1. Introduction

The emission of the greenhouse gases due to burning of fossil fuels (e.g. CO₂, NOₓ and SO₂) has increased since the industrial revolution, and those gasses have accumulated in the atmosphere and ocean to date. Atmospheric CO₂ concentration was predicted to reach more than 700 ppm by the end of the 21st century⁵. The CO₂ gas increase can lead to climate changes such as rapid global warming and ocean acidification. On the other hand, the further usage of the fossil fuels would eventually lead to the depletion of the underground resources. Therefore, natural energy resources have to be changed to alternative energy resources to avoid environmental changes and energy depletion in near future. The biodiesel fuel (BDF) is known as one of the alternative energy sources. The BDF can be made from various bio-materials containing lipids of land plants, aquatic algae and animals⁶. Among them, photosynthetic plants and microalgae can produce carbon-neutral BDF. Chisti (2007)⁷ reported that microalgae would be the only promising source of renewable BDF that could be sufficient for global demands of transport fuels, primarily because of high lipid con-

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Direct Biodiesel Production from Wet Microalgae
*Nannochloropsis oceanica* using Microwave Reactor with Closed Circulation System

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Abstract

This study was made to examine the efficiency of direct conversion from wet microalgae (*Nannochloropsis oceanica*) to crude biodiesel fuel (BDF) using a microwave reactor (MWR) with a closed circulation system in which a glass coil condenser was installed. The concentrated microalgae and SrO catalyst mixed with organic solvents was flowed from a sample bottle outside the MWR to the coil condenser located inside, and was returned to the sample bottle. The MW irradiation time was changed from 0 to 500 sec. The extraction efficiency of crude BDF (BDF/dry weight of samples) was not significantly different (30.8−36.7%) among the irradiation times, whereas the efficiency from triacylglycerol (TAG) to fatty acid methyl ester (FAME), [FAME/(FAME+TAG)] increased with the irradiation time, and reached the maximum of 86±5% at 350 sec. No marked change in the composition of FAME under MW exposure was observed. The results indicated that the present MWR effectively converted from the TAG to FAME within a very short time (<6 min), and will be applicable for the crude BDF production.
tents (~75% of dry weight) and high growth rates relative to land plants.

The production of the BDF usually needs two-step processes of lipid extraction from algal cells and subsequently methyl esterification of the extracted lipids (two-step method). In contrast, a direct method (single-step method) indicates that the two chemical processes mentioned above are completed with one operation. The BDF extraction in either method has been done using traditional organic solvents, autoclaves, ultrasonic, microwave (MW), osmotic shock and supercritical fluid extraction (e.g., 4–6). Recently, Park et al. (2015) (7) indicated the advantages of the direct method with wet biomass that have simple and energy efficient processes without the lipid extraction step. Koberg et al. (2011) (8) succeeded the BDF extraction using MW methods and the BDF yield from dried microalgae was 5-fold higher than that using conventional heating method. Cheng et al. (2013) (9) also showed that the production rates and yields of BDF using the MW method were 6-fold and 1.3-fold higher than the two-step heating method. Furthermore, the concentration of linolenic acid (one of the BDF products) that can be effective in reducing the oxidation stability of the BDF was lower in the direct MW method than in the ultrasonic method (10). Those reports suggested that the direct MW method from wet microalgae was likely the best method to produce the BDF at present, while batch-type MW reactors developed in previous experimental studies (11) might be unsuited for large volume and high-throughput manufactures of BDF.

This study was to examine the extraction efficiency of the BDF and the conversion efficiency from triacylglycerol (TAG) to fatty acid methyl ester (FAME) from wet microalgae, *Nannochloropsis oceanica*, using a newly developed microwave reactor (MWR) with a closed circulation system.

