Optimization of Hydrolysis and Esterification for Biodiesel Production from Wet Microalgae

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Optimization of Hydrolysis and Esterification for Biodiesel Production from Wet Microalgae

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Contents

Summary

It has become obvious that continued dependence on fossil fuel is unsustainable because of global warming by greenhouse gas emission and the future depletion of fossil fuel. Development of renewable energy has attracted much interest for energy sustainability. Biodiesel is a renewable fuel which is produced from oils derived from plants, animals or microbes. It is non-toxic and biodegradable, and has lower emission of greenhouse gas when burned in diesel engine. Various methods such as transesterification, blending, cracking, microemulsification and pyrolysis have been developed to convert oil into biodiesel which is comparable to diesel fuel. Transesterification is the most common method for the production of biodiesel and consists of a number of consecutive reversible reactions. Triglyceride is converted stepwise to diglyceride, monoglyceride and finally glycerol and a mole of fatty acid methyl ester (FAME) named as biodiesel is liberated at each step. Generally, alcohol and catalyst are needed for transesterification of oil.

Microalgae are unicellular microscopic (2–200 μm) autotrophic organisms which grow by photosynthesis and are the eukaryotic representatives, though the prokaryotic cyanobacteria are often included in algae. Some species contain more than 70% lipid

(dry weight basis). They also grow extremely rapidly under optimal conditions and their growth rates are 100 times faster than terrestrial plants. Oil yield of microalgae containing 70% oil content is 58,700 L/ha year and much higher than other crops (e.g., soybean 446 L/ha year and palm 5950 L/ha year). In addition, microalgal cultivation does not encroach on arable land suitable for food production.

The extraction of lipid from microalgae and their conversion into biodiesel in a single step would be highly valuable as it will bypass the use (and cost) of large quantities of organic solvents. However, such direct transesterification approach has an issue, namely that the existence of water inhibits the reaction. In our study, hydrolysis of lipid to free fatty acid (FFA) from microalgae under high water content was investigated as a pretreatment of direct esterification. Results indicated that the hydrolysis process reduced the inhibition by water in FAME production. Also, FAME obtained by esterification of hydrolysates was increased by 181.7% compared to FAME obtained by direct transesterification under the same amount of water content (80%). Therefore, it was confirmed that hydrolysis process can reduce the negative effect of water on biodiesel production from wet microalgae.

In addition, hydrolysis of wet microalgal lipid to FFA followed by esterification of FFA using acid in one-step process was investigated. The investigation of simultaneous

hydrolysis-esterification (SHE) of wet microalgal lipid was conducted by using L27 orthogonal design and the effects of water content, volume of sulphuric acid, volume of methanol, temperature and time on SHE were examined. As a result, water content was found to be the most effective factor. The effects of various parameters on FAME content and equilibrium relation between FAME and FFA were also examined under water content 80%. Equimolar amounts of sulphuric acid and hydrochloric acid showed similar results. When two-step and simultaneous processes were compared, total reaction time in the two-step process was found to be faster than that seen in the simultaneous process. These methods have great potential in terms of biodiesel production from microalgae since no organic solvents are used, simultaneously reducing the drying cost and lowering the operating cost compared to other traditional methods.

Chapter 1 Background

1. Introduction

Climate change is the most critical global environmental problem. The potential threat of global climate change has increased, and much of this risk has been attributed to greenhouse gas (GHG) emissions by fossil fuel usage. It has become necessary to develop techniques and adopt policies to minimize impacts of global warming which results from the increase in anthropogenic GHG emissions. In 1997, the Kyoto Protocol called for a 5.2% reduction in GHG emissions from 1990 (Wang et al., 2008) and various technologies has been investigated to meet the value. Another problem is the future energy crisis due to depletion of fossil fuels. As shown in Fig. 1-1 (Ahmad et al., 2011), world energy consumption has been gradually increasing. Also, Fig. 1-2 shows energy consumption of each country in 2005 (Saito, 2010). The continuous use of fossil fuels as a primary source of energy is widely recognized to be unsustainable. Therefore, it is absolutely necessary to ensure new energy resources before the world will be confronted with an energy crisis. Currently, various technologies to allow substitution of fossil fuel with renewable energy have been developed.

Biodiesel has been widely known as one of alternative fuel. It can be produced from oils derived from plants, animals or microbes (Graboski and McCormick, 1998), which represents 82% of total biofuel production (Bozbas, 2008). Some methods are currently available and have been adopted for the production of biodiesel fuel. There are four primary ways to produce biodiesel (Table 1-1). Especially, transesterification, which produces the monoalkyl ester of long-chain fatty acid is the most general method. Transesterification consists of a number of consecutive reversible reactions (Freedman et al., 1986). Triglyceride (TAG) is commonly reacted with methanol and is converted stepwise to diglyceride, monoglyceride and finally glycerol as shown in equation (1) and (2). A mole of fatty acid methyl ester (FAME) is liberated at each step.

$$\begin{aligned}
&\text{Triglyceride} + \text{Methanol} \leftrightarrow \text{Diglyceride} + \text{Fattyacid methylester} \\
&\text{Diglyceride} + \text{Methanol} \leftrightarrow \text{Monoglyceride} + \text{Fattyacid methylester} \\
&\text{Monoglyceride} + \text{Methanol} \leftrightarrow \text{Glycerol} + \text{Fattyacid methylester}
\end{aligned} \quad (1)$$

$$\begin{array}{lcl}
\begin{array}{l} CH_2-COOR_1 \\ | \\ CH-COOR_2 \\ | \\ CH_2-COOR_3 \end{array} & + & \begin{array}{l} CH_3OH \\ \\ CH_3OH \\ \\ CH_3OH \end{array} \\
\text{(Triglyceride)} & & \text{(Methanol)}
\end{array}
\leftrightarrow
\begin{array}{lcl}
\begin{array}{l} CH_3-COOR_1 \\ \\ CH_3-COOR_2 \\ \\ CH_3-COOR_3 \end{array} & + & \begin{array}{l} CH_2-OH \\ | \\ CH-OH \\ | \\ CH_2-OH \end{array} \\
\text{(Fattyacid methylester)} & & \text{(Glycerol)}
\end{array}
\quad (2)$$

There are some reasons chosen as an attractive renewable energy. Biodiesel is biodegradable and less toxic. Also, it can be used in existing diesel engines with little or no modification (Demirbas, 2002). Furthermore, it can be blended in any ratio with traditional petroleum-based diesel fuel (Peterson, 1991). On the other hand, there are some drawbacks in the current biodiesel production technology. The cultivation area of crops for biodiesel production is growing with the increased biodiesel production. Increasing demand for biodiesel crop cultivation may result in the destruction of natural habitats. Additionally, the increment of the use of vegetable oil for biodiesel production accelerates the competition with food production. Therefore, new alternative feedstock is eagerly anticipated to meet the biodiesel production demand.

2. Microalgae as a biodiesel feedstock

2.1. Microalgal biology

Microalgae are prokaryotic or eukaryotic thallophytes, have no sterile covering of cells around the reproductive cells and have chlorophyll *a* as photosynthetic pigment (Lee, 1980). Microalgae are present in a wide range of environmental conditions and it is estimated that more than 50,000 species exist (Richmond, 2004). Prokaryotic cells (cyanobacteria, blue-green algae) lack membrane-bound organelles and are more similar

to bacteria rather than algae. On the other hand, eukaryotic cells have these organelles that control the functions of the cell (Brennan and Owende, 2010). Eukaryotes are categorized into a variety of classes mainly defined by their pigmentation, life cycle and basic cellular structure (Khan et al., 2009). The most important classes include green algae, red algae and diatoms. Algae can either be autotrophic or heterotrophic. Some algae are mixotrophic (Lee, 1980). The biosynthesis route of TAG in microalgae may consist of the following three steps: (a) the formation of acetyl coenzyme A (acetyl-coA) in the cytoplasm; (b) the elongation and desaturation of the carbon chain of fatty acid; and (c) the biosynthesis of TAG (Huang et al., 2010). In general, l-α-phosphoglycerol and acetyl-coA are two major elements required for the biosynthesis of TAG. Microalgae form and accumulate more TAG, which is the main lipid, under stress conditions. Lipid accumulation begins when microalgae exhaust nitrogen from the medium. Also, the excess supply of carbon is assimilated by the cells and is converted into TAG. Synthesized lipid was stored within their cells which can no longer divide (Meng et al., 2009). TAG does not perform a structural role but serve as a carbon energy source.

2.2. Microalgal advantages

There are many advantages to using microalgae as a biodiesel feedstock. The most important point is their higher growth rates and oil productivity compared to conventional crops (Minowa et al, 1995). Generally, microalgae have oil levels in the range of 20 to 50% by weight of dry biomass (Table 1-2), but higher productivities can be also reached (Mata et al., 2010). Microalgae commonly double their biomass within 24 h, and algal cells divide as frequently as 3.5 h during the exponential growth phase (Chisti, 2007). Table 1-3 shows comparison of some sources of biodiesel. Oil yield is 58,700 L ha^{-1} $year^{-1}$ for microalgae containing 30% oil by weight, compared with 636 L ha^{-1} $year^{-1}$ for soybean and 5366 L ha^{-1} $year^{-1}$ for palm (Mata et al., 2010). If microalgae contain 70% oil by weight, oil of 136,900 L ha^{-1} $year^{-1}$ can be produced. In terms of the cultivation area, microalgae are clearly advantageous because of their higher biomass productivity and oil yield. Also, microalgae do not compete for land with crops used for food production, fodder and other products (Huang et al., 2010). Microalgae can be grown in a number of environments that are unsuitable for growing other crops, such as fresh, brackish or salt water or non-arable lands (Patil et al., 2008). CO_2, which is essential to the autotrophic cultivation of microalgae, can be provided by industrial facilities such as power plants and boilers where the CO_2 concentration in emitted gases

may reach 15 % or more (Salih, 2011 and Zhaoa et al., 2011). Microalgae can also be used to treat wastewater. Microalgae have been shown to be efficient in nitrogen and phosphorus removal (Mallick, 2002), and the combination of microalgal cultivation with wastewater treatment can generate the environmental benefit. Furthermore, microalgae produce valuable co-products or by-products such as biopolymers, proteins, carbohydrates and residual biomass. They can be used as energy such as ethanol and methane by fermentation, and can be supplied as livestock feed and fertilizer. They can also produce a variety of chemical products such as pharmaceuticals and platform chemicals; highly unsaturated fatty acids such as docosahexaenoic acid (Molina Grima et al., 2003); proteins and carbohydrates, which can be used as gross nutrients (Knuckey et al., 2006); specific compounds such as pigments (Lorenz & Cysewski, 2000); or silica derived from diatom cell walls (Gordon et al., 2009).

3. Biodiesel production from microalgae

3.1. Traditional biodiesel production from microalgae

Fig. 1-3 shows flow diagram of biodiesel production from microalgae. In the cultivation step, it is important to consider different factors which influence algal growth: light, temperature, nutrient concentration, CO_2, pH, salinity, contamination and

so on. Cultivated microalgae are harvested and concentrated by sedimentation, centrifugation, flocculation or membrane filtration. Though centrifugation is often employed, the process energy is high. The development of more cost-viable and energy-efficient method is needed. After drying and cell disruption of harvested biomass are implemented as appropriate, it is subject to oil extraction step. There are three common methods to extract oil from microalgae: (1) expeller/press, (2) solvent extraction and (3) supercritical fluid extraction. Currently, the most popular extraction method is Soxhlet extraction using hexane as a solvent. Extracted oil is converted to biodiesel through transesterification. Finally, crude biodiesel become a biodiesel product after purification.

3.2. Direct transesterification of microalgal lipid

Currently, the most common biodiesel production method from microalgae is the extraction of lipid using organic solvents (e.g., hexanes, chloroform, and methanol), followed by the FAME generation from extracted lipid by transesterification. This results from the traditional method using terrestrial feedstock such as soybean or rapeseed. However, the extraction of oil from microalgal cells is prevented by their rigid cell walls. Mechanically crushing algal biomass to extract oil is not easy to be

performed using the existing crushing equipments. Also, life cycle analysis conducted on the process of biodiesel production from microalgae indicates that 90% of the process energy is consumed by oil extraction, indicating that any improvement in lipid extraction will have a significant impact on the economics of the process (Lardon et al., 2009). Therefore, the extraction of lipid from microalgae and its conversion into biodiesel in a single step would be highly valuable as it will bypass the use (and cost) of large quantities of organic solvent and the distillation cost to recovery solvent (Fig. 1-3). Alcohol can be simultaneously used for the extraction of oil and as acyl acceptor for transesterification. Such direct transesterification can simplify the fuel conversion process, potentially reducing the overall process cost, hence reducing the final fuel product costs as well. Direct transesterification has been used as an analytical technique to determine the fatty acid composition of lipid containing tissue (Lepage and Roy, 1984, Park and Goins, 1994, and Rodríguez-Ruiz et al., 1998). Also, it has been reported that direct method can result in greater FAME yield than the extraction followed by transesterification approach (Lepage and Roy, 1984, Siler-Marinkovic and Tomasevic, 1998, Lewis et al., 2000). Direct approach has been shown to be effective in making biodiesel from both pure (Johnson and Wen, 2009 and Vicente et al., 2009) and mixed cultures of microorganisms (Dufreche et al., 2007 and Mondala et al., 2009). In

addition, this method confirmed that TAG, free fatty acid (FFA) and phospholipid all contributed to the formation of FAME (Wahlen et al., 2011). Furthermore, process wastes and pollution could also be reduced by this method (Haas et al., 2007).

