S) - \alpha_R A(T_\infty - T_R) \qquad (2.11)$$

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. (2.13),

$$-\alpha_S A \Delta T = A(\alpha_S - \alpha_R)(T_\infty - T_R) + \mathrm{d}(M_R C_{P,R} T_R)/\mathrm{d}t - \mathrm{d}(MH)/\mathrm{d}t \qquad (2.14)$$

In the mass-difference baseline method (Yang and Roy, 1996), 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:

$$\alpha_S A(\Delta T_L - \Delta T_S) = \mathrm{d}(M_S H_S - M_L H_L)/\mathrm{d}t \qquad (2.15)$$

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. (2.15),

$$d(M_S H_S - M_L H_L) = \alpha\,(DTA_L - DTA_S)dT \qquad (2.16)$$

Integrating Eq. (2.16) from T_1 to T_2 yields:

$$\{M_S(T_2)\,H_S(T_2) - M_L(T_2)\,H_L(T_2)\} - \{M_S(T_1)\,H_S(T_1) - M_L(T_1)\,H_L(T_1)\}$$

$$= \alpha \int_{T_1}^{T_2}(DTA_L - DTA_S)dT = \alpha\Sigma \qquad (2.17)$$

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:

$$Q = [\{M_L(T_1)\,H_L(T_1) - M_S(T_1)\,H_S(T_1)\} - \{M_L(T_2)\,H_L(T_2) - M_S(T_2)\,H_S(T_2)\}]/\{M_L(T_1) - M_S(T_1)\}$$

$$= \alpha\,\Sigma\,/\,\{M_L(T_1) - M_S(T_1)\} \qquad (2.18)$$

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2.2 Experimental procedures

2.2.1   Temperature program

Shimadzu DTG 60/60H (Kyoto, Japan) was used for the simultaneous TG/DTA analysis of 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($\pm$0.04) and 10.02($\pm$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 (Yang and Roy, 1996), the heat evolved in the temperature range from T_1 to T_2 (°C), Q (J/g), was calculated using Equation (2.18). The overall conversion coefficient ,α ,was determined using the melting heat of indum (28.4 J/g at 157 °C). The standard material, indium, was purchased from the National Institute of Standards and Technology (Gaithersburg, USA).

In the two-step linear temperature program, the furnace temperature was linearly increased

from 30 to 280 °C, decreased to 30 °C, linearly increased from 30 to 360 °C, and isothermally

held at 360 °C for 30 min (see Fig. 4.1 later).

2.2.2 Preparation of microbial samples

E. coli JM109 (Takara Bio Inc., Japan) and *R. opacus* B-4 (Na *et al.*, 2005) were grown in

Lysogeny Broth (LB) medium with shaking at 170 rpm at 37 and 30 °C, respectively. *S.*

cerevisiae (Oriental Yeast Co., Ltd., Tokyo, Japan) was grown in GYP medium containing 2.0%

glucose, 1.0% yeast extracts, and 2.0% tryptone with shaking at 170 rpm at 30 °C.

The oleaginous fungi, *M. alpina* IFO32281 (Yokoyama *et al.*, 1989) and *M. alliacea* YN-15

(Aki *et al.*, 2001), were maintained on Czapek-Dox agar plates (Oxiod Ltd., Cambridge)

supplemented with 50 μg mL^{-1} uridine. Czapek-Dox agar plates were inoculated with fungal

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 spore suspension was filtrated with a sterile

polyallomer wool. The spores were collected by centrifugation at 140×*g* for 10 min at 4 °C and

resuspended in a small volume of 0.1% v/v Tween-80. The spore was counted using a

hemacytometer (Erma Co. Ltd., Tokyo). The density of spores was maintained at 1,600 mm^{-3} in

0.1% v/v Tween-80. The spore suspension 100 μl was inoculated into a 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 6-12 days.

The oleaginous yeast, *R. toruloides* DMKU3-TK 16 (Kraisintu *et al.*, 2010), was precultured in a 20-ml test tube containing 5 ml of nitrogen-limited medium I (30 g glucose, 1.5 g yeast extract, 0.5 g NH_4Cl, 7.0 g KH_2PO_4, 5.0 g $Na_2HPO_4 \cdot 12H_2O$, 1.5 g $MgSO_4 \cdot 7H_2O$, 0.08 g $FeCl_3 \cdot 6H_2O$, 0.01 g $ZnSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1 mg $MnSO_4 \cdot 5H_2O$, and 0.1 mg $CuSO_4 \cdot 5H_2O$ per liter and pH 3.8) with shaking at 150 rpm and 28 °C for one day. The precultured yeast cells were transferred to a 500-ml Erlenmeyer flask containing 100 ml of nitrogen-limited medium II (70.0 g glucose, 0.75 g yeast extract, 0.55 g $(NH_4)_2SO_4$, 0.4 g KH_2PO_4, 2.0 g $MgSO_4 \cdot 7H_2O$, 0.22 g $CaCl_2 \cdot 2H_2O$, 0.55 g $ZnSO_4 \cdot 7H_2O$, 24.2 µg $MnCl_2 \cdot 4H_2O$, and 25 µg $CuSO_4 \cdot 5H_2O$ per liter and pH 5.5) and grown with shaking at 150 rpm and 28 °C for 7 days.

The oleaginous microalga, *Aurantiochytrium* sp. CB 15-5 (Aki *et al.*, 2010), was precultured in a 20-ml test tube containing 5 ml of GPY medium (30 g glucose, 6.0 g polypeptone, 2 g yeast extract, and 20 g seasalt (Sigma-Aldrich Co., USA) per liter) with shaking at 300 rpm and 28 °C for 7 days. The precultured cells were transferred to a 250-ml Erlenmeyer flask containing 50 ml of fresh GPY medium and cultivated with shaking at 160 rpm and 28 °C for 7 days.

Microbial cells were harvested by centrifugation at $6000 \times g$ for 5min, washed three times with distilled water and dried overnight in an incubator at 30, 40, and 70 °C. Dried samples were pulverized in a bowl prior to analytical experiments. This gave satisfactory reproducibility of TG/DTA curves. All chemicals used were of analytical grade.

2.2.3 Lipid extraction

Dried microbial cells contained approximately 15~32 wt % water were ground into fine powder (particle size: 70~100 μ m) using a mortar and pestle (Iwaki Co. Ltd). Approximately 100 mg of the powder was mixed with 450-µl chloroform-methanol (2:1 v/v) in a 2-ml Eppendorf tube (Bligh and Dyer, 1959). The mixture was vortexed for 20 min at room temperature and centrifuged at 20,000$\times g$ for 5 min to recover the solvent phase. The solvent phase was transferred to a preweighed 15-ml Pyrex glass tube (Iwaki Co. Ltd., Japan). This extraction was repeated three times. After solvent evaporation, the weight of the crude lipid was determined by weighing the glass tube using an electronic scale. At least three measurements were performed with each microbial sample. Triolein (2,3-Bis[[(z)-octadec-9-enoyl]oxy]propyl (z)-octadec-9-enoate) was used as a naturally occurring TAG (60% purity; Wako Co. Ltd., Tokyo).

Chapter 3

Simultaneous TG/DTA analysis of microbial samples using the mass-difference-baseline method

3.1 Purpose of study

Simultaneous TG/DTA was applied to oleaginous and non- oleaginous microorganisms for assessing their burning characteristics. The microorganisms examined were an oleaginous fungus, *Mortirella alpina* IFO32281 (Yokoyama *et al.*, 1989), and non-oleaginous species including *Escherichia coli* JM109 (Takara Bio Inc., Japan), *Rhodococcus opacus* B-4 (Na *et al.*, 2005), and *Saccharomyces cerevisiae* (Oriental Yeast Co., Ltd., Tokyo, Japan). The mass-difference baseline method was employed to quantitatively determine 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.