### 2. Material and methods

#### 2.1. Cultivation and harvesting of *Nannochloropsis oceanica*

An acrylic columnar photobioreactor (hereafter PBR, diameter: 190 mm; height: 1,500 mm) with a bubbling stone (Φ 132 mm, S104-A, SUDO & CAMpany Inc.) on the bottom was stood by the window from which natural light was transmitted. Cold cathode fluorescent lamps (E-COOL OPT-S40C-BN, Optrom Inc.) were also placed to the opposite side of the window to keep growing of *N. oceanica* throughout 24 hours. Thirty-five liters of filtered seawater (0.2 μm pore-size membrane cartridge filter, TCS-G020, ADVANTEC) were poured into the PBR. Stock solutions of f/2 medium (12) without Na₂SiO₃・9H₂O were added into the PBR, and the medium was mixed with the aeration. After few hours, pre-incubated *N. oceanica* was inoculated into PBR.

The harvesting operation was carried out using a centrifuge (CR-22N, Hitachi Koki Co., Ltd.) with a sealed continuous rotor (R18C-848, Hitachi Koki Co., Ltd.). The condensed pellets of *N. oceanica* were collected in a plastic container, and were stored in a freezer (−20℃) until lipid extraction experiments.

#### 2.2. Dry weight determinations of *N. oceanica*

The wet weight of the pellets was measured with an electronic balance (FZ-500i, A&T Co., Ltd.). The sample was dried for 48 hours in a drying chamber (60℃), and the DW were also determined with the electronic balance. The conversion factor from the wet weight to DW of *N. oceanica* was calculated to be 0.31 ± 0.04 (average ± standard deviation, N = 9).

#### 2.3. Construction of the MWR system

The present MWR was modified from a commercial MW oven (frequency: 2.45 GHz; power dissipation: 1,310 W; power output of MW: 700W; RE-T2, SHARP Corp.). Two holes of 15 mm
were made at the top plate of the MW oven. A Dimorth-type glass coil condenser (82-4073, Sansyo Co., Ltd.) was placed into the MW oven, and the coupling ports of cooling hoses of the condenser were thrust out from inside the MW oven to outside through the holes. Those coupling ports were connected with Teflon tubes (TYGON SE-200, Saint-Gobain Inc.), and the junctions were sealed with the wire bands. One port of the Teflon tubes was jointed with a diaphragm-type pump (DPE-800-7P-Y1, Nitto Kohki Co., Ltd.), and the other was linked up with a three-way branch pipe of Teflon. To measure the temperature of the reactant exposed to the MW, a thermometer probe (SN-3400-04, Netsuken Co., Ltd.) was inserted to the port of the three-way branch pipe, and the temperature was monitored and recorded with a thermometer (SN-3400, Netsuken Co., Ltd.). The remaining ports of the pump and the three-way branch pipe were put into a sample bottle on the stirrer.

An outline of the process with this MWR system is as follows. (1): the slurry algal sample mixed by the stirrer in the bottle is transported to the cooling-pipe in the MWR through the pump (Fig. 1a). (2): In the MWR, algal lipids (e.g., TAG) are converted to FAME by the MW irradiation (Fig. 1b) . (3): The thermometer immediately measures the temperature of the slurry algal sample after the reaction, and the sample is returned to the sample bottle (Fig. 1c).

The surface area and volume of the glass-cooling pipe and the sample flowing velocity were measured as 503 cm², 45 mL and 6.4 mL sec⁻¹, respectively.

2.4. Preparation of samples

The wet N. oceanica (ca. 3 g) was mixed with methanol (100 mL) and chloroform (200 mL). As catalyst for methanolysis, SrO (0.1 g, Mitsuwa Chemicals Co., Ltd.) was added just before the MWR activated. The sample bottle was set on the stirrer, and was mixed during the experiment (Fig. 1). In this study, the times of the MW irradiation were 0 (control), 50, 150, 250, 350 and 500 sec. In the control experiment, the sample was mixed with the stirrer at room temperature (ca. 23℃) during 10 min without MW irradiation. The initial and maximum temperatures of the slurry sample, and the elapsed time from initial temperature to 50℃ were measured to estimate the rate of temperature increase (℃ sec⁻¹) for all experiments.