4. Various direct transesterification methods

Various catalysts are used in order to perform direct transesterification of microalgal lipid. Advantages and disadvantages of the main types of catalysts used for transesterification are listed in Table 1-4. In addition to them, new methods such as supercritical method and microwave-assisted transesterification have also been investigated. They are described in detail below.

4.1. Alkaline catalyzed method

There are few researches of direct transesterification using homogenous alkaline catalyst (Xu and Mi, 2011, Velasquez-Orta et al., 2012 and Velasquez-Orta et al., 2013). Many microalgae have high FFA contents. FFA reacts with alkaline catalyst to form soap, consumes the catalyst, and results in the low tranesterification reaction as shown in equation (3) (Al-Zuhair, 2007).

$$\underset{\text{(Free fatty acid)}}{R-COOH} + \underset{\text{(Potassium hydroxide)}}{KOH} \rightarrow \underset{\text{(Soap)}}{R-COOK} + \underset{\text{(Water)}}{H_2O} \quad (3)$$

Therefore, alkaline catalyst is usually not recommended for the direct tranesterification from microalgae. However, if microalgae have low FFA content, alkaline catalyst is the most suitable because transesterification proceeds faster than with acid catalyst. Alkaline catalysts are also less corrosive to equipment than acid catalysts (Freedman, 1986). Moreover, alkaline catalyst has a higher tolerance for water than acid catalyst (Kusdiana and Saka, 2004). Also, presumably as the sole paper of direct transesterification using heterogenous catalyst, Li et al. (2011) reported a maximum conversion of 28% on the direct transesterification of dried *Nannochloropsis* sp. by reacting microalgae with 10% of an alkaline heterogeneous catalyst (Mg-Zr) in the volume ratio of methanol to dichloromethane 2: 1 at 65 °C for 4 h.

4.2. Acid catalyzed method

Direct transesterification from microalgae has been mainly conducted using homogenous acid catalyst because of their high FFA content. Acid catalyst can convert FFA into FAME as opposed to alkaline catalyst as shown in equation (4) (Ehimen et al.,

2010).

$$\underset{\text{(Free fatty acid)}}{R-COOH} + \underset{\text{(Methanol)}}{CH_3OH} \rightarrow \underset{\text{(Fatty acid mety lester)}}{CH_3-COOR} + \underset{\text{(Water)}}{H_2O} \quad (4)$$

In fact, when Nagle and Lemke (1990) examined the effect of acid and alkaline catalysts for the conversion of microalgae oil, acid catalyst resulted in higher FAME yield than alkaline catalyst under the same reaction conditions. However, direct transesterification with acid catalyst is weak in the water existence. FAME yield was reduced with an increment in water content for direct transesterification using *Chlorella* biomass (Ehimen et al., 2010). FAME yield of 81.7 % was observed for the biomass with 0.7% water content, while only the yield of 19.5 % was for the biomass with 73 % water content.

4.3. Enzyme catalyzed method

As far as I know, the report of Tran et al (2013) is only direct method using enzyme catalyst. In the work, the direct conversion of the lipid in *Chlorella vulgaris* ESP-31 into FAME was performed using immobilized *Burkholderia* lipase as the catalyst after the microalgal biomass (water content of 86–91% and oil content 14–63%) was

pretreated by sonication to disrupt the cell walls. Lipase produced by an isolated strain *Burkholderia* sp. C20 was immobilized on hybrid nanomaterials (Fe_3O_4–SiO_2) grafted to a long chain alkyl group as supporters (Liu et al., 2012 and Tran et al., 2012). The immobilized lipase worked well with wet microalgal biomass. In this method, it is important that the microalgal biomass has a high lipid content to achieve over 90% biodiesel conversion with a lower biocatalyst loading and better lipase recycle efficiency.

4.4. Supercritical method

The catalyst free method has been recently developed by employing supercritical methanol (Demirbas, 2009). This process is conducted at high reaction temperature and pressure (Kusdiana and Saka, 2004). Supercritical methanol can form a single phase with lipids, in contrast to the two phases at the normal conditions. This can be achieved because the decrease in the dielectric constant of methanol at supercritical state. In such supercritical method, the reaction was achieved in a very short time within 2–4 min, and FFA and TAG can be simultaneously esterified and transesterified. Also, supercritical method has a possibility of an alternative process to reduce cost associated with drying microalgae as the reaction is not inhibited under high water content. When this method

was using wet algal biomass containing about 90 % water, 90% of FAME yield was reached (Patil et al., 2011a). Two-step process was also reported (Levine et al., 2010); in the first step, wet microalgal biomass reacts to hydrolyze intracellular lipid to FFA under subcritical water condition, and in the second step, the wet FFA rich solid are subjected to supercritical direct transesterification. However, as it now stands, this method may be disadvantageous due to the adverse process economics as well as safety concerns (Marchetti and Errazu, 2008).

4.5. Microwave- and ultrasound-assisted method

Microwave radiation is a non-ionizing radiation that influences molecular motions. A molecule with a dipole moment is sensitive to external electric fields. In microwave-assisted transesterification, methanol absorbs microwave radiation, redirecting its dipole quickly. This enables the destruction of the methanol-lipid interface (Patil et al., 2011b). The microwave transfers energy in an electromagnetic form and the oscillating microwave field tends to move continuously to polar ends of molecules or ions (Azcan and Danisman, 2008). Consequently, collisions between the moving molecules produce heat (Marra et al., 2010) and it can shorten reaction time (Lidstrom et al., 2001). Ultrasonic technology is also an effective method to enhance

mass transfer rate between immiscible phases (Pan et al., 2002). This high frequency sound wave compresses and stretches the molecular spacing of media in which it passes through, and these molecules remain continuously vibrating with the formation of fine micro-bubbles or micro-cavities (Ji et al., 2006 and Lam et al., 2010). Generally, homogenous alkaline catalysts have been used in these approaches.

5. Conclusions

Biodiesel production from microalgal lipids holds great potential for a new energy industry because some microalgae have high productivity of biomass and oil. There are mainly two approaches which produce FAME from lipids such as TAG and FFA; one is the organic solvent–extraction of lipid from microalgae followed by transesterification of the lipid extracts, and the other is direct transesterification from microalgal biomass. The former has the disadvantage of the increased cost caused by using organic solvent. On the other hand, the latter considerably can reduce the process energy because it produces biodiesel without the need for organic solvent. In addition, other research showed that direct transesterification could convert phospholipid into FAME as well as TAG and FFA. Therefore, direct transesterification should be considered as a promising biodiesel production process from microalgae. There are many approaches of direct

transesterification as mentioned above. It is considered that the important keys to commercialization are the process energy. For the future, further researches are desired in order to develop the cost-effective technology.

Table 1-1. Different methods of biodiesel production (Leung et al., 2010)

Methods	Definition	Advantage	Disadvantage	Problems of using in engines
Direct use and blending	Direct use as diesel fuel or blend with diesel fuel	Liquid nature-portability	Higher viscosity	Coking and trumpet formation
		Heat content (~80% of diesel fuel)	Lower volatility	Carbon deposits
		Readily available; renewability	Reactivity of unsaturated hydrocarbon chains	Oil ring sticking; thickening and gelling of the lubricating oil
Micro-emulsions	A colloidal equilibrium dispersion of optically isotropic fluid microstructures with dimensions	Better spray patterns during combustion	Lower cetane number	Irregular injector needle sticking; incomplete combustion
	generally in the 1–150 nm range formed spontaneously from two immiscible liquids and one or more ionic or non-ionic amphiphiles	Lower fuel viscosities	Lower energy content	Heavy carbon deposits; increase lubrication oil viscosity
Thermal cracking (pyrolysis)	The conversion of long-chain and saturated substance (biomass basis) to biodiesel by means of heat	Chemically similar to petroleum-derived gasoline and diesel fuel	Energy intensive and hence higher cost	–
Transesterification	The reaction of a fat or oil with an alcohol in the presence of catalyst to form esters and glycerol	Renewability; higher cetane number; lower emissions; higher combustion efficiency	Disposal of by-product (glycerol and waste water)	–

Table 1-2. Lipid content and productivities of different microalgae species (Mata et al., 2010).

Marine and freshwater microalgae species	Lipid content (% dry weight biomass)
Ankistrodesmus sp.	24.0–31.0
Botryococcus braunii	25.0–75.0
Chaetoceros muelleri	33.6
Chlorella emersonii	25.0–63.0
Chlorella protothecoides	14.6–57.8
Chlorella vulgaris	5.0–58.0
Chlorella sp.	10.0–48.0
Chlorococcum sp.	19.3
Crypthecodinium cohnii	20.0–51.1
Dunaliella salina	6.0–25.0
Dunaliella primolecta	23.1
Dunaliella tertiolecta	16.7–71.0
Dunaliella sp.	17.5–67.0
Ellipsoidion sp.	27.4
Euglena gracilis	14.0–20.0
Haematococcus pluvialis	25.0
Monallanthus salina	20.0–22.0
Nannochloris sp.	20.0–56.0
Nannochloropsis oculata.	22.7–29.7
Nannochloropsis sp.	12.0–53.0
Neochloris oleoabundans	29.0–65.0
Nitzschia sp.	16.0–47.0
Oocystis pusilla	10.5
Pavlova lutheri	35.5
Phaeodactylum tricornutum	18.0–57.0
Scenedesmus obliquus	11.0–55.0
Scenedesmus quadricauda	1.9–18.4
Scenedesmus sp.	19.6–21.1
Skeletonema costatum	13.5–51.3
Spirulina platensis	4.0–16.6
Thalassiosira pseudonana	20.6
Tetraselmis suecica	8.5–23.0

Table 1-3. Comparison of microalgae with other biodiesel feedstocks (Mata et al., 2010).

Plant source	Oil content (% dry weight biomass)	Oil yield (L oil/ha year)	Land use (m^2 year/kg biodiesel)
Corn	44	172	66
Hemp	33	363	31
Soybean	18	636	18
Jatropha	28	741	15
Camelina	42	915	12
Canola/Rapeseed	41	974	12
Sunflower	40	1070	11
Castor	48	1307	9
Palm oil	36	5366	2
Microalgae (low oil content)	30	58,700	0.2
Microalgae (medium oil content)	50	97,800	0.1
Microalgae (high oil content)	70	136,900	0.1

Table 1-4. Advantages and disadvantages of the main types of catalysts used for transesterification (Atadashi et al., 2013)

Type of catalyst	Example	Merits	Demerits
Homogeneous catalyst			
Alkaline catalysts:	NaOH, KOH, CH_3ONa, CH_3OK	Less corrosive, high reaction rate	Formation of saponified product, emulsion formation, high water and energy consumption, huge, high wastewater discharges, high purification cost. Feedstocks are limited to 0.5 wt FFAs, not recycle
Acid catalysts:	H_2SO_4	Zero soap formation, the catalyst can be used to catalyze both esterification and transesterification simultaneously	More waste as a result of neutralization, recycling difficulty, high purification cost, energy consuming, low reaction rates
Heterogeneous catalysts			
Solid alkaline catalysts and solid acid catalyst	MgO, CaO, ZnO KOH/NaY, CaO/MgO, Al_2O_3–SnO, KOH/K_2CO_3, Al_2O_3–ZnO, $Ca(NO_3)_2/Al_2O_3$, CaO/Al_2O_3, KOH/Al_2O_3, Al_2O_3/KI $Sr(NO_3)_2$/ZnO, ZrO_2/SO_4^{2-} TiO_2/SO_4^{2-}. ETS-10 zeolite, zeolite HY, and zeolite X	Environmentally friendly, easily ecycle, less discharges, less separation difficulty, high purity glycerol, lower cost of separation Insignificant leaching of CaO/Al_2O_3	Leaching effects, catalysts preparation is complicated and expense relatively slow rates
Enzymes catalysts:	*Candida antarctica* B lipase, Rhizomucor meihei lipase, candida rugosa Pseudonas cepacia, M. meihei (Lypozyme), M. meihei (Lypozyme IM60), Aspergillus niger.	Zero saponification products nonpolluting, easily separable, lesser separation cost, high purity glycerol and biodiesel products, environmentally benign	Catalysts inhibition by water
	P. fluorescens, R. Oryzae	Simple glycerol recovery	High cost of enzymes

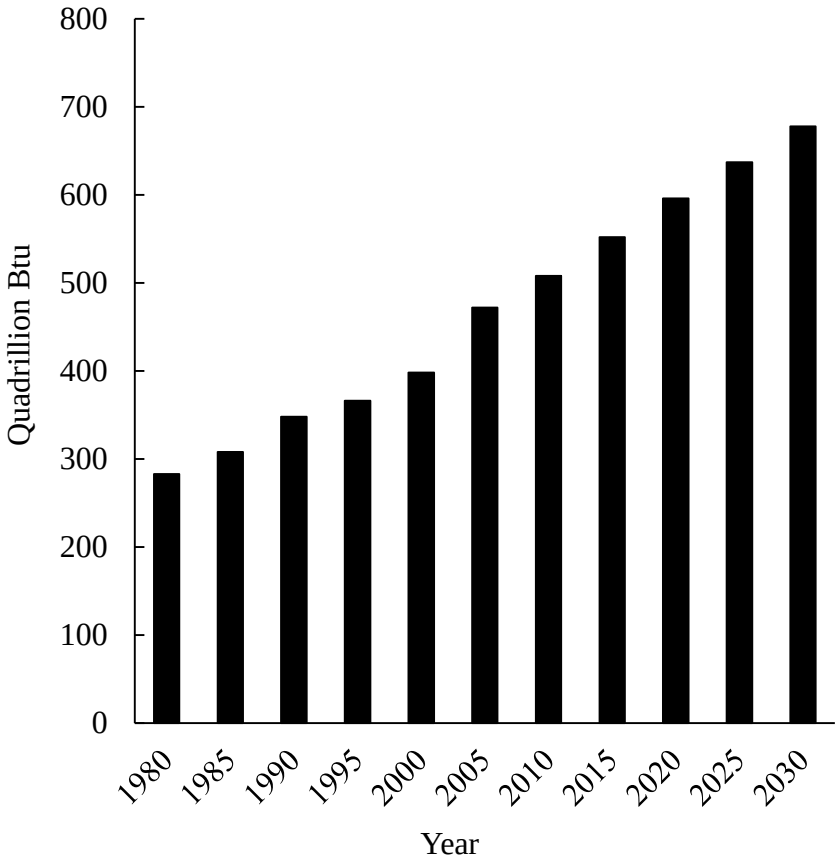

Fig. 1-1. World marketed energy consumption (Ahmad et al., 2011).