3.2 Results

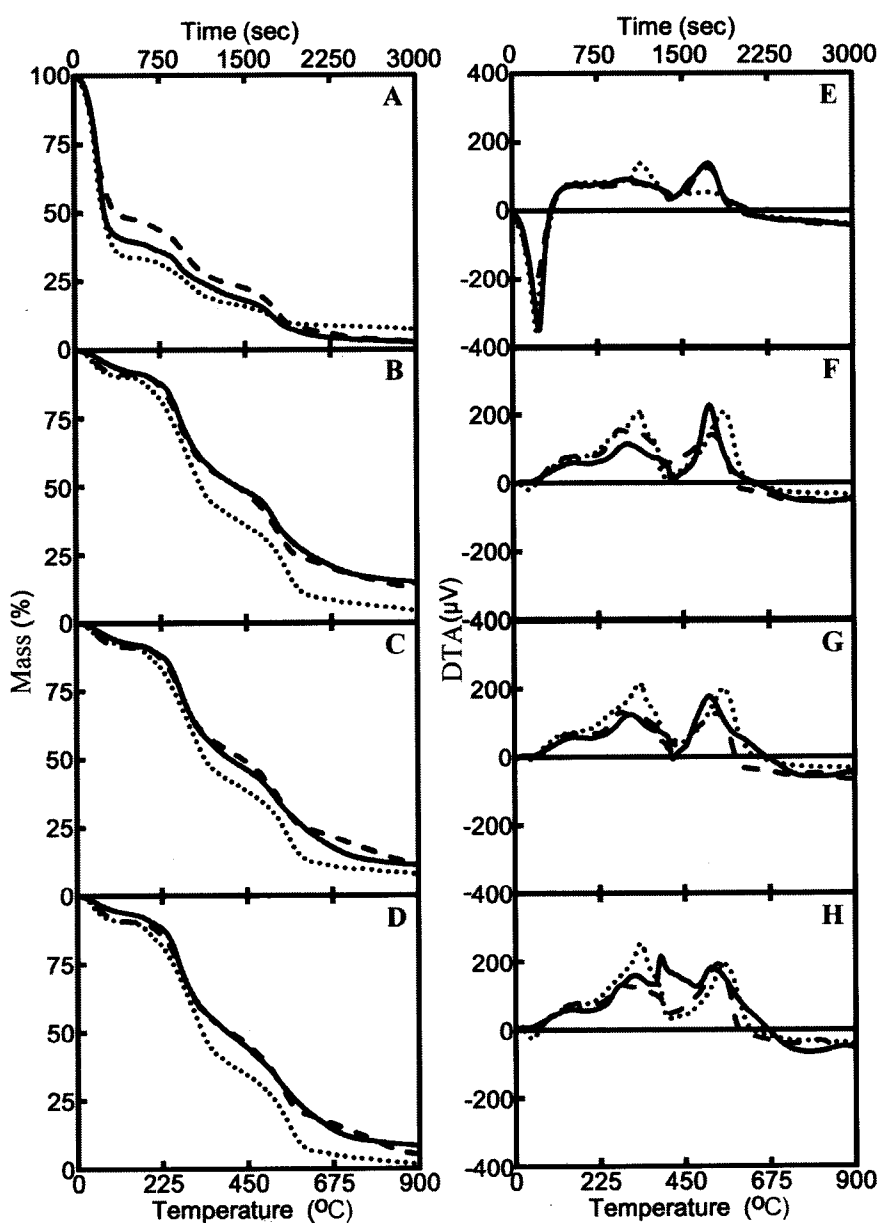### 3.2.1 TG and DTA curves of microbial samples

Typical TG/DTA curves of *E. coli* JM109, *R. opacus* B-4, and *S. cerevisiae* are shown in Fig. 3.1. Previous workers suggested that the moisture content of biological samples could significantly affect the results of TG/DTA measurements (Mackenzie and Mitchell, 1970; Alcázar *et al.*, 2000; Vozárová, 2005). 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. 3. 1A). The DTA curve showed a large endothermic peak at approximately 75 °C because of water evaporation (Fig. 3. 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 to 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. 3. 1F) were similar to those of the samples pretreated at 40 °C (Fig. 3. 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. 3. 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. 3. 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, microbial samples were pretreated overnight at 30°C for further study.

Fig. 3. 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.

3.2.2 Estimation of heat evolved from microbial samples

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 (Yang and Roy, 1996). 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 (Yang and Roy, 1996). 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 (Yang and Roy, 1996), was employed for quantifying the peak area under DTA curves in the present study. Figs. 3. 2A and 3. 2B show 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. 3. 2B) and the non-oleaginous microorganisms (Figs. 3. 2E, 3. 2H, and 3. 2K). Although the non-oleaginous species showed the two-peak system, their second 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 = d*DTA*/d*T*) showed that the remarkable change in the DTA signals of *M. alpina* IFO32281 occurred in the temperature range from 280 to 360 °C (Fig. 3. 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.
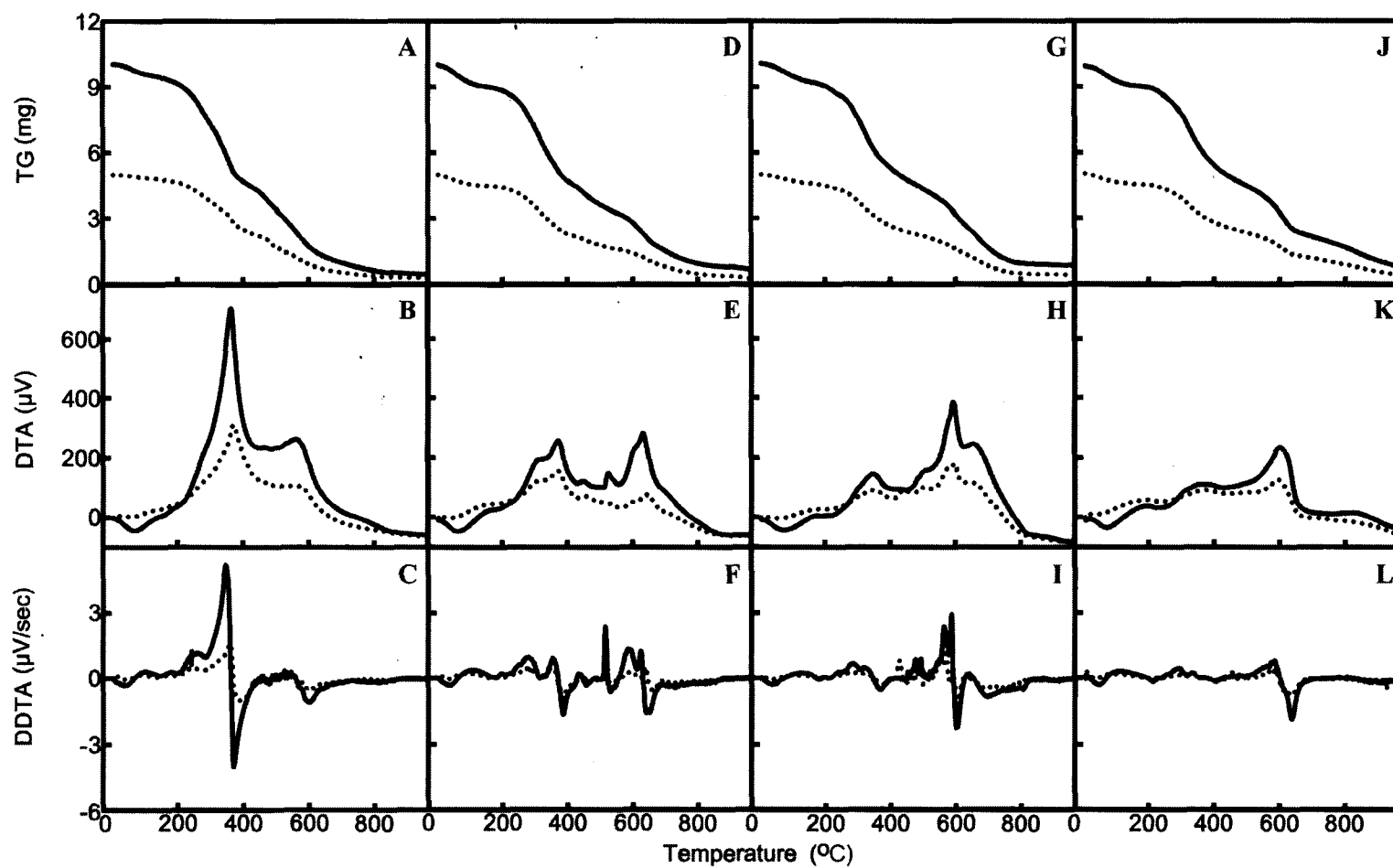
Fig. 3. 2. TG, DTA, and differential DTA (DDTA) curves for microbial samples of 5 mg (broken line) and 10 mg (closed line). The microorganisms examined were *M. alpina* IFO32281 (A, B, and C), *R. opacus* B-4 (D, E, and F), *S. cerevisiae* (G, H, and I), and *E. coli* JM109 (J, K, and L).