The slurry samples after the MW irradiation were filtered (0.8 μm pore-size), and chloroform–methanol fraction of the filtrate was evaporated with a rotary evaporator (N-1110V-W, Tokyo Rikakikai Co., Ltd.) under N₂ gas purge. The crude BDF containing FAME was dissolved with a small amount of chloroform, and was transferred to a pre-weighed bottle.
The chloroform was completely evaporated in a vacuum oven (FVM-303D, TGK Co., Ltd.) at 60℃ and 0.02-0.04 MPa under N2 gas purge. The weight of the crude BDF was determined with the electronic balance. The extraction efficiency was calculated based on Eq. 1.

\[ e_{\text{ex}} = \frac{W_{\text{tissue}}}{D W_{\text{algae}}} \times 100(\%) \]  

(1)

where \( e_{\text{ex}} \) is the extraction efficiency of the crude BDF (%), \( W_{\text{tissue}} \) is the weight of the crude BDF extracted from \( N. \text{oceanica} \) (g), and \( D W_{\text{algae}} \) is the dry weight of the algae (g).

2.5. Separation of lipids with thin layer chromatography

The mixed solution (developer) of hexane:diethyl ether:acetic acid (90:10:1) was poured into a developing chamber, and was stood at least 1 hour to saturate the chamber inside with the vapor. One micro litter of the crude BDF dissolved in chloroform was spotted onto the base point of the thin layer chromatograph (TLC, Silica gel 60F254, 1.05554.0001, Merck Co., Ltd.). To confirm the positions of FAME and TAG after the development, the methyl stearate and trilinolein of the gas chromatography (GC) grade were also spotted to both sides of the sample. The sheet of TLC after the development was dried for 15 min in a draft chamber at room temperature. The coloration was carried out with iodine vapor for 15 min, and the colored sheet was immediately photographed with a digital camera (EOS Kiss digital N, Canon Inc.). The image analysis was made using the Image J (http://imagej.nih.gov/ij/). The color element of the TLC image was split to red, green and blue. The monochromatic intensities of FAME and TAG spots on the blue image were converted to the spectral data, and the values of those areas were used as an indicator of the BDF Conversion Efficiency (BCE). The \( \text{BCE}_{\text{ex}} \) (%) was estimated as the following equation (Eq. 2).

\[ \text{BCE}_{\text{ex}} = \frac{\text{FAME}_{\text{area}}}{(\text{FAME} + \text{TAG})_{\text{area}}} \times 100(\%) \]  

(2)

where \( \text{FAME}_{\text{area}} \) and \( \text{TAG}_{\text{area}} \) are estimated areas of FAME and TAG, respectively.

2.6. Analysis of FAME

2.6.1. Purification of FAME with column chromatography

Column chromatography (CC) was carried out to separate the FAME from the crude BDF. Ten grams of silica gel (37565-79, Kanto chemical Co., Inc.) were mixed with the TLC developer above, and the slurry was poured into a column (Φ 12 mm). After the top layer of the poured silica gel was calmed down sufficiently, the crude BDF (50–100 mg) was gently injected into the top of the silica gel. The liquid fraction separated by the CC was dispensed to vials and were analyzed with TLC using the same method above. The fraction of FAME was evaporated with the rotary evaporator and dried in the vacuum oven at 60℃ and 0.02-0.04 MPa under N2 gas purge.

2.6.2. Determination of FAME with gas chromatography

The pure sample of FAME obtained with the CC was dissolved with methyl acetate including methyl pentadecanoate (C15:0) as the internal standard. The composition of the FAME was determined with the GC (7890A GC system, Agilent Technologies Co., Ltd.) equipped with a flame ionization detector (FID) and a DB-23 Agilent column (length: 60 m, internal diameter: 0.25 mm, film: 0.15 mm). The analytical condition was as follows. The flow rate of carrier gas (helium) was 1.4 mL min−1, the injection temperature was 250℃ and the detector temperature was 280℃. The temperature of the column oven was programed as 0℃ (1 min), 50–175℃ (25℃ min−1), 175–230℃ (4℃ min−1) and 230℃ (5 min). The concentrations of seven fatty acids (C14:0 myristic acid, C16:0 palmitic acid, C16:1 palmitoleic acid, C18:0 stearic acid, C18:1 n−9...
oleic acid, C20:4 n-6 arachidonic acid, and C20:5 n-3 eicosapentaenoic acid, EPA) were determined in this study, and the percentages of each fatty acid to the total amount were calculated.