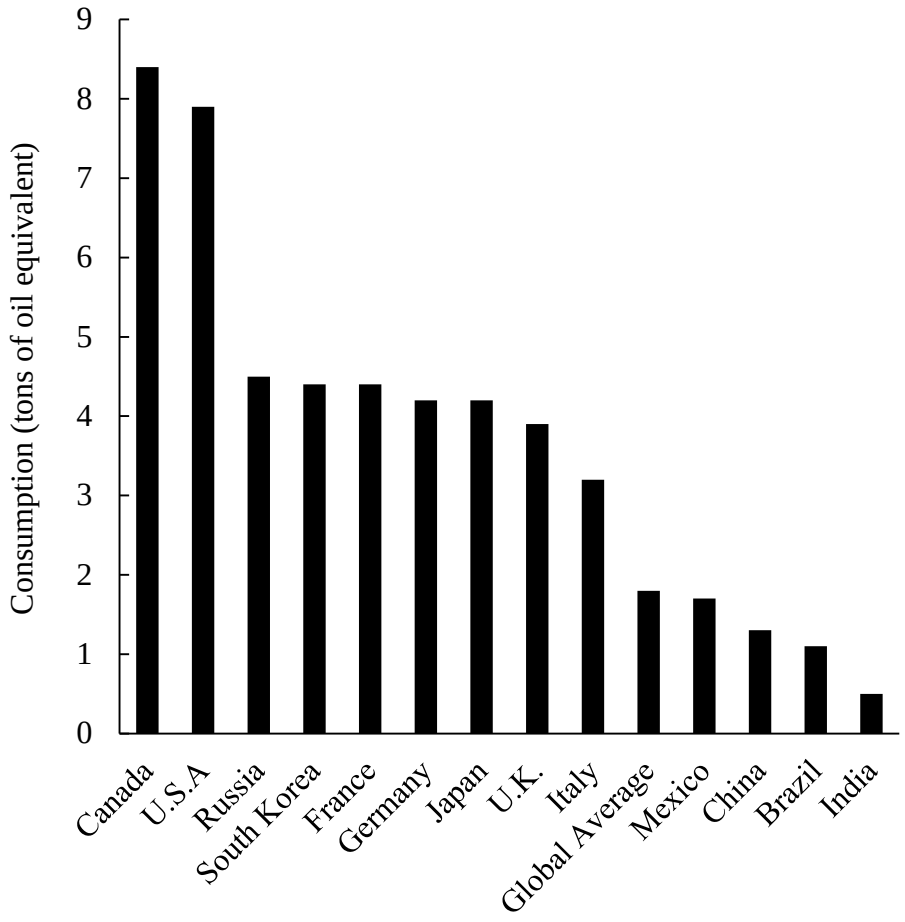

Fig. 1-2. Energy consumption of each country (Saito, 2010).

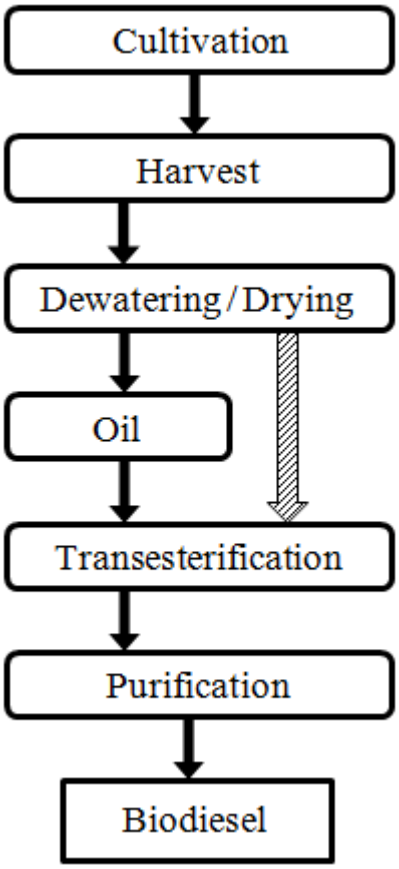

Fig. 1-3. Common biodiesel production procedure from microalgae.

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Chapter 2 Two-step hydrolysis and esterification from wet microalgae

1. Introduction

Biodiesel is a renewable fuel, which can be generated from oils derived from plants, animals or microbes (Graboski and McCormick, 1998 and Leung et al., 2010). A major problem in the growth of the biodiesel industry is supply and price of feedstock (Greenwell et al., 2010). Particularly, biodiesel production from microalgal lipids holds great potential for a new energy industry because some microalgae have high productivity of biomass and oil (Hu et al., 2008 and Williams and Laurens, 2010).

As shown in Fig. 2-1, there are mainly two approaches which produce FAME from lipids such as TAG, FFA, etc.; one is the organic solvent–extraction of lipids from microalgae followed by transesterification of the lipid extracts (Nagle and Lemke, 1990) and the other is direct transesterification from microalgal biomass (Liu and Zhao, 2007 and Johnson and Wen, 2009). The former has the disadvantage of the increased cost caused by using organic solvent. Lardon et al. (2009) reported that life cycle analysis on the biodiesel production process from dry microalgae indicated that 90% of all the process energy is consumed by the extraction process (70% when considering the wet extraction). On the other hand, the latter considerably can reduce the process energy

because it produces biodiesel without the need for organic solvent. In addition, other research showed that direct transesterification could convert phospholipids into FAME as well as TAG (Vicente et al., 2009). Therefore, direct transesterification should be considered as a promising biodiesel production process from microalgae.

Acid catalysts are typically used in direct transesterification processes because of their inability to form soap, unlike alkali catalysts. However, the direct transesterification using acid catalysts has a problem that water inhibits the reaction (Ehimen et al., 2010 and Wahlen et al., 2011). According to Kusdiana and Saka (2004), supercritical transesterification is the method where the influence of water on FAME yield is insignificant. When they investigated the effect of water content (0−5%) using rapeseed oil under supercritical conditions (350 °C, 43 MPa for 4 min) treatment with a molar ratio of 42 in methanol, the amount of water wasn't found to have any significant effect on the conversion. Additionally, supercritical or subcritical hydrolysis followed by esterification with wet microalgae (water content 80−90%) was also investigated (Levine et al., 2010, Patil et al., 2011 and Tsiqie et al., 2012). However, according to Marchetti and Errazuthis (2008), while this method has the benefit of no catalyst required, it may be disadvantageous due to the adverse process economics as well as safety concerns related to the reaction conditions.

Sathish and Sims (2012) reported wet lipid extraction from wet algal biomass (84% water content) via acid and base hydrolysis at 90 °C. However, two acid processes and one alkali process are required in this method. Besides, it leaves much room for discussions about whether hydrolysis was actually accomplished. Therefore, in our study, the detailed investigation of the effect of hydrolysis followed by esterification with methanol using acid as a catalyst was conducted to explore a new process to reduce inhibition by high water content (70−90%) in FAME production. First, the difference in the inhibition of FAME yield by adding water in TAG and FFA was examined, and then the hydrolysis condition of wet microalgal lipids was investigated. Finally, the effect of hydrolysis on FAME yield was investigated.

2. Material and Methods

2.1. Materials

Palmitic acid (95%, Wako Pure Chemical Industries, Ltd., Japan) and tripalmitin (99%, Acros Organics, USA) were used as feeding material in the experiment for the effect of water on transesterification and esterification. For the following experiments, commercially available dried *Chlorella* powder was provided by Natural Health Inc., Japan. The powder was dried at 80 °C for 24 h and stored at −20 °C.

Distilled water was added into microalgal powder in order to reproduce harvested and concentrated microalgae where microalgal water content was assumed as 70−90%. Methanol (99.8%), sulphuric acid (95%) and hexane (96%) were purchased from Wako Chemical Industries Ltd. (Japan). Hexane was used to extraction FFA and FAME in our laboratory experiments. However, it could not be used as a solvent at large scale because FAME becomes partitioned into the upper layer.

2.2. Experimental procedures

2.2.1. Effect of water on transesterification and esterification

Palmitic acid or tripalmitin (10 mg) was mixed with various volumes (0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 mL) of water and 2 mL of methanol containing 2% sulphuric acid in a test tube. Each tube was sealed using PTFE lined screw cap. Transesterification and esterification reactions were performed at 120 °C for 1 h. After the reaction, 4 mL of hexane was added to the reaction mixture and vortexed. Each tube was centrifuged at 3000 rpm for 5 min to accelerate phase separation. The FAME in hexane was analysed.

2.2.2. Orthogonal experiment

The experiments using a L_{27} orthogonal array were implemented to inspect the effects

of different parameters on hydrolysis of lipids from microalgae. Microalgal powder (0.3 g) was mixed with water and sulphuric acid in a test tube. Each tube was sealed using PTFE lined screw cap. After the hydrolysis, 3 mL of hexane was added to the reaction mixture and vortexed. After the tube was centrifuged at 3000 rpm for 5 min, the hexane layer was removed. Further extractions with hexane were performed twice in the remaining water layer and FFA in the hexane was analysed.

As the conditional factors of the orthogonal experiment, temperature, water content, volume of sulphuric acid and time were chosen. Each factor was given at three levels as shown in Table 2-1 and these factors and levels were assigned to the L_{27} orthogonal design as shown in Table 2-2. The extra columns were used to represent an experimental error. The test runs were performed in a random order. Data analysis for the investigation of the optimal hydrolysis condition was carried out through the range analysis and analysis of variance (ANOVA).

2.2.3. Effect of temperature and volume of sulphuric acid on hydrolysis

For investigating the optimal hydrolysis temperature, 0.3 g of microalgal powder was mixed with 1.2 mL of water and volume of sulphuric acid 200 mL/kg-dry algae in a test tube and each tube was sealed using PTFE lined screw cap. Hydrolysis was performed

at 120, 130, 140, 150 and 160 °C, respectively. Also, for investigating the optimal hydrolysis volume of sulphuric acid, 0.3 g of microalgal powder was added with 1.2 mL of water and volume of sulphuric acid 100, 200, 300 and 400 mL/kg-dry algae in a test tube and the hydrolysis was performed at 140 °C. After the reaction, 3 mL of hexane was added to the reaction mixture, and the resulting mixture was vortexed. After the tube was centrifuged at 3000 rpm for 5 min, hexane layer was removed. Further extractions with hexane were performed twice in the remaining water layer and FFA in the hexane was analysed.

2.2.4. Maximum FAME content

Dry microalgal powder (0.3 g) was mixed with 4 mL of methanol containing 2% sulphuric acid in a test tube. Each tube was sealed using PTFE lined screw cap and the microalgal lipids were transesterified at 80 °C for 6 h. After the transesterification, 3 mL of hexane was added to the reaction mixture and vortexed. After the tube was centrifuged at 3000 rpm for 5 min, the hexane layer was removed. Further extractions with hexane were performed twice in the remaining water layer and FAME in the hexane was analysed.

2.2.5. The effect of hydrolysis on FAME yield

FAME content of the sample obtained by esterification of FFA after hydrolysis of lipids from microalgae was compared to that of samples obtained by the direct transesterification under the same amount of water content to examine the influence of hydrolysis. In the control, 0.3 g of microalgal powder was mixed with 1.2 mL of water, volume of sulphuric acid 300 mL/kg-dry algae and 4 mL of methanol in a test tube, and direct transesterification was performed at 80 °C for 1 h. In Run 1, direct transesterification without the addition of water was implemented. In Run 2, 4 mL of methanol (wet algae to methanol (wt./vol.) ratio of 1: 2.67) was added to hydrolysates and esterified at 80 °C for 1 h after the hydrolysis with water and sulphuric acid at 140 °C for 30 min. After the reaction, 3 mL of hexane was added to the reaction mixture and vortexed. After the tube was centrifuged at 3000 rpm for 5 min, the hexane layer was removed. Further extractions with hexane were performed twice in the remaining water layer and FAME in the hexane was analysed.

2.2.6. The effect of methanol volume on esterification of hydrolysates

After 0.3 g of dry microalgae powder was hydrolysed with 1.2 mL of water and sulphuric acid 400 mL/kg-dry algae in a test tube at 140 °C for 20 min, 2,4 or 6 mL of

methanol (methanol to wet algae (vol./wt.) ratio of 1.33, 2.67and 4) was added to hydrolysates and esterified at 80 °C. After the reaction, 3 mL of hexane was added to the reaction mixture and vortexed. After the tube was centrifuged at 3000 rpm for 5 min, the hexane layer was removed. Further extractions with hexane were performed twice in the remaining water layer and FAME in the hexane was analysed.