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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. 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. 3. 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. 3. 3A). However, the difference in the mass-difference TG curve among the microorganisms examined was smaller than in the mass-difference DTA curve.

The heat evolved from the microbial samples was estimated from the area under the mass-difference DTA curve using Equation (2.18). 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 $\times 10^{-7}$ (J/μV°C).

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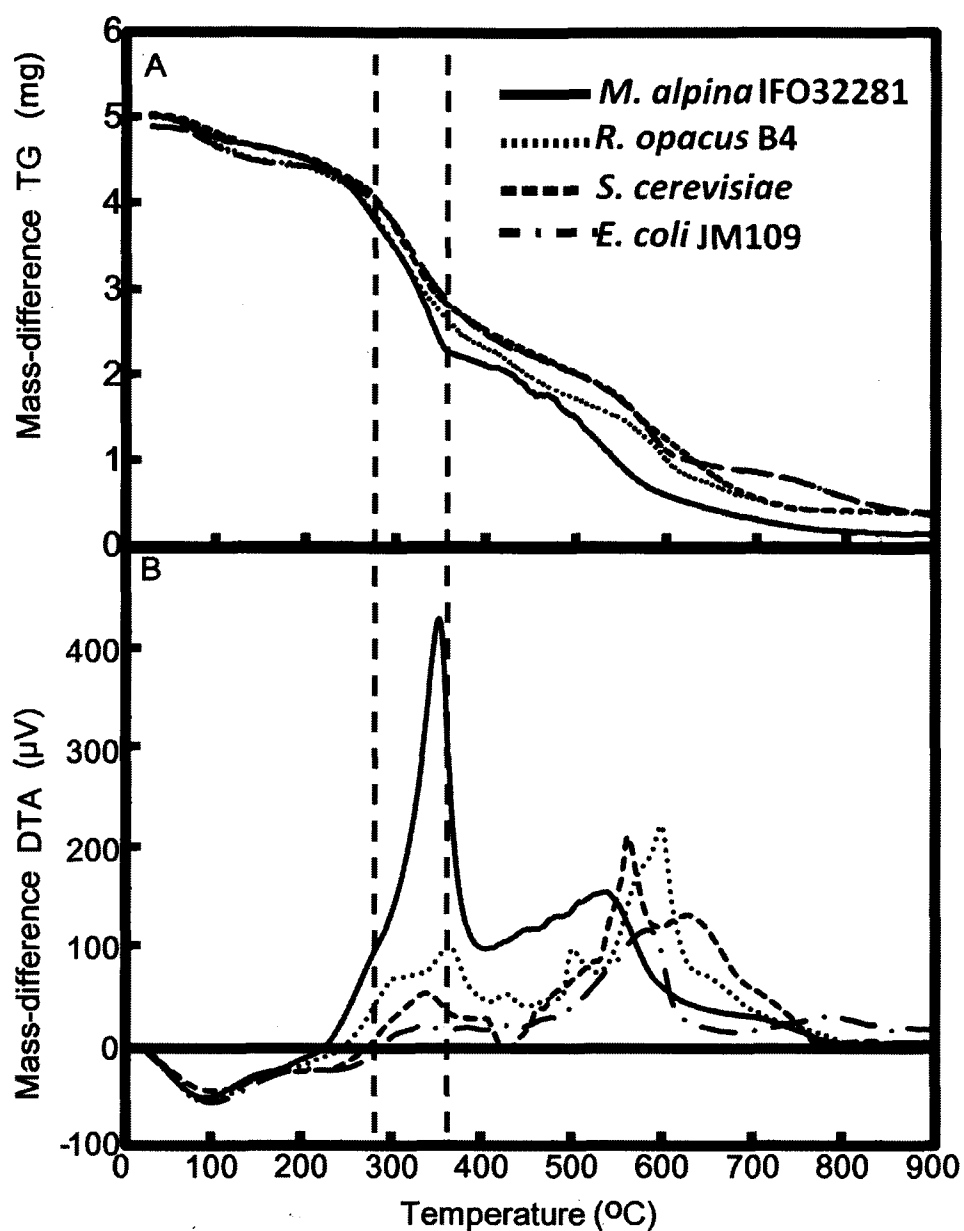
Fig. 3. 3. Mass-difference TG (A) and DTA (B) curves of *E. coli* JM109, *S. cerevisiae*, *R. opacus* B-4, and *M. alpina* IFO32281. The mass-difference curves were obtained by subtracting the TG and DTA curves for the small-mass sample (approximately 5 mg) from those for large-mass sample (approximately 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).

Table 3. 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.

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

Microorganisms	Temperature range (°C)	Peak temperature* (°C)	Heat evolved* (kJ/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

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

3.3 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 microorganims. Quantitative thermal analysis

has been generally conducted using differential scanning calorimetry (DSC) (Tunick *et al.*, 2006). 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. 3. 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 (Yang and Roy, 1996). 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 Equation (2.18). One major difficulty in DTA measurement is that the overall conversion factor α is rarely constant in a wide temperature range (Yang and Roy, 1996). To overcome this problem, the mass-difference baseline method has been proposed by Yang and Roy (Yang and Roy, 1996).

The complete-combustion curves of four microorganisms examined showed two exothermic peaks at approximately 350 and 550 °C (Fig. 3. 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 (Mitchell and Birnie, 1970). 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 (Mitchell and Knight, 1963). DTA curves have been obtained for simple organic

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materials, including hydrocarbons, carbohydrates, and proteins (Mitchell and Knight, 1963). 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 (Ratledge., 1991). 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. 3). Consequently, a relatively large exothermic heat was evolved from this organism in the temperature range of 280 to 360 °C among the non-oleaginous species (Table 3. 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 (Sameshima *et al.*, 2008).

As can be seen in Fig. 3. 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 microorganims 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 $^\circ$C was approximately 3.3- to 11-fold greater than those from the non-oleaginous microorganisms (Table 3. 1).