3. Result and discussion

3.1. Temperature of slurry samples

The initial (before MW irradiation) and maximum temperatures, times for temperature increase to 50°C and the temperature increase rates during the MWR operations were shown in Table 1. The initial temperature ranged between 18.3 and 31.3°C, and the average was 25.5±4.2°C. The maximum temperature slightly changed (53.5-57.8°C, 54.5±1.3°C). The temperature reached the maximum temperature within 30 sec, and the temperature maintained to the end of the experiment. No significant relationship was shown in the temperature and irradiation time (Pearson’s correlation test, p=0.12, n=14, data not shown), indicating that the elongation of the irradiation time of the present MWR does not affect the maximum temperature. The elapsed times for temperature increase to 50°C (15-25 sec, 19±3 sec in average) probably influenced by the initial temperature. The range and average of the temperature increase rates were 1.1-1.7°C sec⁻¹ and 1.3±0.2°C sec⁻¹.

3.2. Extraction efficiency of crude BDF

The extraction efficiency of crude BDF to DW was shown in Fig. 2. The average value slightly increased from 0 (control, 32.9%) sec to 150 sec (36.7%), and decreased from 150 sec to 500 sec (30.8%). No significant difference was obtained between the extraction efficiency and the MW irradiation time (One-way ANOVA, p=0.24). This means that the lipid extraction was not affected by the time of MW irradiation but was affected by the solute solubility of the solvent as described by Prommuak et al. (2012) (13).

Table 1 List of temperature parameters in the microwave reactor (MWR). The initial and the maximum temperatures of slurry samples were measured before and during the MW irradiation, respectively. Elapsed time from the initial temperature to 50°C and rate of temperature increase were also shown.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range</th>
<th>Average ± S.D.</th>
<th>Sample number (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial temperature (°C)</td>
<td>18.3–31.3</td>
<td>25.5 ± 4.2</td>
<td>14</td>
</tr>
<tr>
<td>Maximum temperature (°C)</td>
<td>53.5–57.8</td>
<td>54.5 ± 1.3</td>
<td>14</td>
</tr>
<tr>
<td>Elapsed time from initial temperature to 50°C (sec)</td>
<td>15–25</td>
<td>19 ± 3</td>
<td>12</td>
</tr>
<tr>
<td>Rate of temperature increase (°C sec⁻¹)</td>
<td>1.1–1.7</td>
<td>1.3 ± 0.2</td>
<td>12</td>
</tr>
</tbody>
</table>
3.3. Fatty acid compositions and MW irradiation time

Table 2 showed the compositions and percentages (weight%) of seven FAME determined with the GC. Six fatty acid compositions, except for stearic acid, were not significantly different from the irradiation times (One-way ANOVA, myristic acid: \( p = 0.57 \), palmitic acid: \( p = 0.21 \), palmitoleic acid: \( p = 0.19 \), oleic acid: \( p = 0.50 \), arachidonic acid: \( p = 0.11 \), EPA: \( p = 0.22 \)). Stearic acid was undetectable in this study. The result indicated that the composition of FAME produced with the MWR did not depend on the irradiation times. The average values of three fatty acids of EPA, palmitic acid and palmitoleic acid for 0-500 sec irradiation time accounted for 74% of the total. The relative dominancy of the three fatty acids was correspondent with other reports\(^{14-16}\). According to the present compositions of seven fatty acids, the percentage of the unsaturated fatty acids (palmitoleic acid, oleic acid, arachidonic acid and EPA) were higher than that the saturated fatty acids (myristic acid, palmitic acid and stearic acid) (Table 2). Viswanathan et al. (2012)\(^{5}\) reported that the percentage of unsaturated fatty acids under higher temperature (>90℃) trended to decrease due to oxidation causing cleavage of unsaturated bonds. In the present study, as the maximum temperature of the reactant exposed to the MW was 53.5-57.8℃, and the high percentage of the unsaturated fatty acids might be obtained. It is known that higher percentage of unsaturated fatty acids contributes to oxidative instability during storage and to low temperature-resistant BDF\(^{5,10}\). Therefore, the BDF produced in this study can be suitable for the usage under cold conditions.