2.3. FAME and FFA analysis

To quantify FAME and FFA, the samples dissolved in hexane were mixed with an internal standard and analysed by gas chromatography (GC-2010, Shimadzu Corporation, Japan) with a 30 m length × 0.25 mm i.d. × 0.5 μm film thickness capillary column (Stabilwax, Restek Corporation, USA) and a flame ionisation detector. Operating conditions in FAME analysis were as follows: carrier gas N_2, injection temperature 250 °C, oven temperature 225 °C, detector temperature 250 °C and linear velocity 30 cm/s. Also, FFA analysis conditions were as: carrier gas N_2, injection temperature 250 °C, oven temperature 240 °C, detector temperature 250 °C and linear velocity 60 cm/s.

3. Results and Discussions

3.1. Effect of water on transesterification and esterification

Fig. 2-2 shows the effect of added water volume on FAME yield of transesterification and esterification, where FAME yield was assumed to be 100% without the added water. As added water volume was increased, esterification of FFA gave a higher FAME yield compared to transesterification of TAG. FAME yields of transesterification of TAG were 89.6, 64.7, 15.0 and 4.4% when added water volumes were 0.2, 0.4, 0.8 and 1.6 mL, respectively. FAME yields of esterification of FFA were 99.6, 91.1, 84.8 and 79.0% when added water volumes were 0.2, 0.4, 0.8 and 1.6 mL, respectively. This result was similar to the report by G. Lepage et al. (1984). When experimenters varied the amounts (10−30%) of water added to solutions of tripalmitin and palmitic acid in transesterification and esterification, respectively, FAME yield of tripalmitin were 98.9, 86.7 and 58.1% at 10, 20 and 30%, respectively and that of palmitic acid were 99.4, 94.8 and 91.8% at 10, 20 and 30%, respectively. In addition, they reported that the methylation of longer-chain fatty acids was inhibited by the addition of water. These results have been supported by experiments by Warabi et al. (2004), where supercritical transesterification of rapeseed oil and supercritical esterification of FFA were compared. It was found that esterification was faster than transesterification for all alcohols used

(methanol, ethanol, 1-propanol and 1-butanol). They provided two reasons: the first is that the esterification is easier to perform because FFA is more soluble in alcohol than TAG. The second is that transesterification of TAG includes three-step reactions, while esterification of FFA is s one-step reaction. TAG initially reacts with methanol to produce FAME and diglyceride, which then further reacts with methanol to generate FAME and monoglyceride. Finally, monoglyceride reacts with methanol to give FAME and glycerin. Monoglyceride is the most stable intermediate compound, and is considered as the step that determines the reaction rate. Meanwhile, in esterification of FFA, FFA only reacts with methanol to generate FAME and water. This difference in reaction steps likely corresponds to the difference in reaction rates. Also, it was considered that methylation of the longer-chain fatty acid in methanol was inhibited by the addition of water because of reduced solubility imparted by the carbon chain length of fatty acid.

This result suggests that the hydrolysis of TAG to FFA and the esterification reaction of FFA reduced the inhibition effect of water and enabled the higher FAME yield.

3.2. Investigation of effective parameters on hydrolysis with orthogonal experiment

The mean values of each experimental factor and the two-factor interactions are shown in Fig. 2-3. According to Fig. 2-3a, the optimal hydrolysis condition was as follows: temperature 140 °C, water content 80%, volume of sulphuric acid 200 mL/kg-dry algae and time 60 min. The ANOVA result is shown in Table 3. It shows that temperature was the most important factor with contributing ratio 26.76% (significant at 1% level), followed by time with contributing ratio 12.36% and volume of sulphuric acid with contributing ratio 11.38% (both significant at 5% level). As a single effect, water content between 70% and 90% did not affect the hydrolysis of lipids significantly. However, there were significant interactions of water content with temperature and volume of sulphuric acid at 5% level as shown in Fig. 2-3b.

Based on the orthogonal experiment results, the further detailed hydrolysis conditions need to be investigated. These experiments were conducted by varying temperature or volume of sulphuric acid and fixing other parameters.

3.3. Effect of temperature and acid on hydrolysis

In these experiments, water content was set at 80% because the effect of water content as a single variable was insignificant. Fig. 2-4 shows the time courses of

hydrolysis at various temperatures. FFA reached the maximum value at 140, 150 and 160 °C in 40, 20 and 10 min, respectively. This result suggests higher temperature provides faster hydrolysis. Also, FFA decreased after reaching the maximum value and higher temperature showed more reduction. This is because excess heating degrades FFA. Maximum FFA at 150 °C was lower than that at 160 °C. It can be considered that this is because the hydrolysis to FFA and the degradation of FFA simultaneously occurred with time. The time courses of hydrolysis using various volumes of sulphuric acid are shown in Fig. 2-5. FFA reached the maximum value at 200, 300 and 400 mL/kg-dry algae for 40, 30 and 20 min, respectively. It was found that more input of sulphuric acid shortened hydrolysis time and provided higher maximum FFA. FFA also decreased after reaching the maximum value. This result is thought to be due to the degradation of FFA by the addition of excess sulphuric acid. No further degradation of FFA in the enzymatic hydrolysis of soybean oil to FFA has been observed (Ting et al., 2006). Therefore, time, temperature and acid concentration must be controlled more carefully compared to enzymatic hydrolysis.

3.4. Esterification of hydrolysates

Esterification of hydrolysates was conducted to investigate the effect of the

hydrolysis process on FAME. Fig. 2-6 shows that FAME of Run 1 and Run 2 were increased by 135.4 and 181.7%, respectively, compared to the control. An increase of FAME in Run 1 is attributed to the fact that the reaction isn't inhibited by water, while the hydrolysis process resulted in a further increase of FAME as shown in Run 2. This indicates that esterification of wet hydrolysates is faster in FAME production than direct transesterification of dry microalgal lipids. Therefore, the positive effect of hydrolysis was confirmed.

In supercritical method, Saka and Kusdiana (2004) reported that the optimal condition in two step reactions was 270 ℃ and 20 min for hydrolysis and methyl esterification, respectively. Our method could be performed at much lower temperature.

FAME yields of Control, Run1 and Run2 were 26.5, 62.4 and 74.5%, respectively, compared to maximum FAME content (5.80%). Further investigation is necessary for the optimisation of the esterification of hydrolysates. Currently, the optimisation of esterification of hydrolysates is being investigated by varying parameters such as volume of methanol, temperature and time. In addition, it is important to recover methanol from the reaction mixture in terms of recycle. It could be considered that remaining heat used in hydrolysis and esterification must be utilized in the recovery of methanol. The utilization of remaining heat could save the recovery cost. The detailed

research focused on energy consumption must be performed.

3.5. Effect of methanol volume on esterification

Fig. 2-7 shows effect of methanol volume on esterification of hydrolysates. FAME conversion rates between the ratios of methanol to wet biomass 1.33 and 2.67 were similar. However, under the ratio of methanol to wet biomass 4, FAME conversion rate was faster and FAME almost reached equilibrium value for 1 h. this suggests that the increase of methanol volume reduces the negative effect of water and accelerates esterification of hydrolysates.

4. Conclusions

The effect of acid hydrolysis of lipids from microalgae on FAME production was investigated. This research showed that the hydrolysis process reduced the inhibition by water in FAME production and enabled an increase in FAME under high water content, which was increased by 181.7% compared to FAME obtained by direct transesterification under the same amount of water content. This method would reduce the drying cost which is a problem in industrial biodiesel production from microalgae. Currently, the optimisation of esterification of hydrolysates is being investigated.

Table 2-1. Factors and levels for the L_{27} orthogonal experiment.

Factors	Level 1	Level 2	Level 3
A: Temperature (°C)	100	120	140
B: Water content (%)	70	80	90
C: Volume of sulphuric acid (mL/kg-dry algae)	100	200	300
D: Time (min)	20	40	60

Table 2-2. L_{27} orthogonal design for the investigation of hydrolysis condition.

No.	A	B	A × B	A × B	C	A × C	A × C	B × C	D	e	B × C	e	e	FFA(%)
1	100	70	3	2	30	3	2	1	60	2	1	3	2	0.22
2	100	70	3	2	60	1	3	2	20	3	2	1	3	0.34
3	100	70	3	2	90	2	1	3	40	1	3	2	1	0.93
4	100	80	1	3	30	3	2	2	20	3	3	2	1	0.11
5	100	80	1	3	60	1	3	3	40	1	1	3	2	0.26
6	100	80	1	3	90	2	1	1	60	2	2	1	3	0.55
7	100	90	2	1	30	3	2	3	40	1	2	1	3	0.15
8	100	90	2	1	60	1	3	1	60	2	3	2	1	0.36
9	100	90	2	1	90	2	1	2	20	3	1	3	2	0.19
10	120	70	1	1	30	1	1	1	20	1	1	1	1	0.21
11	120	70	1	1	60	2	2	2	40	2	2	2	2	0.98
12	120	70	1	1	90	3	3	3	60	3	3	3	3	1.20
13	120	80	2	2	30	1	1	2	40	2	3	3	3	0.44
14	120	80	2	2	60	2	2	3	60	3	1	1	1	1.02
15	120	80	2	2	90	3	3	1	20	1	2	2	2	0.28
16	120	90	3	3	30	1	1	3	60	3	2	2	2	0.64
17	120	90	3	3	60	2	2	1	20	1	3	3	3	0.35
18	120	90	3	3	90	3	3	2	40	2	1	1	1	0.59
19	140	70	2	3	30	2	3	1	40	3	1	2	3	0.34
20	140	70	2	3	60	3	1	2	60	1	2	3	1	0.78
21	140	70	2	3	90	1	2	3	20	2	3	1	2	0.48
22	140	80	3	1	30	2	3	2	60	1	3	1	2	1.02
23	140	80	3	1	60	3	1	3	20	2	1	2	3	1.34
24	140	80	3	1	90	1	2	1	40	3	2	3	1	0.78
25	140	90	1	2	30	2	3	3	20	2	2	3	1	0.61
26	140	90	1	2	60	3	1	1	40	3	3	1	2	0.75
27	140	90	1	2	90	1	2	2	60	1	1	2	3	0.90

Table 2-3. ANOVA analysis of the orthogonal experiment.

Factors	Variance ratios	Significances	Contributing ratios (%)
A: Temperature (°C)	20.31	**	26.76
B: Water content (%)	2.25		1.73
C: Volume of sulphuric acid (mL/kg-dry algae)	9.21	*	11.38
D: Time (min)	9.92	*	12.36
A×B	6.26	*	14.57
A×C	2.23		3.40
B×C	5.26	*	11.80
e: Error			18.01

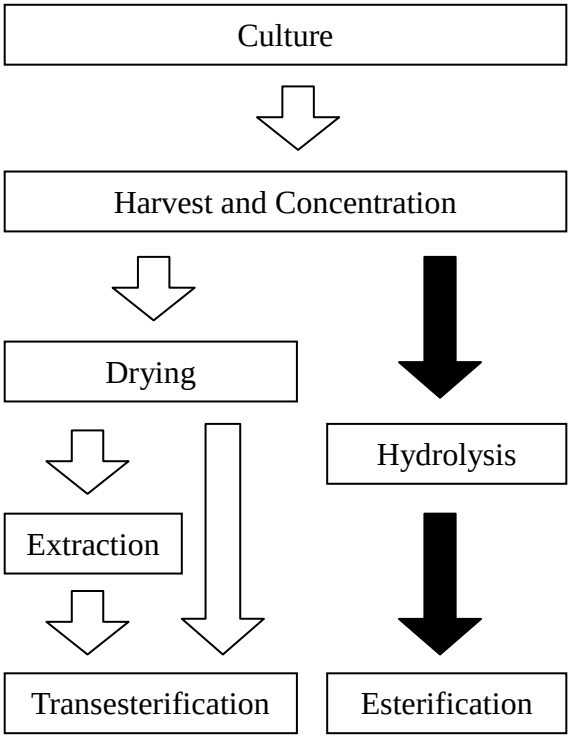

Fig. 2-1. Biodiesel production flow chart.

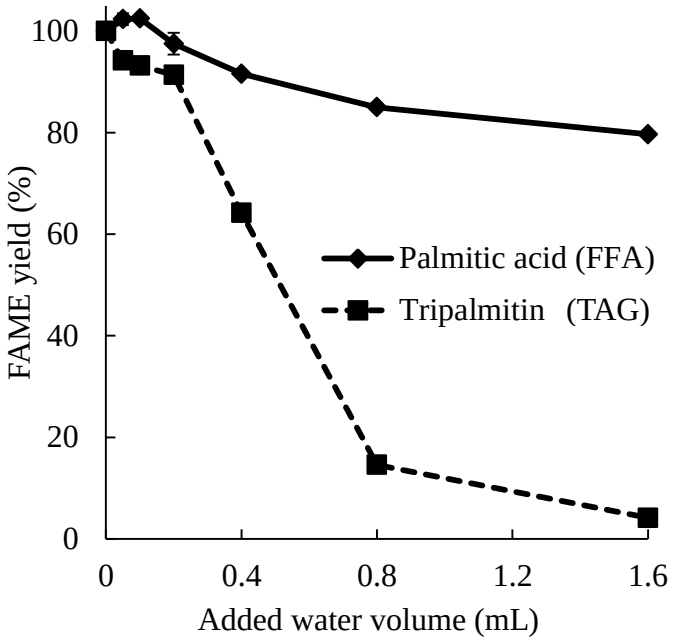

Fig. 2-2. Effect of water on transesterification and esterification.

