Biodiesel Production from *Scenedesmus sp.* through Optimized *in situ* Acidic Transesterification Process

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Biodiesel production from *Scenedesmus* sp. was optimized through *in situ* transesterification via acid catalysis associated with high pressure homogenization pretreatment. Response Surface Methodogy (RSM) was applied to investigate the influence of catalyst amount and biomass to solvent ratio as two major factors for *in situ* transesterification by central composite method. Optimized reaction conditions were determined as H_2SO_4 amount of 5.46 % and biomass to methanol ratio of 1:22.07 at 70 ºC for 10 hrs reaction time. The Fatty Acid Methyl Ester (FAME) from this process was confirmed to be almost identical to a commercial standard biodiesel by TLC analysis. Biodiesel yield from the quadratic response surface model was also calculated as 69.36 % (base on lipid weight) of the maximum yield under these conditions, and this theoretical yield showed good agreement with 69.11±1.16 % of experimental result. From GC analysis of biodiesel, ten species of C_{16}–C_{22} with saturated and unsaturated fatty acid were identified, and predominate fatty acids were C_{16} and C_{18} methyl esters. These results confirm that an efficient production of biodiesel from mciroalge, *Scenedesmus* sp. could be possible through an optimized *in situ* acidic transesterification process.

Key words:
optimization, biodiesel, *in situ* transesterification, RSM, *Scenedesmus* sp.

Introduction

Biodiesel, one of renewable bioenergy, has been produced from various resources such as vegetable oil, animal fats, used frying oils and microbial oils etc.[1–3] Extensive study has been conducted on using edible oils, which limited by the availability of oil inventories, but these materials result in high sensitivity of prices to oil demand from industry.[4–7] In order to not compete with edible oils, the low-cost and profitable biodiesel should be produced from low-cost feedstocks such as non-edible oils (used frying oils, animal fats and greases etc.). However the available quantities of waste oils and animal fats are not enough to match the today demands for biodiesel. Therefore, other biofuel feedstock, microalgae, has been focused since it would not much require the agricultural land and has higher energy yields per hectare as well as very short cell cycles (within 24 hours).[8] In addition, liquid culture of the microalgae can be controlled easily and even used for a waste treatment as a new source having renewable, environmental and economical sustainability.[4,6,9–11]

However, in making biodiesel, actually Fatty Acid Methyl Esters (FAME), generally lipid extraction and its transesterification process produce large amount of hazardous solvent waste and are also very cumbersome.[6,12,13] Automated extraction equipment such as the Soxhlet apparatus has been designed, but they require the long time of extraction process. To overcome this limitation, one step to make biodiesel, *in situ* transesterification (or direct transesterification from the biomass) process, has been considered where intact biomas rather than pre-extracted oil directly contacts with acidified or alkalized alcohol that acts as both an extraction solvent and an esterification reagent. This process coul reduce the long process time and also maximize biodiesel yield as well as use of reagents an solvents, etc.[13–16] However, overall process of directly producing biodiesel (FAME) from fresh microalgae has not been well investigated and did not identify even key parameters in employing *in situ* transesterification for microalgae that contain relatively hard and rigid cell membranes, which require longer process time and larger extraction solvents, etc.[17]

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Therefore, in this work, *Scenedesmus* sp. were selected because this agla has not been much studied for biodiesel production and its lipid composition and extraction yield were very feasible for economic production from our previous study.[18–20] For *in situ* transesterification, important variables such as ratio of biomass to solvent and amount of catalyst will be optimized by using Response Surface Methodology (RSM), and where high pressure homogenization pretreatment process and strong acid catalyst will be simultanesously applied to maintain high biodiesel yield due to rigid cell walls of microalgae.[21,22]

Materials and methods

Materials

Marine microalga, *Scenedesmus* sp. was provided from KORDI (Korea Ocean Research & Development Institute), and was cultured with BG11 under controlled conditions with continous 12/12hr cyclic, light intensity at 10–25 μmol photons/m^2/s, CO_2 supply at a rate of 250 mL min^{-1} in a 5 L photobioreactor. After 30 days cultivation, the algal biomass was harvested by centrifugation at 3000 rpm for 10 min. Then, the cells were freeze-dried, ground down to 100 mesh size, and stored in the freezer. The freeze dried powder was kept under desiccation of anhydrous sodium sulfate (Daejung Co., Ltd, Shi heung, Korea) for overnight, before to use.