The data presented in this study suggest that the measurement of the heat evolved in the temperature range from 280 to 360 $^\circ$C is a promising 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 $^\circ$C and 322 to 367 $^\circ$C, respectively. Microbial samples of 5-10 mg may be held isothermally in the temperature range from 280 to 360 $^\circ$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 $^\circ$C.

3.4 Summary

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 $^\circ$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.

Chapter 4

Differentiatiation between oleaginous and non-oleaginous microorganisms using a two-step linear temperature program

4.1 Purpose of study

If thermal analysis is proved to be a promising technique, it may open the way for the development of a simple physical technique to assess the burning characteristics of microorganisms and to screen oleaginous species for oil production. The potential of thermal analysis for differentiating between oleaginous and non-oleaginous microorganisms was examined by simultaneous TG and DTA. A two-step linear temperature program was employed for the TG/DTA analysis to resolve overlapping peaks in the DTA curves. The oleaginous microorganisms examined in this study were the fungi, *Mortirella alpina* IFO32281 (Yokoyama *et al.*, 1989) and *Mortirella alliacea* YN-15 (Aki *et al.*, 2001), the microalga, *Aurantiochytrium* sp. CB 15-5 (Aki *et al.*, 2010), and the yeast, *Rhodosporidium toruloides* DMKU3-TK 16 (Kraisintu *et al.*, 2010). For comparison, *Escherichia coli* JM109 (Takara Bio Inc., Japan), *Rhodococcus opacus* B-4 (Na *et al.*, 2005), and *Saccharomyces cerevisiae* (Oriental Yeast Co., Ltd., Japan), were used as the non-oleaginous microorganisms.

4.2 Results

4.2.1 TG/DTA of microbial samples with a two-step linear temperature program

When TG/DTA analysis of a microbial sample was first performed using a linear temperature program (Fig. 4. 1A). A distinct reduction in sample mass was detected in the temperature range from 280 to 360 °C in the linear temperature program (Fig. 4. 1B). However, the DTA curve showed overlapping peaks above 280 °C (Fig. 4. 1C). The largest peak appeared in the DTA curve between 280 and 360 °C. The size and sharpness of the exothermic peak between 280 and 360 °C differed between oleaginous and non-oleaginous microorganims (data not shown).

To resolve the overlapping peaks in the temperature range from 280 to 360 °C, TG/DTA analysis was performed with a two-step linear temperature program where furnace temperature was linearly increased from 30 to 280 °C (phase I), decreased to 30 °C (phase II), linearly increased from 30 to 360 °C (phase III), and isothermally held at 360 °C for 30 min (phase IV) (Fig. 1D). In phase II, furnace temperature was decreased at a rate of 10 °C min^{-1} until the temperature was decreased to 30 °C. A small endothermic peak occurring at approximately 75 °C in phase I was due to water evaporation. An approximately 25% reduction in sample mass was detected when furnace temperature was increased from 30 to 280 °C (phase I) (Fig. 4. 1E). This mass reduction in phase I was accompanied by an exothermic peak in the DTA curve (Fig. 1F). Similar exothermic peaks occurred between 200 and 280 °C, regardless of the type of the microorganism, i.e., oleaginous or non-oleaginous (data not shown). Neither mass reduction nor exothermic peak was detected when the furnace temperature was decreased from 280 to 30 °C (phase II). When the furnace temperature was increased again in phase III, another exothermic

peak was observed with the DTA curve above 280 °C (Fig. 4. 1F). The DTA peak was

accompanied by a sharp decrease in sample mass (Fig. 4. 1E). This exothermic peak disappeared

in the isothermic section at 360 °C (phase IV) (Fig. 4. 1F). No other exothermic peak was

observed when the furnace temperature was isothermally held at 360 °C for 30 min.

The two-step linear temperature program was used for comparing the thermal behavior

between oleaginous and non-oleaginous microorganims (Fig. 4. 2). As can be seen in Fig. 4. 2,

an exothermic peak was observed between 280 and 360 °C, regardless of the type of the

microorganisms. Each peak was accompanied by a significant reduction in sample mass.

Obviously, the size and sharpness of the exothermic peak were more distinct for the oleaginous

microorganisms (Figs. 4. 2F, 4. 2G, and 4. 2H) compared to the non-oleaginous species (Figs. 4.

2B, 4. 2C, and 4. 2D). These findings suggested that the heat evolved from oleaginous

microorganisms was larger than that from non-oleaginous species in the temperature range from
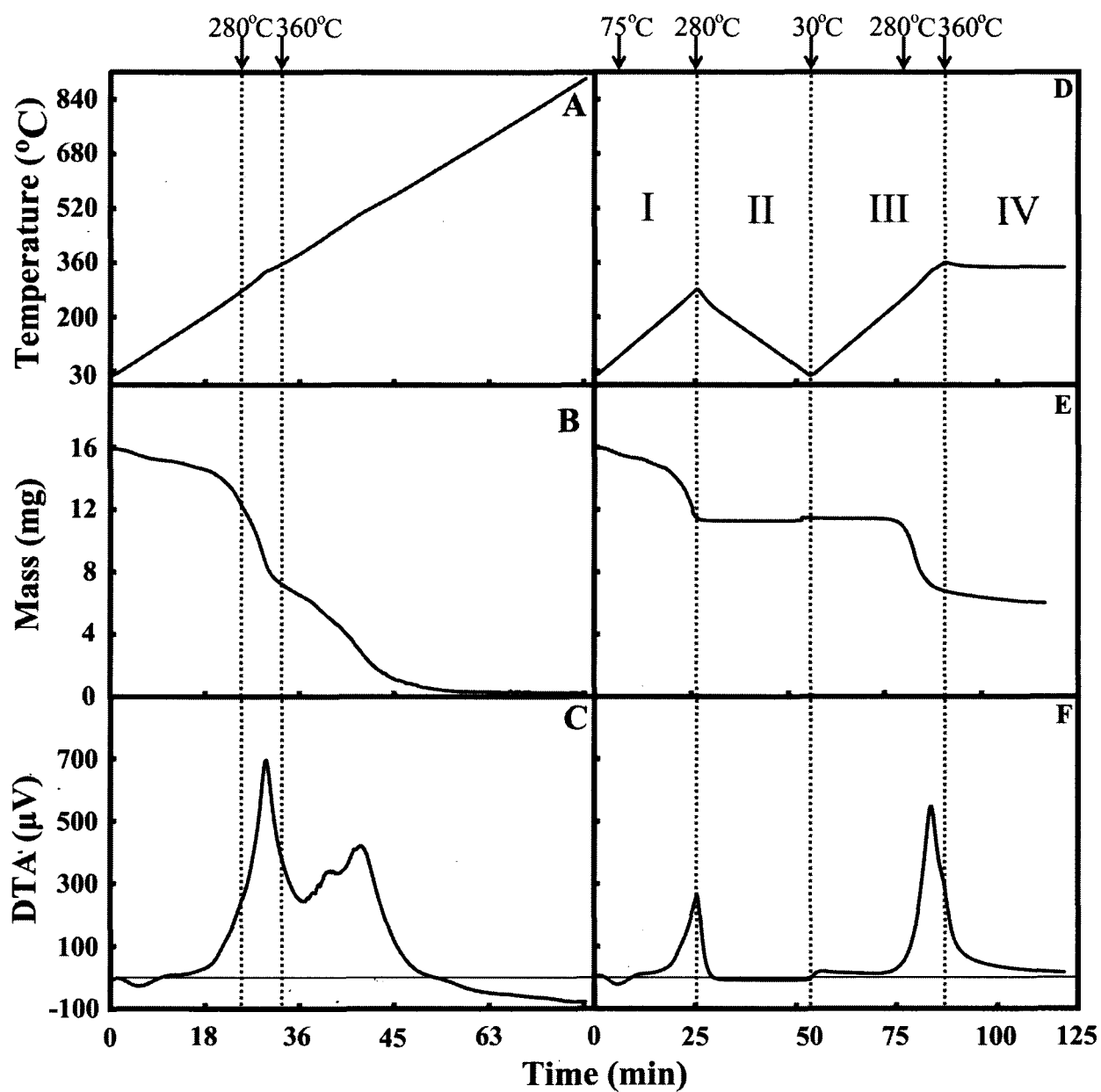
280 to 360 °C.