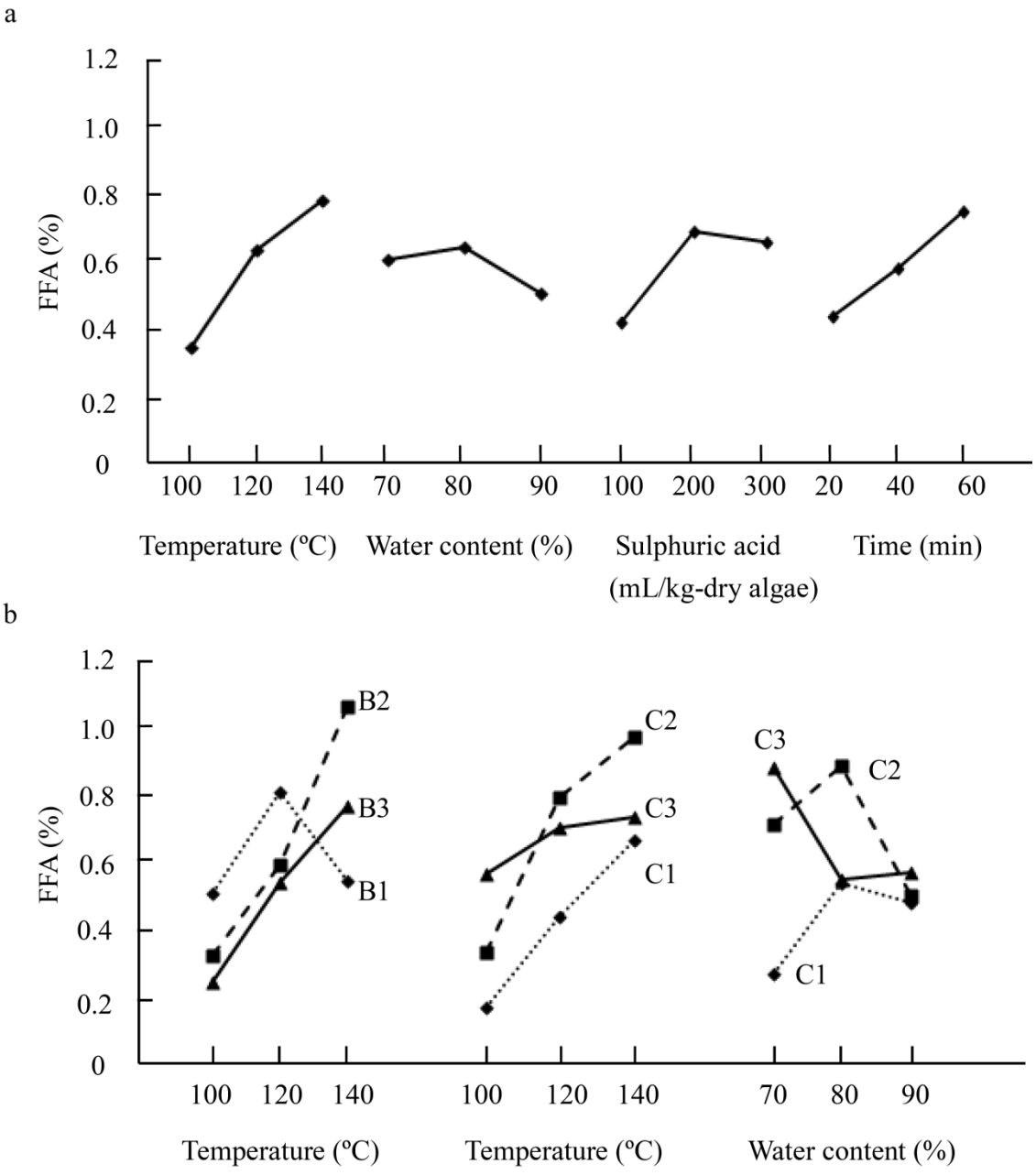

Fig. 2-3. Effect of each parameter on hydrolysis; a: Main effects. b: Interaction. B1, B2 and B3, water content 70, 80 and 90%; C1, C2 and C3, volume of sulphuric acid 100, 200 and 300 (mL/kg-dry algae)

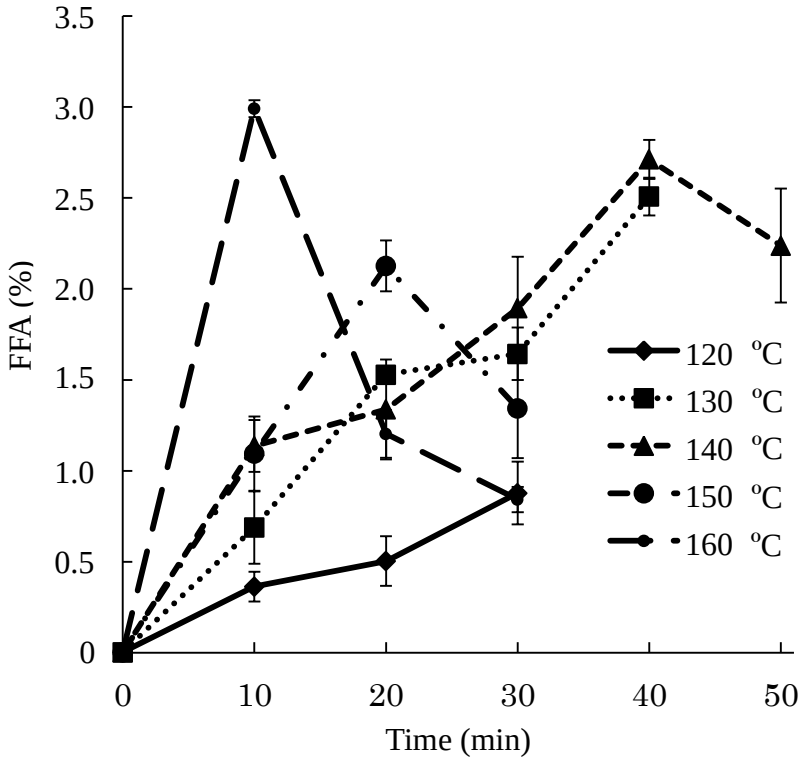

Fig. 2-4. Effect of temperature on hydrolysis. The reaction condition is as follows: microalgae 0.3 g, water content 80%, sulphuric acid 200 mL/kg-dry algae.

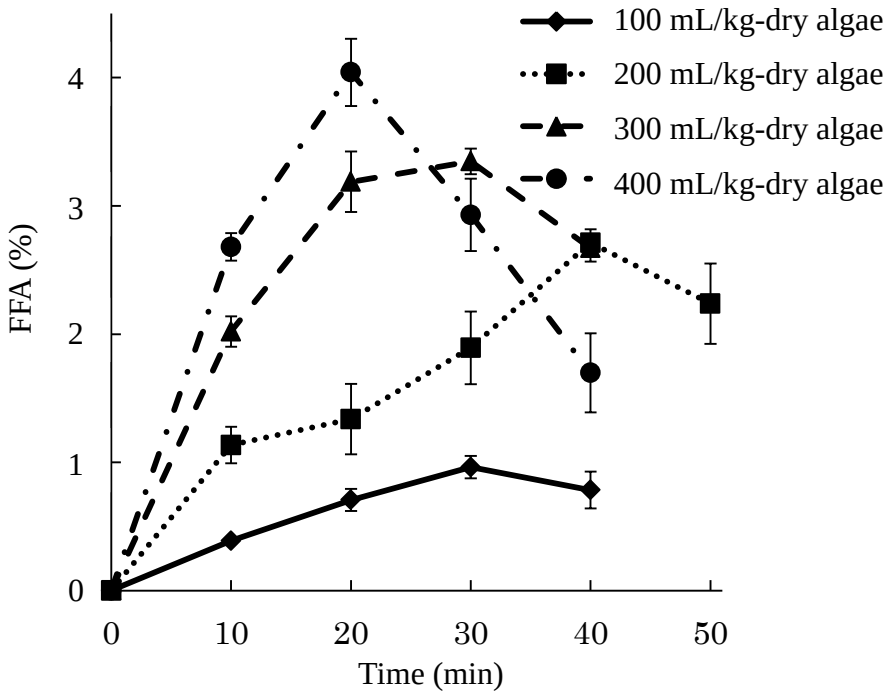

Fig. 2-5. Effect of sulphuric acid volume on hydrolysis. The reaction condition is as follows: microalgae 0.3 g, water content 80%, reaction temperature 140 °C.

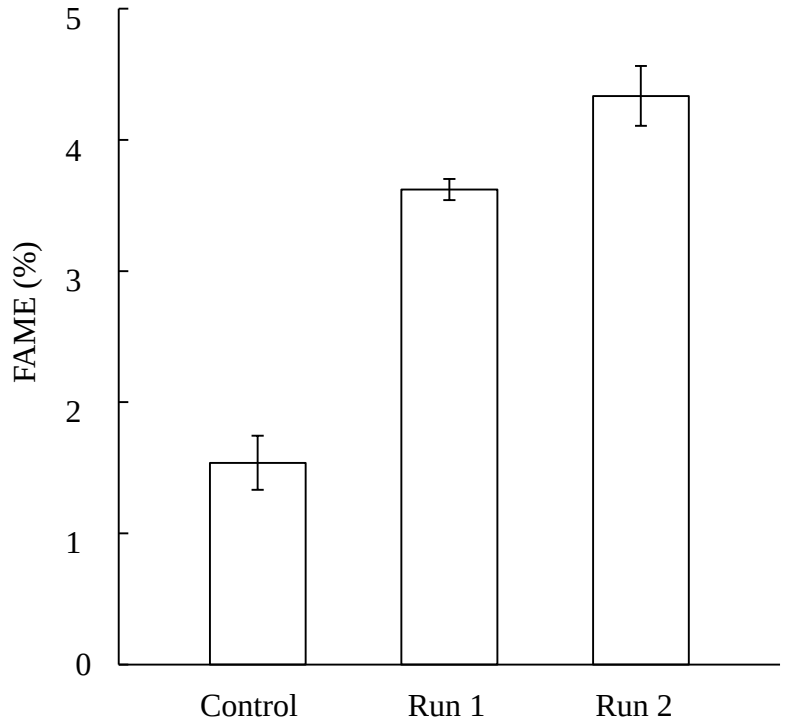

Fig. 2-6. Effect of hydrolysis on FAME yield. Control, direct transesterification of wet microalgal lipids; Run 1, direct transesterification of dry microalgal lipids; Run 2, esterification of wet hydrolysates.

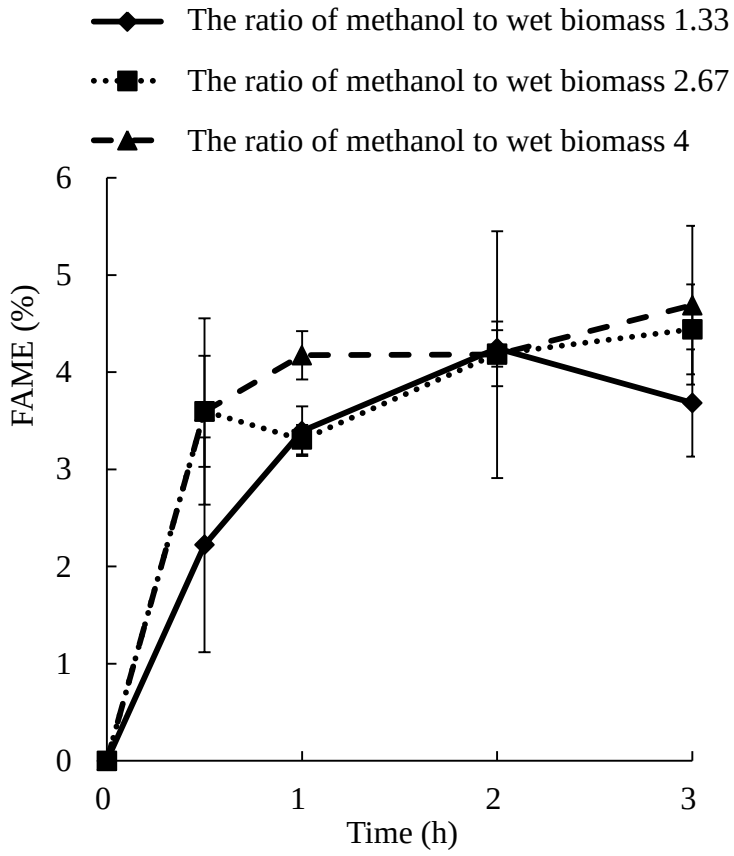

Fig. 2-7 Effect of methanol volume on esterification of hydrolysates. Microalgae powder (0.3 g) was hydrolysed with 1.2 mL of water and sulphuric acid 400 mL/kg-dry algae at 140 °C for 20 min. 2,4 or 6 mL of methanol (methanol to wet algae (vol./wt.) ratio of 1.33, 2.67and 4) was added to hydrolysates and esterified at 80 °C.

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Chapter 3 Simultaneous hydrolysis-esterification from wet microalgae

1. Introduction

Biodiesel is receiving much attention due to its potential as a viable alternative to fossil fuel. It can be produced from oils derived from plants, animals or microbes (Graboski and McCormick, 1998). Biodiesel is generated through various techniques such as direct/oil blends, microemulsion, pyrolysis and transesterification (Ma and Hanna, 1999). Especially, transesterification is the most common process for making biodiesel (Baroutian et al., 2008). Transesterification consists of a number of consecutive reversible reactions (Freedman et al., 1986). TAG is converted stepwise to diglyceride, monoglyceride and finally glycerol. A mole of FAME is liberated at each step.

Microalgae are considered as advantageous materials to produce biodiesel over other sources. Microalgae have higher growth rates of biomass and oil productivities than conventional crops because of their simple cellular structure (Becker, 1994) and have been claimed to be up to 20 times more productive per unit area than palm oil (Chisti, 2008). In addition, microalgae do not compete for land used for food production, fodder

and other products (Huang et al., 2010). Furthermore, microalgae can be grown in a number of environments that are unsuitable for growing other crops (Patil et al., 2008).