Pretreatment of microalga

The freeze dried microalga was soaked with the 30 times (w/v) distilled water and agitated at 500 rpm for 12~24 hrs. Then, the microalga was immediately passed through a high pressure homogenizer (HPH-mini Model 200, Micronox Inc., Seongnam, Korea) by one time at 6.8~8.3 MPa of pressure. After the high pressure homogenization, the cell were collected by centrifugation at 5000x*g* for 15 min and freeze dried to make the powder (100 mesh size). The powdered biomass was stored at freezer of 4 ºC before *in situ* transesterification.[19]

Transmission Electron Microscopy (TEM) of pretreated or fresh microalga

To compare the morphology of the microalga with or without high pressure homogenization, the freeze-dried alga with or without pretreatment was fixed by placing in 4 % glutaraldehyde in 0.1 mol L^{-1} phosphate buffer (pH 7.4) for 4 hr. The cells were then rinsed twice for 15 min in phosphate buffer and post-fixed in 1 % osmium tetroxide (OsO_4) in 0.1 mol L^{-1} phosphate buffer for 2 hr. The samples were then run through a graded dehydration series (50~100 %) of EtOH and rinsed twice in propylene oxide for another 20 min. The agar plug and cells were then embedded in EPON mixture (EMS, Fort Washington, PA, USA) and thin sections obtained using an ultramicotome (Leica Ultracup, UCP, Germany). Thin sections were placed on formvar black grid, stained with uranyl acetate and lead citrate and viewed under the electron microscopy (Carl Zeiss, Oberkochen, Germany).

In situ transesterification with acid catalysis

The *in situ* transesterification process was followed by Johnson *et al.*[16] in a 250 mL or 30 L working voume of laboratory-scale setup under the following conditions: Biomass to solvent ratio from 1:7.93 to 1:22.07 (w/v) and concentrations of sulfuric acid from 2.17 % to 7.83 % (v/v of solvent); reaction temperature of 70 ºC and reaction time of 10 hr. For more detail explanation of *in situ* transesterification process, 5 g of pretreated algal biomass was placed in a 500 mL round bottom flask with a reflux condenser and mixed with methanolic solution containing sulfuric acid, which was prepared freshly in order to maintain the catalytic activity, as shown in a diagram of Fig. 1. Hexane (95 % purity,

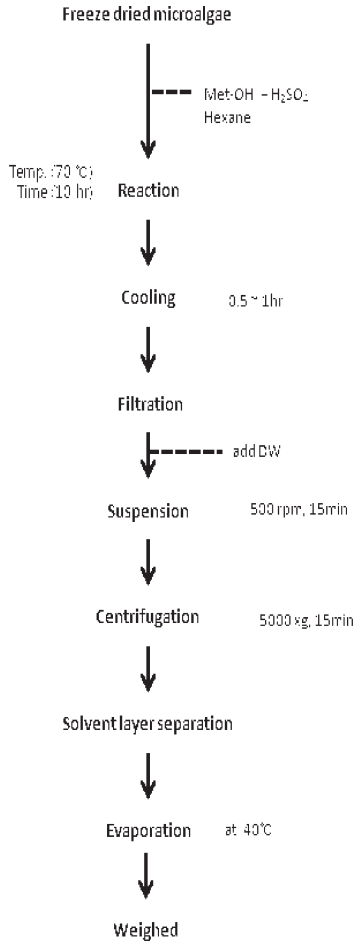

Fig. 1 – *Flow diagram for production of crude biodiesel from Scenedesmus sp. by in situ transesterification via acid catalysis*