Fig. 4. 1. Simultaneous TG/DTA analysis of the oleaginous fungus, *M. alpina* IFO 32281, using

single-step linear temperature program and two-step linear temperature program. In the single-

step linear temperature program (A), furnace temperature was linearly increased from 30 to 900

^{o}C at a rate of 10 ^{o}C min^{-1}. The temperature range between 280 and 360 ^{o}C is indicated by two

vertical dotted lines. In the two-step linear temperature program (D), furnace temperature was linearly increased from 30 to 280 °C (phase I), decreased to 30 °C (phase II), linearly increased from 30 to 360 °C (phase III), and isothermally held at 360 °C for 30 min (phase IV). The heating rate in the phases I and III was set at 10 °C min^{-1}. In phase II, furnace temperature was decreased at a rate of 10 °C min^{-1}. TG (B and E) and DTA (C and F) curves of *M. alpina* IFO 32281 were obtained using a simultaneous TG/DTA instrument (model DTG 60/60H, Shimadzu, Kyoto, Japan). The zero baselines for the DTA curves (0 μV) are indicated by horizontal lines.
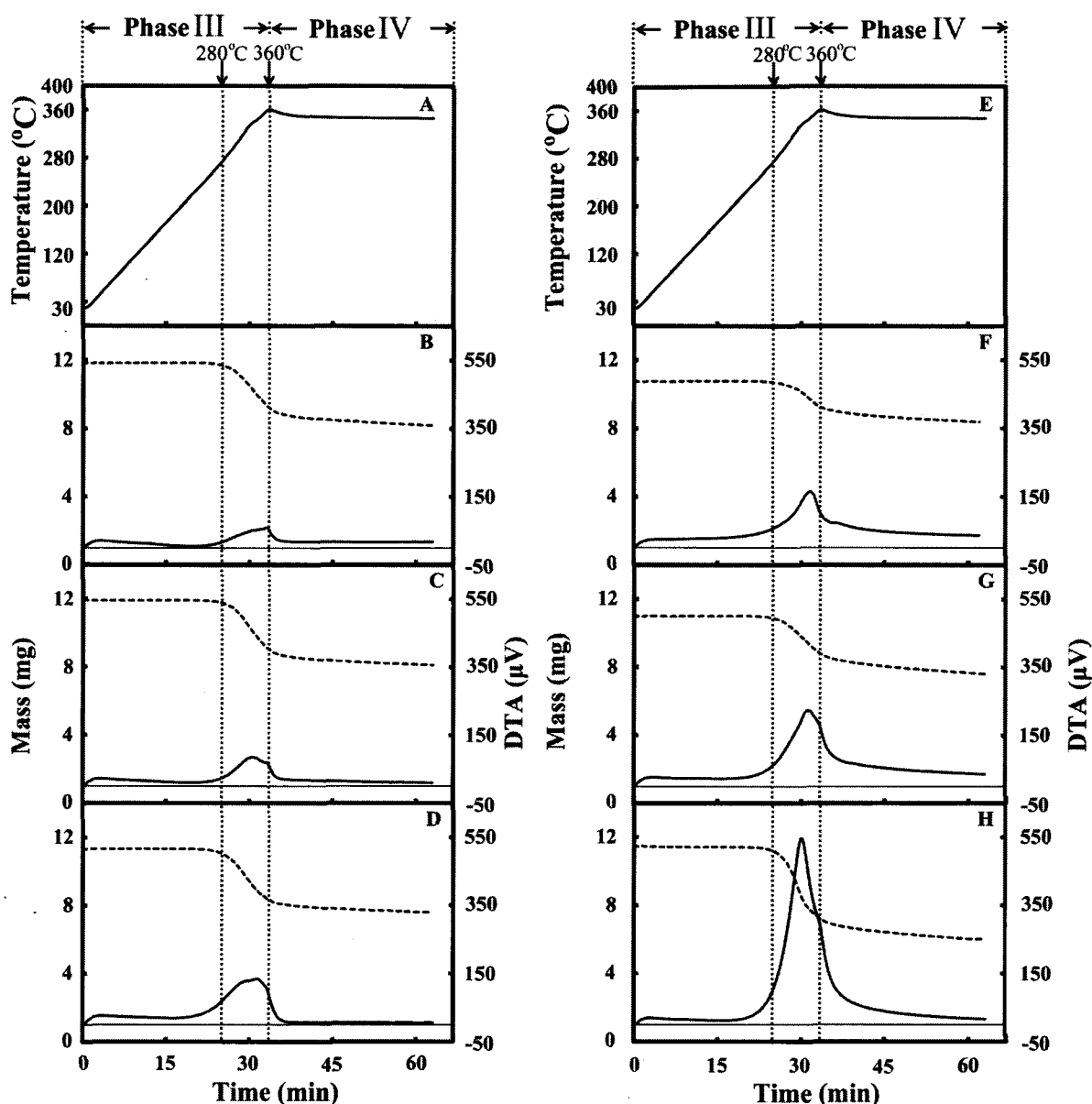
Fig. 4. 2. TG and DTA curves of *E. coli* JM109 (B), *S. cerevisiae* (C), *R. opacus* B-4 (D),

Aurantiochytrium sp. CB 15-5 (F), *R. toruloides* DMKU3-TK 16 (G), and *M. alpina* IFO32281

(H). The TG (broken line) and DTA (closed line) curves were obtained using the two-step linear

temperature program (A and E). Only the data relevant to phases III and IV are shown. The

temperature range between 280 and 360°C is indicated by two vertical dotted lines. The zero

base lines for the DTA curves (0 μV) are indicated by horizontal lines.

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4.2.2 TG/DTA of total lipids extracted from oleaginous fungi

To determine whether the exothermic peak occurring between 280 and 360 °C reflects the burning of cellular lipids, total lipids were extracted from the oleaginous fungi, *M. alpina* IFO32281 and *M. alliacea* YN-15, using the conventional technique (Bligh and Dyer, 1959). TG/DTA analysis of the total lipids was performed using a temperature program in which the furnace temperature was linearly increased from 30 to 360 °C and isothermally held at 360 °C for 30 min (Fig. 4. 3A). Because no significant peaks were detected below 280 °C, it was unnecessary to use a two-step linear temperature program for the analysis of total lipids. For comparison, TG/DTA analysis was also performed using 60% triolein, which is a naturally occurring glyceride formed from oleic acid.

As expected, the total lipids showed a distinct mass reduction between 280 and 360 °C (Fig. 4. 3B). This mass reduction was associated with a sharp exothermic peak in the DTA curve (Fig. 4. 3C). A similar exothermic peak was observed with 60% triolein. However, the exothermic peak of 60% triolein appeared at a temperature somewhat lower than that detected with total lipids. The peak temperature, where the DTA curve reached the maximum, was approximately 300 °C for 60% triolein, whereas that of the total lipids occurred at approximately 350 °C (Fig. 4. 3C). Previous workers have demonstrated that DTA curves were modified by the presence of impurities (An and Sabbah, 1991). The difference in the peak temperature between 60% triolein and the total lipids was likely attributable to the amount of imprities present in the samples.