There are various methods which produce FAME from microalgal lipid such as oil extraction followed by transesterification, direct transesterification, supercritical method, and so on. Direct transesterification is typically the method which generates FAME from microbial biomass by using acid catalyst. It eliminates the need to extract and refine lipid before converting it to biodiesel, which could provide a reduction in the cost of biodiesel production (Haas and Wagner, 2011). In addition, direct transesterification can convert not only TAG but also other lipids such as phospholipid into FAME (Vicente et al., 2009). Therefore, direct transesterification is a promising biodiesel production process from microalgae. However, it has a problem that water inhibits the reaction (Ehimen et al., 2010 and Wahlen et al., 2011).

FFA is more resistant to the FAME production inhibition caused by water existence than TAG (Lepage and Roy, 1984). Hence, hydrolysis of lipid to FFA followed by esterification could be a valid method, which could reduce the inhibition and result in a decrease of the drying cost. There are some reports where hydrolysis followed by esterification of wet microalgal lipid was implemented under supercritical or subcritical condition. Two-step process from wet microalgal biomass was conducted by Levine et

al. (2010). In their report, wet microalgal biomass (water content 80%) reacted in subcritical water to hydrolyze intracellular lipid in the first step and the wet fatty acid-rich solids underwent supercritical in situ transesterification with ethanol to produce FAME in the second step. Simultaneous hydrolysis-esterification (SHE) was also achieved under supercritical or subcritical condition (Patil et al., 2011 and Tsigie et al., 2012). However, this method may be disadvantageous due to the adverse process economics as well as safety concerns (Marchetti and Errazu, 2008).

Only a few papers have dealt with hydrolysis followed by esterification using acid without application of pressure which is necessary for supercritical or subcritical condition. Wet lipid extraction from wet algal biomass (84%) via acid and base hydrolysis at 90 °C was performed by Sathish and Sims (2012). In our previous research, two-step hydrolysis followed by esterification was carried out (Takisawa et al., 2013). Acid hydrolysis provokes microalgal cell disruption and sugar extraction. When algal biomass was processed with physical methods (sonication, bead-beating, autoclaving and homogenization) and with physicochemical processes consisting of alkaline and acid hydrolysis (NaOH, HCl and H_2SO_4) in autoclave, acid process was the most effective hydrolysis method in all the processes (Miranda et al., 2012). In addition, when the effects of enzymatic hydrolysis and acid hydrolysis on ethanol fermentation

were compared, acid hydrolysis showed higher ethanol yield than enzyme hydrolysis (Ho et al., 2013). Thus, acid hydrolysis can give a positive effect on bioethanol production as well as biodiesel production. In this study, the optimisation of SHE of wet microalgal biomass using acid was performed.

2. Materials and methods

2.1. Materials

Hydrolysis is sensitive to microalgal cell wall. It is necessary to select the species with tough cell wall in order to hydrolyse any species cell. *Chlorella* is known to have rigid wall components embedded within a more plastic polymeric matrix (Gerken et al., 2013). This is why *Chlorella* was selected as a material in this study. Commercially available dried *Chlorella* powder was provided by Natural Health Inc., Japan. The powder was dried at 80 °C for 24 h and stored at −20 °C.

Distilled water was added into microalgal powder to reproduce harvested and concentrated microalgae where microalgal water content was assumed as 70−90%. Water content is shown on the basis of the weight of wet algal biomass (% (w/w)). Methanol (99.8%), ethanol (99.8), sulphuric acid (95%) and hexane (96%) were purchased from Wako Chemical Industries Ltd. (Japan). As the internal standard of

FAME and FFA, methyl pentadecanoate (>98%) and pentadecanoic acid (>98%) were bought from Tokyo Chemical Industry Co., Lid., Japan.

2.2. Experimental procedures

2.2.1. Maximum FAME content

Maximum FAME content was determined by reference to the method of Wahlen et al. (2011). Dry microalgal powder (0.3 g) was mixed with 4 mL of methanol containing 2% sulphuric acid in a test tube. Each tube was sealed using PTFE lined screw cap and the microalgal lipid was transesterified by using dry block bath (MG-2200, EYELA, Japan) at 80 °C for 6 h. After the transesterification, 3 mL of hexane was added to the reaction mixture and vortexed. After the tube was centrifuged at 3000 rpm for 5 min, the hexane layer was removed. Further extractions with hexane were performed twice in the remaining water layer and FAME in the hexane was analysed.

2.2.2. SHE with orthogonal experiment

The experiment using L_{27} orthogonal array was implemented to inspect the effects of various parameters on SHE of microalgal lipid. Microalgal powder (0.3 g) was added with water, sulphuric acid and methanol in a test tube. Microalgal lipid simultaneously

hydrolysed and esterified. After cooled at room temperature for 30 min, FAME extractions using hexane were performed as mentioned above and FAME was analysed.

Water content (Factor A), volume of sulphuric acid (Factor B), volume of methanol (Factor C), temperature (Factor D) and time (Factor E) were chosen as the conditional factors of the orthogonal experiment. Each factor was given at three levels as shown in Table 3-1. Water content was set at 70, 80 and 90%, which are the possible values of centrifuged microalgae. Volume of sulphuric acid and temperature are set by considering the report by Takisawa et al. (2013). Volume of methanol was set based on the report of Tsigie et al. (2012), where the optimum condition of the ratio of methanol to wet biomass is 4 (vol./wt.). All the factors and levels were assigned to L_{27} orthogonal design as shown in Table 3-2. The interactions between water content and other parameters (A×B, A×C, A×D and A×E) were examined as well as main effects. The test runs were performed in random order and data analysis was carried out through analysis of variance (ANOVA).

2.2.3. Investigation of effect of water content on SHE

Further investigation of effect of water content was performed, which was the most effective parameter. Microalgal powder (0.3 g) was added with 1.2 mL of water, volume

of sulphuric acid 200 mL/kg-dry algae and methanol in the ratio of methanol to wet biomass 1.33 (vol./wt.). SHE was performed at 130 °C for 1, 2, 3 and 4h. After cooled at room temperature for 30 min, FAME and FFA extractions using hexane were performed as mentioned above and FAME and FFA were analysed.

2.2.4. Effects of various parameters on SHE

Water content gave the highest effect on FAME production. Therefore, to investigate significant effects of various parameters (reaction temperature, volumes of methanol and sulphuric acid and kinds of catalyst and alcohol) in detail, water content was fixed as 80% and further experiments were implemented. The experimental procedures are same as previous experiment.

2.3. FAME and FFA analysis

To quantify FAME and FFA, the samples dissolved in hexane were mixed with an internal standard and analysed by gas chromatography (GC-2010, Shimadzu Corporation, Japan) with a 30 m length × 0.25 mm i.d. × 0.5 μm film thickness capillary column (Stabilwax, Restek Corporation, USA) and a flame ionisation detector. Operating conditions in FAME and FFA analysis were as follows: carrier gas N_2,

injection temperature 250 °C, detector temperature 250 °C and linear velocity 60 cm/s. Oven temperature was operated at 220 °C (hold 10.2 min) and raised at 10 °C /min to 240 °C (hold 17.8 min). FAME is calculated as a percentage of dry algal biomass (% (w/w)) from equation (1).

$$\mathrm{FAME}(\%) = \frac{(\sum \mathrm{A}) - \mathrm{As}}{\mathrm{As}} \times \frac{\mathrm{Cs} \times \mathrm{Vs}}{\mathrm{m}} \times 100 \quad (1)$$

where ΣA is the total peak area of FAMEs; A_S is the peak area of internal standard; C_S is the concentration of internal standard (g/L); V_S is the volume of internal standard (L); m is the dry weight of microalgae (g).

FAME yield is determined by comparing the mass of FAME obtained from the experiment with the mass of maximum FAME as shown in equation (2).

$$\mathrm{FAME\ yield}\ (\%) = \frac{\text{mass of FAME obtained (g)}}{\text{mass of maximum FAME(g)}} \times 100 \quad (2)$$

3. Results and discussion

3.1. Effects of various parameters on SHE with orthogonal experiment

The mean values of each experimental factor are shown in Fig. 3-1a. This figure

clearly shows that the increase of water reduces FAME production and suggests that water content is the most significant factor. When the investigation of microalgal hydrolysis as a pretreatment of esterification was performed by Takisawa et al. (2013), water content (70−90%) doesn't provide significant effect on hydrolysis of microalgal lipid. Therefore, this result might be obtained because of the inhibition of hydrolysis by methanol and (or) the inhibition of esterification by water. Two-factor interactions are shown in Fig. 3-1b. Less methanol was enough in lower water content while more methanol was required in higher water content. Also, Lower temperature was enough in lower water content while higher temperature was needed in higher water content. ANOVA was carried out to investigate the effects of each factor and interaction as shown in Table 3-3. A×B and A×E were used as the experimental error to indicate the reliability of the experiments as a whole because their effects were very small. It was found that water content was the most important factor with contribution ratio 70.90%, followed by time with contribution ratio 10.36% (both significant at 1% level). As main effects, volume of sulphuric acid, volume of methanol and temperature did not affect FAME production significantly. However, there were significant interactions of water content with temperature and volume of methanol at 1% and 5% level, respectively.

Based on the orthogonal experiment results, the effect of water content which was the

most effective factor on SHE was investigated in detail.

3.2. Effect of water content

Fig. 3-2a shows the time courses of SHE at various water content levels. The increase of water content significantly reduced FAME production as previously explained. FAMEs under water content 60%, 70%, 80% and 90% were 5.51%, 5.00%, 2.89% and 0.86%, respectively for 4 h, and then FAME yields were 95.00%, 86.24%, 49.79% and 14.85%, respectively, based on maximum FAME content 5.80%. Ehimen et al. (2010) reported that the direct transesterification process was investigated for different moisture levels on a basis of 15 g-dry algae (for example, 25.88, 23.40 and 17.95 g were used for the 72.5%, 56.0% and 19.5% moisture levels, respectively) using 60 ml of methanol containing 0.04 mol of sulphuric acid at 60 °C for 6 h. Their result indicated the reactions were almost inhibited under water contents greater than 31.7% (dry basis). Also, when 0.1 g of microalgae were transesterified with 2 mL of methanol containing 1.8% sulphuric acid by heating the mixture at 80 °C for 20 min by Wahlen et al. (2011), FAME yield was 50% under water content 100% (dry basis). Though it is difficult to compare with the results of other researches because the reaction conditions are different, it was suggested that FAME yield increased by hydrolysis (FAME yield 95%

under water content 60%, ie., 150% on dry basis).

Fig. 3-2b shows equilibrium between FAME and FFA. This figure means that the increase of water content reduces the conversion rate of FFA to FAME. This is because esterification is a reversible reaction. The addition of water could push the equilibrium toward hydrolysis to FFA.

3.3. Effect of various parameters

3.3.1. Effect of temperature

Microalgae (0.3 g), water content 80%, sulphuric acid 200 mL/kg-dry algae and methanol in the ratio of methanol to wet biomass 1.33 (vol./wt.) were used as materials. The reaction temperature levels were changed at 120, 130, 140 and 150 °C. Fig. 3-3 shows the effect of reaction temperature on SHE. It was found that the increase of temperature accelerated the initial rate of FAME production. However, FAME contents at various temperature levels for 6h are similar (except 150 °C). Excess heating might result in the degradation of FFA in the hydrolysis process (Takisawa et al., 2013).

3.3.2. Effect of methanol volume

Microalgae (0.3 g), water content 80%, sulphuric acid 200 mL/kg-dry algae were

reacted with methanol in methanol to wet algae (vol./wt.) ratio of 1.33, 2.67 and 4 at 140 °C. Increasing volume of methanol accelerated the reaction, whereas excess input of methanol reduced FAME production rate (Fig. 3-4a). It can be considered that lack of methanol causes the esterification inhibition and the surplus of methanol leads to the slower hydrolysis. Fig. 3-4b shows equilibrium between FAME and FFA. The increase of methanol could push the equilibrium towards FAME production.

3.3.3. Effect of sulphuric acid volume

Microalgae (0.3 g), water content 80% and methanol in the ratio of methanol to wet biomass 2.67 (vol./wt.) were mixed with sulphuric acid 200, 300, 400 mL/kg-dry algae and reacted at 140 °C. Though similar results were obtained among all the experiments as shown in Fig. 3-5, the reaction using sulphuric acid 400 mL/kg-dry algae reduced FAME production slightly. This may be because of a side reaction. Overloaded acid causes side reaction, i.e. acid-promoted polymerization can occur for unsaturated fatty acid with one or more double bonds (Erhan and Bagby, 1994). This could be also considered to be due to the degradation of FFA by the addition of excess sulphuric acid (Takisawa, 2013).

3.3.4. Difference of catalysts and alcohols

Microalgae (0.3 g), water content 80%, and methanol in the ratio of methanol to wet biomass 2.67 (vol./wt.) were reacted with sulphuric acid 300 mL/kg-dry algae or the equimolar amount of hydrochloric acid at 140 °C. As shown in Fig. 3-6, the results of FAME production by using two catalysts were similar. When biodiesel synthesis via esterification of enzyme-hydrolyzed FFAs and methanol was conducted using several homogeneous acid catalysts including nitric, sulfuric, and hydrochloric acids, hydrochloric acid is the only recoverable and reusable catalyst because it can be completely retained in the separated methanol phase (Su, 2013). Hydrochloric acid could be a promising catalyst in terms of the purification of biodiesel and the recovery of catalyst.