3.4. Conversion efficiency from triacylglycerol to fatty acid methyl ester

The BCE changed with the MW irradiation time (Fig. 3). The TAG was slightly transformed to FAME at 0 sec under the existence of SrO catalyst and the room temperature (ca. 23℃). The transesterification reaction was significantly accelerated by the MW irradiation (One-way ANOVA, \( p < 0.05 \), Tukey’s HSD test, \( p < 0.05 \), Fig. 3). The acceleration effect due to MW irradiation is also shown by Azcan and Yilmaz (2012)\(^{17}\). In this study, the highest BCE was observed at 350 sec, and then slightly decreased at 500 sec (Tukey’s HSD test, \( p = 0.29 \)). Hindarso et al. (2015)\(^{18}\) also reported a similar result that the yield of FAME from the microalgae-extracted lipid using the MWR with CaO

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>0</th>
<th>50</th>
<th>150</th>
<th>250</th>
<th>350</th>
<th>500</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0 Myristic</td>
<td>6.5</td>
<td>5.4</td>
<td>7.5</td>
<td>5.9</td>
<td>5.9</td>
<td>6.4</td>
<td>6.3</td>
</tr>
<tr>
<td>C16:0 Palmitic</td>
<td>23.3</td>
<td>20.1</td>
<td>22.7</td>
<td>22.4</td>
<td>22.5</td>
<td>22.2</td>
<td>22.2</td>
</tr>
<tr>
<td>C16:1 n-7 Palmitoleic</td>
<td>29.2</td>
<td>23.2</td>
<td>26.1</td>
<td>22.3</td>
<td>22.3</td>
<td>21.6</td>
<td>24.1</td>
</tr>
<tr>
<td>C18:1 n-9 Oleic</td>
<td>5.3</td>
<td>6.4</td>
<td>4.7</td>
<td>5.4</td>
<td>5.3</td>
<td>5.9</td>
<td>5.5</td>
</tr>
<tr>
<td>C20:4 n-6 Arachidonic</td>
<td>12.6</td>
<td>17.7</td>
<td>13.4</td>
<td>13.3</td>
<td>12.8</td>
<td>13.6</td>
<td>13.9</td>
</tr>
<tr>
<td>C20:5 n-3 Eicosapentaenoic</td>
<td>23.0</td>
<td>27.2</td>
<td>25.6</td>
<td>30.8</td>
<td>31.2</td>
<td>30.4</td>
<td>28.0</td>
</tr>
<tr>
<td>Sum of the saturated fatty acids(^{21})</td>
<td>70.2</td>
<td>74.5</td>
<td>69.8</td>
<td>71.4</td>
<td>71.7</td>
<td>71.4</td>
<td>71.5</td>
</tr>
<tr>
<td>Sum of the unsaturated fatty acids(^{22})</td>
<td>( )</td>
<td>( )</td>
<td>( )</td>
<td>( )</td>
<td>( )</td>
<td>( )</td>
<td>( )</td>
</tr>
</tbody>
</table>

U.D. undetectable

\(^{21}\) C14:0+C16:0+C18:0 (undetectable calculated as 0)

\(^{22}\) C16:1 n-7+C18:1 n-9+C20:4 n-6+C20:5 n-3
and MgO catalysts was highest at 180 sec, and decreased toward 300 sec. This phenomenon occurs because transesterification is a reversible–endothermic reaction (Hindarso et al., 2015).