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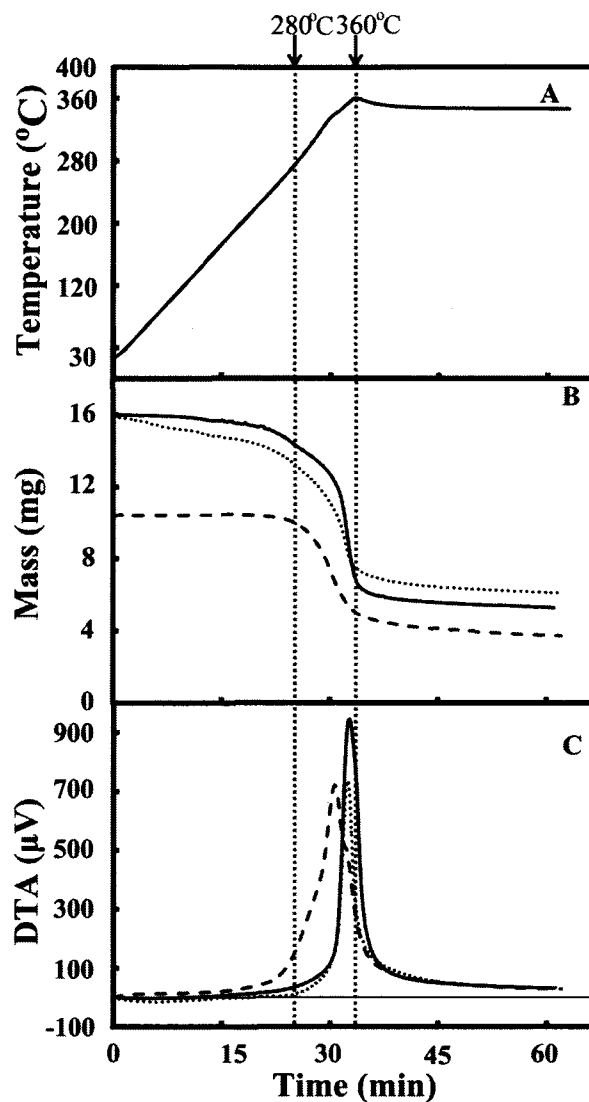
Fig. 4. 3. TG and DTA analysis of 60% triolein and total lipids extracted from oleaginous fungi. Furnace temperature (A) was linearly increased from 30 to 360 °C and isothermally held at 360 °C for 30 min. TG (B) and DTA (C) curves were obtained using about 15 mg of total lipids extracted from *M. alpina* IFO32281 (closed line) and *M. alliacea* YN-15 (dotted line) or about 10 mg of 60% triolein (broken line). The temperature range between 280 and 360 °C is indicated by two vertical dotted lines. The zero baseline for the DTA curves (0 μV) is indicated by a horizontal line.

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4.2.3 Heat evolved from microbial samples

The exothermic heat corresponding to mass loss in the temperature range from 280 to 360 °C was calculated from the area under the DTA curve using Eq. (4.1).

$$Q = \alpha \, \Sigma_{280\text{-}360} / (M_{280} - M_{360}) \tag{4.1}$$

where Q is the exothermic heat per mass loss in the temperature range from 280 to 360 °C (kJ/g); α is the overall conversion coefficient from the DTA signal to the heat evolved (J/μV°C); $\Sigma_{280\text{-}360}$ is the area under the DTA curve in the temperature range from 280 to 360 °C (μV°C); and M_{280} and M_{360} are the TG signals at 280 and 360 °C, respectively (g). To convert a DTA peak area to exothermic heat, indium was employed as a standard material. The DTA curve of indium showed an endothermic peak at the melting temperature of 157 °C (data not shown). The overall conversion coefficient α from DTA signal to heat was estimated to be 1.27×10^{-4} (J/μV°C) from the area above the DTA curve and the melting heat of indum (28.5 J/g).

Figure 4. 4 shows the exothermic heat per mass loss between 280 and 360 °C for oleaginous and non-oleaginous microorganisms. Obviously, the heat evolved per mass loss was larger in the oleaginous microorganisms than in the non-oleaginous species. In particular, the heat evolved from the oleaginous fungi, *M. alpina* IFO 32281 and *M. alliacea* YN-15, was approximately 12 kJ/g, which was about 3- to 4-fold greater than that of the non-oleaginous microorganisms. As has been shown in Fig. 4. 2, the oleaginous and non-oleaginous microorganisms showed similar