Microalgae 0.3 g, water content 80%, sulphuric acid 300 mL/kg-dry algae were reacted with 4 mL of methanol or ethanol at temperature 140 °C. Fig. 3-7a shows effects of methanol and ethanol on FAME production. FAME production rate using ethanol was slightly faster than that using methanol, whereas maximum FAME contents were similar results. Unlike FAME, the results of FFA content were very different between two alcohols. Ethanol extracted more lipid compared to methanol. In contrast, methanol provided higher FAME conversion rate compared to ethanol and FAME

conversion rates by using methanol and ethanol were 80−90% and 55−60%, respectively as shown in Fig. 3-7b.

4. Conclusions

The investigation of SHE of wet microalgal lipid was carried out. It was found that water content was the most influential factor through the experiment with L_{27} orthogonal design. The detailed optimisation of SHE was performed. Also, sulphuric acid and hydrochloric acid as catalysts showed similar effects in equimolar amounts. Methanol provided less total extraction volume and more FAME conversion rate compared to ethanol.

Table 3-1. Factors and levels for the L_{27} orthogonal experiment.

Factors	Level 1	Level 2	Level 3
A: Water content (%)	70	80	90
B: Volume of sulphuric acid (mL/kg-dry algae)	200	300	400
C: Volume of methanol (the ratio of methanol to wet biomass, vol./wt.)	1.33	2	2.67
D: Temperature (°C)	130	140	150
E: Time (h)	1	2	3

Table 3-2. L_{27} orthogonal experimental design.

No.	Water content (%)	Volume of sulphuric acid (mL/kg-dry algae)	Volume of methanol (the ratio of methanol to wet biomass, vol./wt.)	Temperature (°C)	Time (h)	FAME (%)
1	70	200	1.33	130	1	3.93
2	70	200	2	140	2	3.16
3	70	200	2.67	150	3	3.76
4	70	300	1.33	140	3	4.59
5	70	300	2	150	1	2.65
6	70	300	2.67	130	2	3.36
7	70	400	1.33	150	2	3.11
8	70	400	2	130	3	4.01
9	70	400	2.67	140	1	2.82
10	80	200	1.33	130	1	1.43
11	80	200	2	140	2	1.71
12	80	200	2.67	150	3	3.52
13	80	300	1.33	140	3	2.77
14	80	300	2	150	1	2.14
15	80	300	2.67	130	2	2.80
16	80	400	1.33	150	2	1.54
17	80	400	2	130	3	2.79
18	80	400	2.67	140	1	2.18
19	90	200	1.33	130	1	0.03
20	90	200	2	140	2	0.06
21	90	200	2.67	150	3	3.00
22	90	300	1.33	140	3	0.43
23	90	300	2	150	1	1.45
24	90	300	2.67	130	2	0.09
25	90	400	1.33	150	2	0.27
26	90	400	2	130	3	0.23
27	90	400	2.67	140	1	0.04

Table 3-3. ANOVA of the orthogonal experiment.

Factors	Degrees of freedom	Variances	Variance ratios	Significances	P values	Contribution ratios (%)
A: Water content	2	18.686	171.947	**	0	70.90
B: Volume of sulphuric acid	2	0.444	4.083		0.060	1.28
C: Volume of methanol	2	0.433	3.989		0.063	1.24
D: Temperature	2	0.408	3.756		0.071	1.14
E: Time	2	2.822	25.968	**	0	10.36
A×C	4	0.526	4.842	*	0.028	3.19
A×D	4	0.961	8.843	**	0.005	6.51
Error	8	0.109				5.39

**Significant at 1%, * Significant at 5%

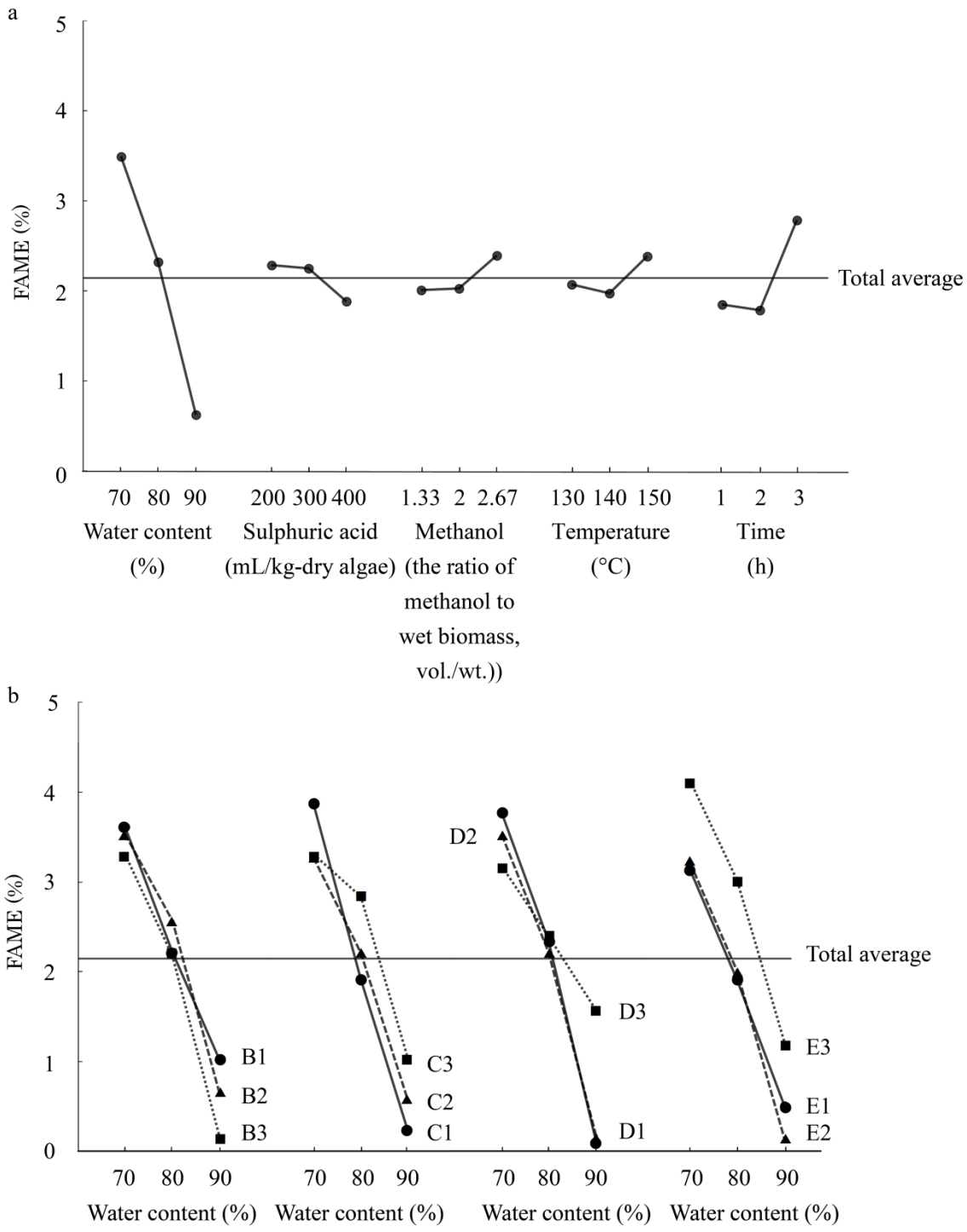

Fig. 3-1. Results of L_{27} orthogonal experiment; a: main effects, b: interaction effects. B1, B2 and B3, volume of sulphuric acid 200, 300 and 400 mL/kg-dry algae; C1, C2 and C3, methanol in methanol to wet algae (vol./wt.) ratio of 1.33, 2 and 2.67; D1, D2 and D3, temperature 130, 140 and 150 °C; E1, E2 and E3, reaction time 1, 2 and 3 h.

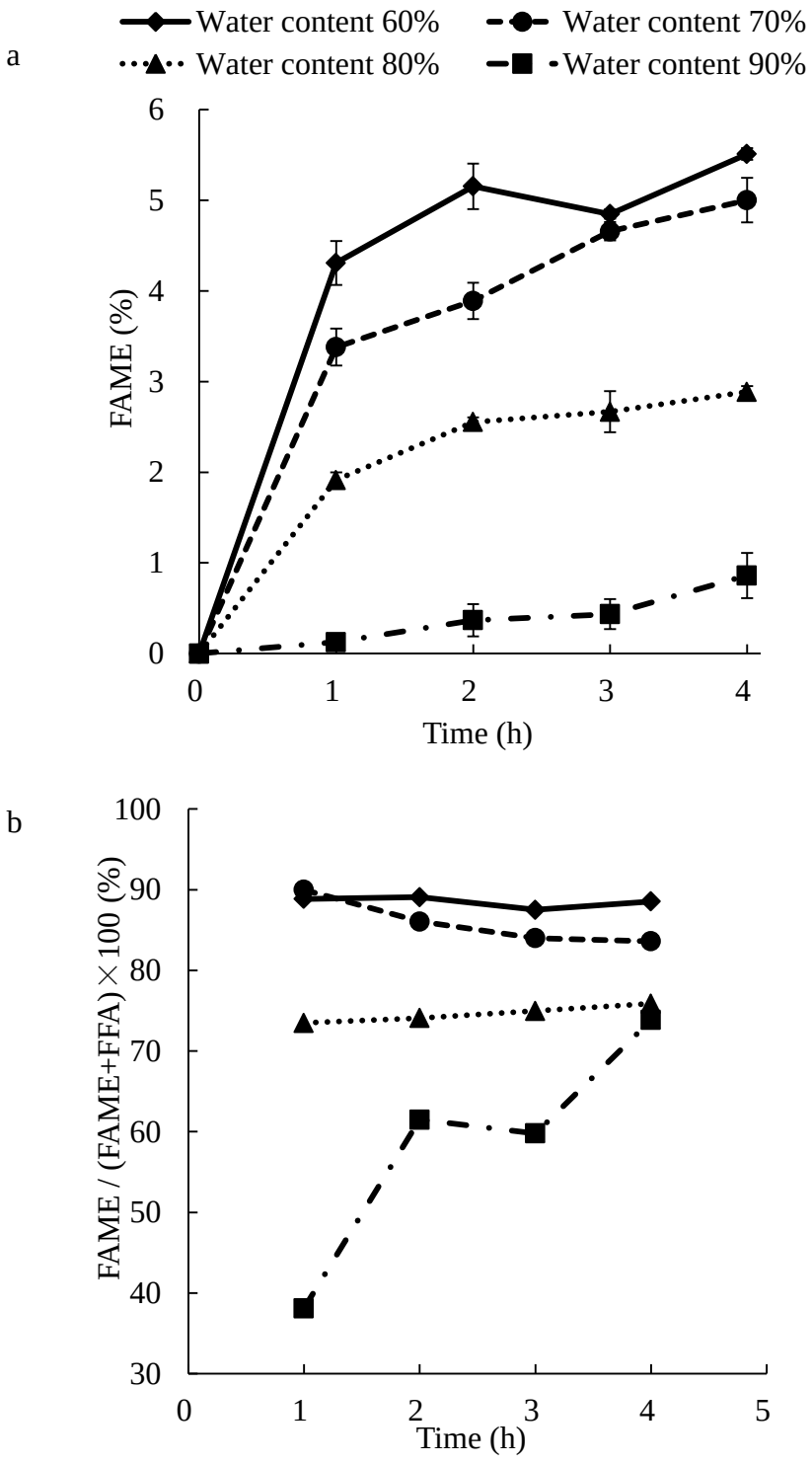

Fig. 3-2. Effect of water content on FAME; a: FAME content, b: equilibrium between FAME and FFA. The reaction condition is as follows: microalgae 0.3 g, sulphuric acid 200 mL/kg-dry algae, methanol in the ratio of methanol to wet biomass 1.33 (vol./wt.) and reaction temperature 130 °C.

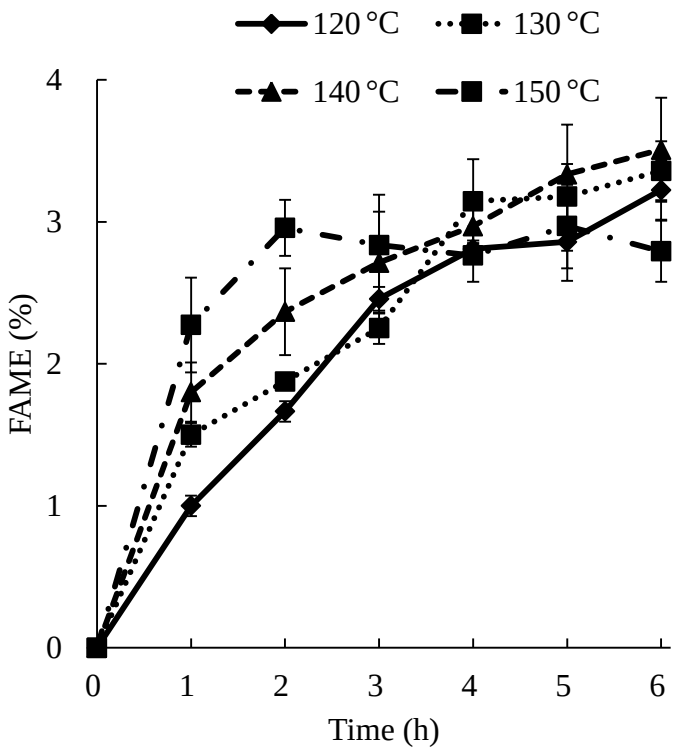

Fig. 3-3. Effect of temperature on FAME. The reaction condition is as follows: microalgae 0.3 g, water content 80%, sulphuric acid 200 mL/kg-dry algae and methanol in the ratio of methanol to wet biomass 1.33 (vol./wt.)