The maximum value of the BCE, in this study did not reach 100%, while Koberg et al. (2011) (8) showed that the conversion from TAG to FAME reached 99.9% at 210 sec using the batch-type MWR with the power output 770W. The method of Koberg et al. (2011) (8) was primarily similar to that of the present study, but there were some differences. They used more powerful MWR and dry biomass with the higher ratio of catalyst to dry biomass (30%) than our study (10%). The lower catalyst to biomass ratio can lead to lower yields of BDF as suggested by Hindarso et al. (2015) (18). To compensate the poorer powered MWR, longer MW irradiation times (>250 sec) than that of Koberg et al. (2011) (8) were applied to improve the BCE, in present study. However, the higher efficiency of BCE, than 86 ± 5% was not obtained for longer irradiation time than 350 sec.

The present MWR is the closed circulation system. Thus, the residence time in the MWR can be estimated to know the effective MW irradiation time for the conversion from TAG to FAME. Under the assumption of perfect diffusion during the process of sample circulation, the volume of MW-unexposed slurry samples was calculated as the following Eq. 3 (19).

\[ V_r = V_0 \exp\left(-\frac{t}{\tau}\right) \quad (3) \]

where \( V_r \) is the MW-unexposed sample volume at any time (mL), \( V_0 \) is the MW-unexposed sample volume at the initial (mL), \( t \) is the MW irradiation time (sec), and \( \tau \) is the residence time (sec\(^{-1}\)) of samples during the experiment which was calculated from Eq. 4.

\[ \tau = \frac{V}{F} \quad (4) \]

where \( V \) is the total volume (mL) of the circulating tube, and \( F \) is the flow rate (mL sec\(^{-1}\)).

The MW-unexposed sample volume decreases exponentially with increasing MW irradiation time (Table 3). The MW-unexposed sample volume and the percentage composition at 350 sec were 0.17 mL and 0.06%, respectively. This showed that the slurry samples were mostly exposed to MW by 350 sec (>99.9%), in the flow rate (6.4 mL sec\(^{-1}\)) of this study and the additional time for MW irradiation more than 350 sec was not effective for further production of FAME in the present MWR. On the other hand, Michel and Wen (2009) (20) reported that FAME yields from wet algal biomass were significantly lower than those from dry algal biomass, suggesting the incomplete conversion from TAG to FAME using wet algae. Therefore, the present maximum BCE, (86 ± 5%) estimated using li-

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Fig. 3. Change in the BDF Conversion Efficiency (BCEi, %) with the MW irradiation time (sec). The different alphabet notations (a, b, c and d) indicates the significant difference between the vertical bar of BCEi (Tukey’s HSD test, \( p < 0.05 \)).

Table 3 The MW irradiation time, the MW-unexposed volume and the percentage compositions of the MW-unexposed samples were estimated from Eq. 3.

<table>
<thead>
<tr>
<th>MW irradiation time (sec)</th>
<th>MW-unexposed volume (mL)</th>
<th>MW-unexposed percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>300</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>103</td>
<td>34</td>
</tr>
<tr>
<td>150</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>250</td>
<td>1.4</td>
<td>0.5</td>
</tr>
<tr>
<td>350</td>
<td>0.17</td>
<td>0.06</td>
</tr>
<tr>
<td>500</td>
<td>0.007</td>
<td>0.002</td>
</tr>
</tbody>
</table>
quid-containing algae was probably the highest value with this method. A significant relationship was found between the MW irradiation time and the BCE from 0 to 350 sec (Pearson’s correlation test, $r = 0.97$, $p < 0.01$, $n = 5$) (Fig. 4). The slope indicated the conversion rate from TAG to FAME as 0.20% sec$^{-1}$.

The present study showed that the MWR with the closed circulation system effectively converted from TAG to FAME of wet algal lipids. The closed circulation system used in this study is thought to be more advantageous for large volume manufacture of BDF than the conventional batch-type reactors, although the conversion efficiency from TAG to FAME has to be improved using more suitable biomass-catalyst ratios with wet biomass.

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