mass loss occurring in the temperature range from 280 to 360 °C. Therefore, the data presented in Fig. 4. 4 is likely a reflection of the difference in the calorific values of cellular materials burnt in the temperature range between 280 and 360 °C.
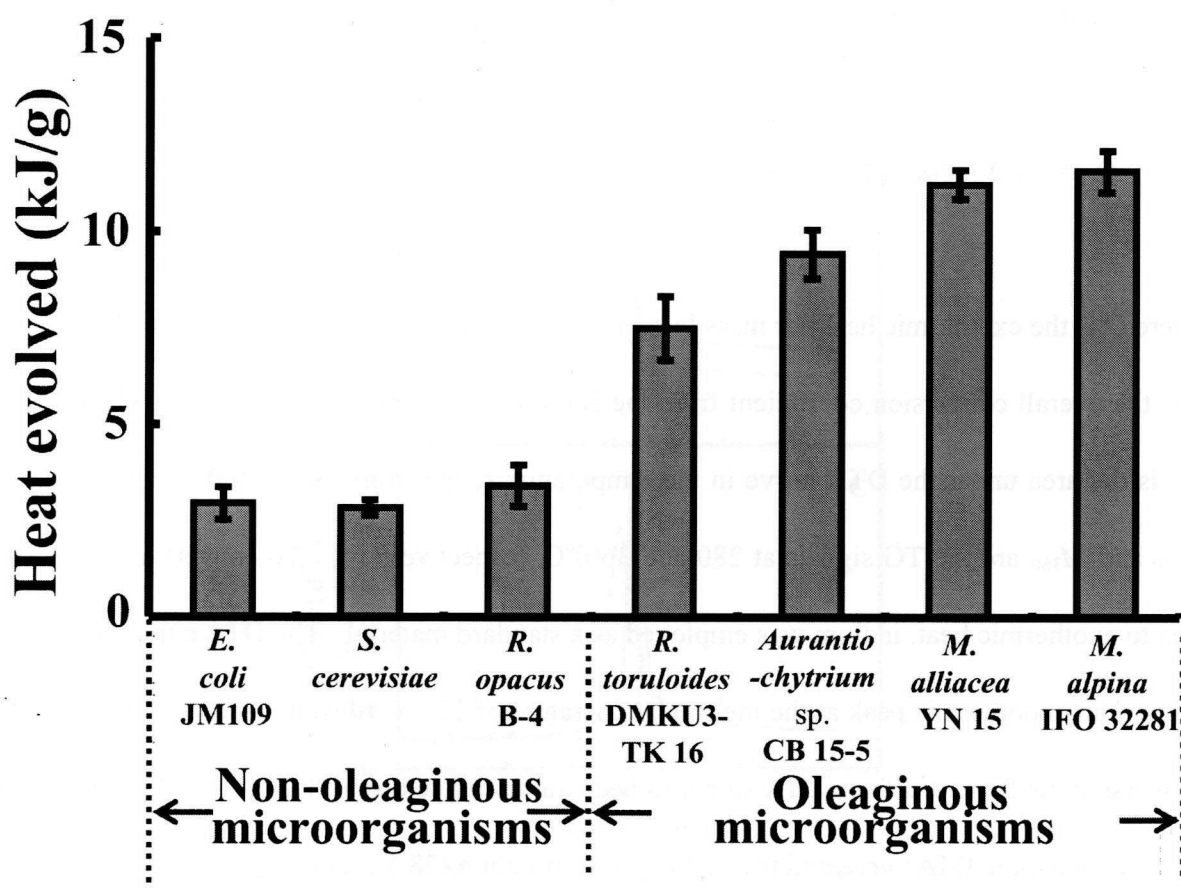
Fig. 4. 4. Heat evolved per mass loss of oleaginous and non-oleaginous microorganisms between 280 and 360 °C. At least three separate measurements were performed for each microbial strain. The data are shown as means±standard deviations.
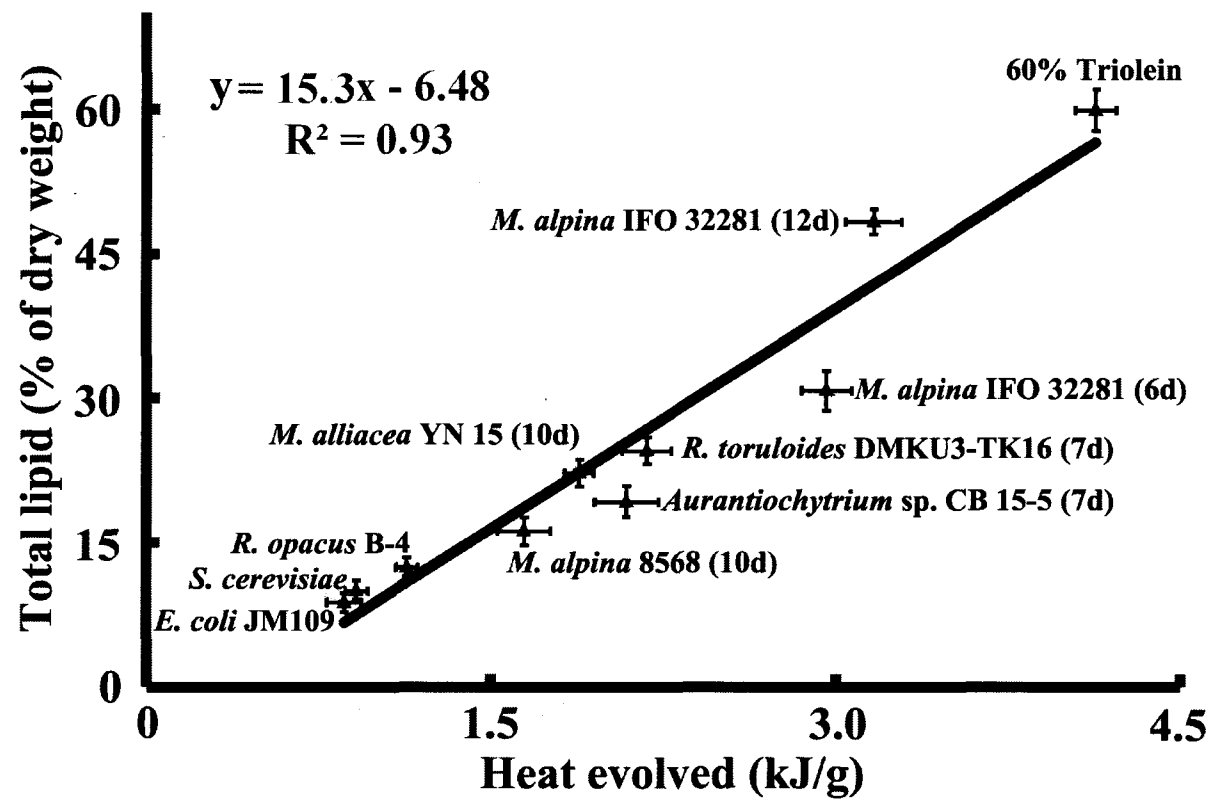
4.2.4 Relationship between exothermic heat and total lipid content of microbial cells

To investigate the relationship between the exothermic heat and total lipid content of microbial cells, exothermic heat per initial sample mass was calculated using Eq. (4. 2).

$$H = \alpha \, \Sigma_{280\text{-}360} / M_{30} \qquad\qquad (4.\,2)$$

where H is the exothermic heat per initial sample mass (kJ/g), and M_{30} is the TG value at 30 °C (g). As shown in Fig. 4. 5, there was a linear relationship between the exothermic heat per initial sample mass and total lipid content of microbial cells ($R^2 = 0.93$). The oleaginous fungus, *M. alpina* IFO 32281, accumulated 31.1 ± 1.9 and $48.5\pm1.0\%$ of dry weight as lipids after 6 and 12 days of cultivation, respectively. Reflecting the lipid accumulation, the heat evolved from *M. alpina* IFO 32281 increased from 2.96 ± 0.10 kJ/g at 6 days to 3.16 ± 0.11 kJ/g at 12 days. The oleaginous fungus, *M. alliacea* YN-15, accumulated $22.6\pm1.2\%$ of the dry weight as lipid after 10 days of cultivation. This fungus exhibited an exothermic heat of 1.88 ± 0.06 kJ/g after 10 days of cultivation. The oleaginous yeast, *R. toruloides* DMKU3-TK 16, and the oleaginous alga, *Aurantiochytrium* sp. CB 15-5, accumulated 24.8 ± 1.2 and $19.6\pm1.3\%$ of their dry weight as lipids, respectively, after 7 days of cultivation. *R. toruloides* DMKU3-TK 16 and *Aurantiochytrium* sp. CB 15-5 evolved exothermic heat of 2.18 ± 0.10 and 2.09 ± 0.13 kJ/g, respectively. By contrast, the non-oleaginous microorganisms, *E. coli* M109, *S. cerevisiae*, and *R. opacus* B-4, accumulated 9.2 ± 0.8, 10.4 ± 0.7, and $12.8\pm0.7\%$ of their dry weight as lipids, respectively. The amounts of heat evolved from *E. coli* M109, *S. cerevisiae*, and *R. opacus* B-4, were 0.86 ± 0.07, 0.92 ± 0.04, and 1.14 ± 0.04 kJ/g, respectively. A relatively large amount of heat

was evolved from *R. opacus* B-4, which is known to have long-chain-length fatty acids (typically mycolic acids) on the cell wall (Na *et al.*, 2005).

Fig. 4. 5. Linear relationship between the exothermic heat and total lipid content of microbial cells. The x-axis represents the heat evolved between 280 and 360 °C per sample mass (kJ/g). The y-axis shows the total lipid content of microbial cells (% of dry weight). The data for 60% triolein are also shown for comparison. The horizontal and vertical bars represent the standard deviations of the exothermic heat and total lipid content of microbial cells, respectively. The linear line is the regression line (R^2 =0.93).

4.3 Discussion

Oleaginous microorganisms, including yeasts, fungi, and microalgae, are actively studied as an alternative source for biodiesel production (Hu *et al.*, 2008; Ratledge, 1991). To improve their prospects of use for biofuel production, the biochemical and physiological characteristics of oleaginous microorganisms have been intensively investigated (Meng *et al.*, 2009). Obviously, knowledge of the thermal behavior of microorganims is fundamentally important for screening potential oleaginous microorganims and developing oil-accumulating strains for biodiesel production. However, little is known about the burning characteristics of oleaginous and non-oleaginous microorganisms.