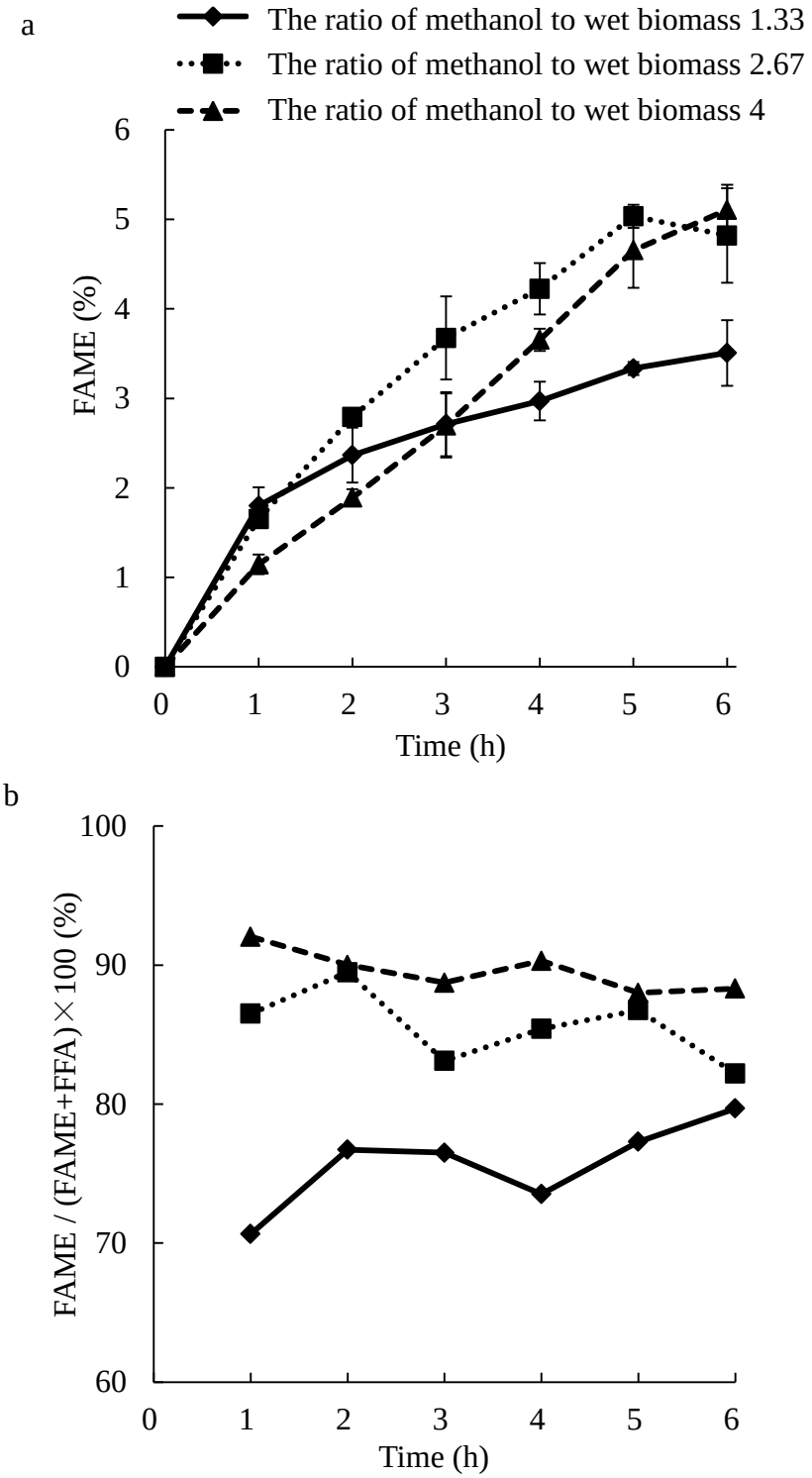

Fig. 3-4. Effect of volume of methanol on FAME; a: FAME content, b: equilibrium between FAME and FFA. The reaction condition is as follows: microalgae 0.3 g, water content 80%, sulphuric acid 200 mL/kg-dry algae and reaction temperature 140 °C.

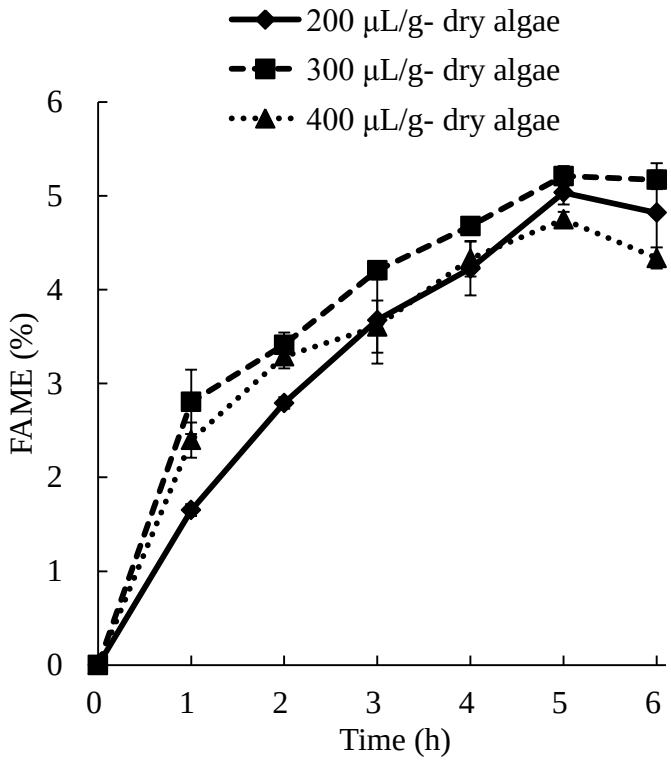

Fig. 3-5. Effect of volume of sulphuric acid on FAME. The reaction condition is as follows: microalgae 0.3 g, water content 80%, methanol in the ratio of methanol to wet biomass 2.67 (vol./wt.) and reaction temperature 140 °C.

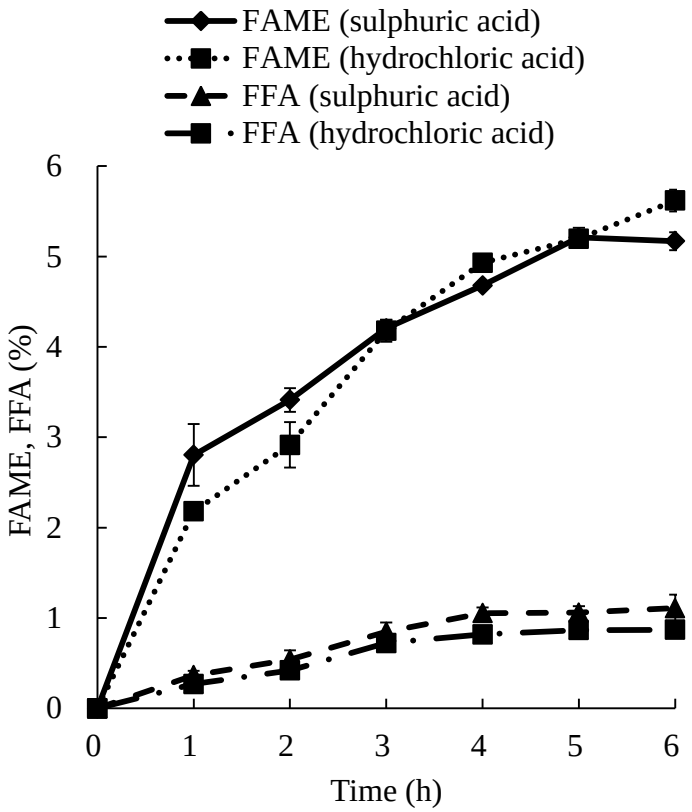

Fig. 3-6. Difference of catalysts. Microalgae 0.3 g, water content 80%, methanol in the ratio of methanol to wet biomass 2.67 (vol./wt.) were mixed with sulphuric acid 300 mL/kg-dry algae or the equimolar amount of hydrochloric acid and reacted at 140 °C.

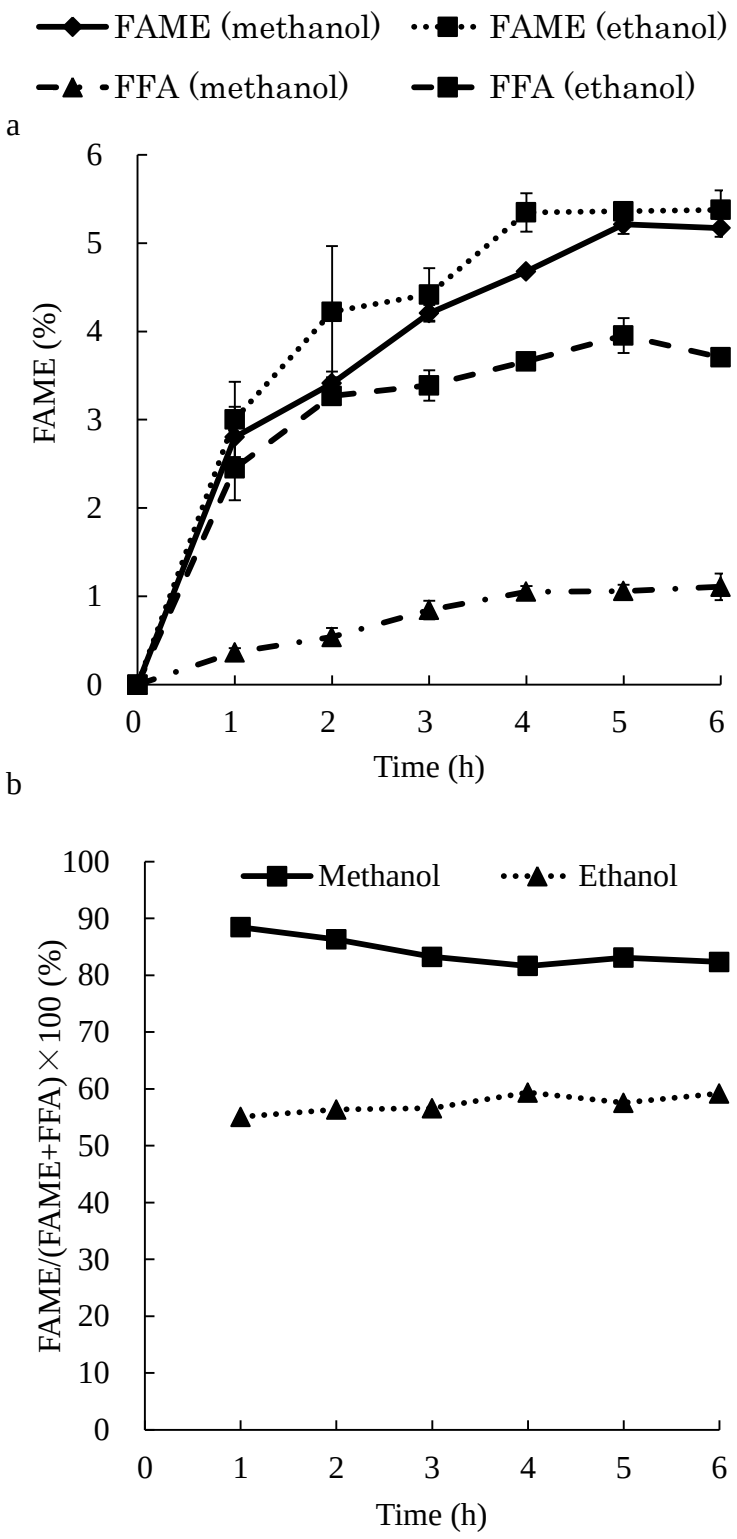

Fig. 3-7. Difference of alcohols. a: FAME content, b: equilibrium between FAME and FFA. Microalgae 0.3 g, water content 80%, sulphuric acid 300 mL/kg-dry algae were reacted with 4 mL of methanol or ethanol at temperature 140 °C.

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Chapter 4 Overall conclusions

This study reviewed various technologies which generate biodiesel from microalgae by transesterification in chapter 1. The performances of alkaline, acidic and enzymatic catalysts were described. Also, modern techniques of development of biodiesel i.e., microwave method and supercritical method were discussed. Hydrolysis of lipids from microalgae under high water content was investigated as a pretreatment of direct esterification in chapter 2. Results indicated that the hydrolysis process reduced the inhibition by water in FAME production; in addition, FAME obtained by esterification of hydrolysates was increased by 181.7% compared to FAME obtained by direct transesterification under the same amount of water content (80%). Finally, chapter 3 demonstrated hydrolysis of wet microalgal lipid and esterification of FFA using acid in one-step process. As a result, water content was found to be the most effective factor. The effects of various parameters on FAME content and equilibrium relation between FAME and FFA were also examined under water content 80%. Equimolar amounts of sulphuric acid and hydrochloric acid showed similar results. Also, methanol provided less total extraction volume and more FAME conversion rate compared to ethanol. This

method has great potential in terms of biodiesel production from microalgae since no organic solvents are used, simultaneously removing the drying cost.

For the future, the process energy evaluation of this method will be compared with the oil extraction from wet microalgae followed by transesterification and the drying of wet microalgae followed by direct transesterification in detail. In addition, the treatment of the hydrolyzed residues must be considered from the point of view of resource circulation. It is estimated that glucose derived from microalgal cell exists in the residues, which could be increased by hydrolysis. Therefore, the bioehanol production from glucose produced after hydrolysis would be suitable. In order to survey the feasibility of the bioethanl production from the hydrolyzed biomass, the investigation of the effect of hydrolysis on glucose production must be achieved.

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