As typically shown in Fig. 4. 1C, microorganisms showed the complete-combustion curves with two overlapping peaks in the DTA curves at approximately 300 and 540 °C. Because this two-peak system reflects a series of discrete chemical reactions, it is difficult to understand the chemical and physical mechanisms underlying the DTA peaks. Both the chemical and physical structures of a biological material affect its burning characteristics (Mitchell and Birnie, 1970). In addition, combustion curves do not necessarily reflect the ultimate thermal decomposition of a sample. The changes in the molecular structure and the rearrangement of functional groups must also be involved (Mitchell and Knight, 1965). The oleaginous microorganisms showed a larger peak at approximately 300 °C than the non-oleaginous species (data not shown). By contrast, the size and sharpness of the second peak at approximately 540 °C were more distinct for the non-oleaginous microorganisms than for the oleaginous ones. Useful information for differentiating

between oleaginous and non-oleaginous microorganisms was provided by the first peak in the DTA curves (Fig. 4. 4).

The two-step linear temperature program could resolve overlapping peaks in the temperature range from 280 and 360 °C (Fig. 1F). As shown in Fig. 4. 3, total lipids extracted from the oleaginous fungi and 60% triolein showed similar sharp exothermic peaks in the DTA curve between 280 and 360 °C. A similar exothermic peak was observed with 60% triolein. Kaisersberger (1989) has reported that the main oxidation peak was detected between 280 and 310 °C for edible oils, including cocofat, lard, butter, and butterfat by differential scanning calorimetery (DSC). TG analysis of soybean, sunflower, and forage turnip biodiesel has also shown a remarkable weight loss between 200 and 300 °C (Soares *et al.*, 2010). Taken together, it is highly likely that the exothermic peak in the DTA curve obtained using the two-step linear temperature program can reflect the total lipid content of microorganisms.

The heat of combustion is one of the most important properties of biomass fuels (Sheng and Azevedo, 2005). The conventional method for determining the calorific value of biomass has been based on the use of a bomb calorimeter (Scragg *et al.*, 2003). The bomb calorimeter is a constant-volume calorimeter used for the direct determination of calorific value when a sample is completely burnt in air. Using this conventional technique, the calorific values of lipid-accumulating microalgae have been shown to be between 18 and 20 kJ/g (Scragg *et al.*, 2003; Bhola *et al.*, 2011). In the present study, the heat evolved per mass loss was estimated from TG and DTA curves between 280 and 360 °C using Eq. (4. 1) for the oleaginous and non-oleaginous microorganisms (Fig. 4. 4). The oleaginous fungi, *M. alpina* IFO 32281 and *M. alliacea* YN-15,

showed 10-12 kJ/g, which was somewhat smaller than the calorific values previously reported for microalgae. Mitchell and Knight (1965) have noted that the area under the exothermic peak of the complete-combustion DTA curve bore a relationship with the calorific value of peat. They have showed an approximate relationship between the calorific values of plant leaves and the areas of the exothermic peak system on the complete-combusion DTA curves. However, DTA curves are affected by factors associated with the apparatus used and sample properties (Kang *et al.*, 2010). Asymmetric heat transfer is caused by the asymmetric location of sample holders in the DTA furnace and the differences in thermal properties between the sample and the reference, for example, emissivity and thermal conductivity. Therefore, it should be noted that heat estimation based on the peak area under a DTA curve has limitations in accuracy and less reliability compared with that using the bomb calorimeter.

4.4 Summary

There was a linear relationship between the exothermic heat and total lipid content of the tested microorganisms. The exothermic heat per unit dry sample mass (kJ/g) in the temperature range from 280 to 360 °C is a promising measure for differentiating between oleaginous and non-oleaginous microorganisms.

Chapter 5

General discussion

The quantification of total lipid in microbial cells has been performed by various analytical techniques. The methods which use a solvent combination, were developed by Folch et al. and Bligh and Dyer. (Schäfer *et al.*, 1998). The fluorophore Nile Red (9-diethylamino-*5H*-benzo[α]phenoxazine-5-one) has been used to determine neutral lipid in microalgal cells (Cooksey *et al.*, 1987). Recently, Supercritical fluid extraction (SFE) was developed for analytical application in the mid-1980s in response to the desire to reduce the use of organic solvents in the laboratory environment (Sahena *et al.*, 2009). However, conventional methods of lipid determination have many complicated steps, i.e., extraction, purification, concentration, and determination, which are time-consuming (Kimura *et al.*, 2004). In recent decades, Nile Red (NR) staining seems preferable in fluorescent measurement of intracellular lipid because the method is quick and simple (Gao *et al.*, 2008). But, it has also been revealed that Nile Red cannot stain dead cells of microalgae (Elsey *et al.*, 2007).

To overcome these problems, the near-infrared spectroscopy has been for the research on the SCOs production in solid-state fermentation (Peng and Chen, 2008). The absorption peaks of the fermented samples in the waveleghth from 4000 to 7500 cm^{-1} was selected to developed the NIR model for total lipids estimation (Peng and Chen, 2008). This method has been advantages of saving time and reagent. The spin-echo NMR pulse sequence has also been applied to separate

the lipid hydrogen nuclei signal from other hydrogen nuclei signals in the TD-NMR (time-domain nuclear magnetic resonance). Especially, the amplitude S_2 represented the lipid signal, which can reflect the lipid content. This NMR can achieve high accuracy and reproducibility in the estimation of total lipids (Gao *et al.*, 2008).

In the present study, the total heat evoled from microbial samples between 280 and 360 $^{\circ}$C was found to be useful for estimating the total lipid contents from microbial cells. There was a linear relationship between the heat evolved per sample mass and total lipid content of the tested microorganisms (Fig. 4. 5). This relationship suggests that the measurement of the heat evolved from microbial cells in the temperature range from 280 to 360 $^{\circ}$C is a promising means of screening oleaginous microorganisms for oil production. The detection of weight loss is unnecessary for the determination of heat evolved per sample mass; and consequently, the instrument design can be based on the requirements for measuring sample temperature. A good resolution may be retained by the pretreatment of a sample by heating up to 280 $^{\circ}$C. After the pretreatment, a sample of 10-15 mg is placed in a furnace isothermally held at 360 $^{\circ}$C. The exothermic heat evolved from the sample can be determined by measuring the temperature difference between the sample and the inert reference material using thermocouples. A simple modification may allow the use of the instrument for assessing a liquid sample. Cells are dried from fluid medium directly by preheating the sample up to 280 $^{\circ}$C. Future study is directed toward the development of an inexpensive, high-throughput instrument for the screening of oil-accumulating microorganisms.

The total heat evolved from microbial samples in the temperature range from 280 to 360 °C appeared to reflect the amount of total cellular lipids. Consequently, the heat estimation is likely for usable for differentiating between oleaginous and non-oleaginous microorganisms. The screening of oleaginous species from naturally isolated ones could be carried out by measuring the exothermic heat per unit dry sample mass (kJ/g) in the temperature range from 280 to 360 °C.

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RELATED PUBLICATIONS

Kang, B. M., Honda, K., Aki, T., Omasa, T., Ohtake, H.; "Simultaneous thermogravimetry and differential thermal analysis for comparing burning characteristics between oleaginous and non-oleaginous microorganisms", *Thermochim. Acta*, 517, 115-120 (2011).

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PRESENTATIONS AT INTERNATIONAL CONFERENCES

Kang, B. M., Honda, K., Aki, T., Omasa, T., Ohtake, H.; "Simultaneous thermogravimetry and differential thermal analysis for comparing burning characteristics between oleaginous and non-oleaginous microorganisms", The Japanese Society for Promotion of Science, JSPS and The Swedish University of Agricultural Sciences, SLU workshop, 2010, June 22-23th, Krusenberg, Uppsala, Sweden (Oral presentation).

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