Daejung Co., Ltd, Shi Heung, Korea) were added to the same volume of sum of methanol and sulfuric acid since hexane was proved to be better solvent and most commonly used for *in situ* transesterfication process.[6,23] The reaction mixture was then heated and maintained at the temperatures of interest for specified periods, and the sample were well-mixed during heating. After the reaction was completed the round bottom flask were allowed to cool to room temperature. The cooled mixture was filtered (Advantec No.2, Toyo Roshi Inc., Tokyo, Japan). Then, half the reaction volume of water was added. These extract mixture was centrifuged (5000x*g*, 15 min), then transferred to a separation funnel. The pooled hexane layer were washed with water (to remove left-over traces of the acidic catalyst and methanol), separated and then dried over 4 % (w/w of biomass) anhydrous sodium sulphate for overnight. The extracted solvent layer was filtered (Advantec No.2, Toyo Roshi Inc., Tokyo, Japan) and checked the volume. The 10 mL of extracted solvent containing FAME (i.e. biodiesel) were transferred to a pre-weighed glass vial. The solvent was evaporated (40–45 ºC) under vacuum condition for 15 min, and cooled inside a desiccator for 30 min. Mass of biodiesel were determined gravimetrically, in duplicated. All the stirred reactions were carried out using a magnetic stirrer system with a rotation speed of 500 rpm was kept constant throughout the duration for the agitated samples.

The crude biodiesel yield from this process was calculated by Eq. (1).

$$\text{Crude biodiesel yield (\%)} = \\ = \frac{\text{Weight of biodiesel (FAME) (g)}}{\text{Algae mass (g)} \cdot \text{Lipid content (\%)}} \qquad (1)$$

where, the weight of biodiesel was the amounts of the crude FAME from *in situ* transesterification, and the production yield was actually estimated based on the total amounts of the lipids existed in the biomass.

Thin Layer Chromatography (TLC) for the identification of FAME

TLC analysis, a reliable and fast method to identify the lipids,[24] was performed on 0.25-mm-thick silica gel G-60 plates (Merck, Darmstadt, Germany) developed with *n*-hexane:diethyl ether (90:10, v/v). To detect FAME spot, plates were visualized by iodine vapor and spraying with 10 % phosphomolybdic acid (98 % purity, Daejung Co., Ltd, Shi Heung, Korea) in ethanol, then dry them in the oven at 105 ºC. The mono-, di- and triglyceride Mix (TAG STD, Supelco, Bellefonte, PA, USA) and commercial biodiesel (FAME STD, S Co., Houston, TX, USA) were used for TLC standards. The actual amounts of FAME was measured by calculating the areas of the FAME and strandard spots on the TLC plate with a calibration curve of the standard biodiesels from a programmed UV scanner.[25]

Gas Chromatography (GC) for profiling fatty acids composition

To obtain a compositional profile of the biodiesel products, quantitative analysis was carried out by GC-FID (Agilent 6980N, Agilent Technologies, Palo Alto, CA) with a HP-INNOWax capillary GC column (30 m × 0.25 mm). The same temperature of injector and detector were 290 ºC, respectively. The column temperature was 140 ºC, then increased by 5 °C min^{-1} to 290 °C and held for 20 min. A 1.95 mL sample of methyl heptadecanoate (10 mg mL^{-1}) (C17:0, ≥99.5 %, Fluka, Buchs, Switzerland) was added as internal standard to 50 μL aliquots of each samples. The ester content, C of the biodiesel samples was also determined by gas chromatography and expressed as mass percentage by the Eq. (2) according to EN 14103:[26]

$$C = \frac{(\Sigma A) - AEI}{AEI} \cdot \frac{CEI - VEI}{m} \cdot 100 \qquad (2)$$

where,

ΣA = total peak area C14:0-C24:1
AEI = internal standard (methyl heptadecanoate) peak area
CEI = concentration of the internal standard solution, in mg mL^{-1}
VEI = volumn of the internal standard solution used, mL
m = mass of the sample, in mg.

Design of Response Surface Methodology (RSM)

To optimize *in situ* transesterification process by using a RSM, two of effectiveness factors were considered: Reaction temperature and solvent quntity in alkali catalysis; catalyst amount and biomass to solvent ratio in acid catalyst were selected from Taguchi method.[26] The condition of biodiesel product were prepared according to a central composite design, consisting of a five-level-two-factorial design,[27] in 13 treatments. The dependent variables; responses were the yield of biodiesel. Then, RSM analysis was carried out by using MINITAB®16 (Minitab Inc., Pennsylvania, USA).

Statistical analysis

The data are expressed as mean ± SD (standard deviation) and the mean is the average of five test results per experiment. The data were analyzed using student *t*-test (SAS 9.1, SAS, Cary, NC, USA). The experiments were repeated at least three times

to confirm the results. The data were analyzed using an analysis of variance, and the mean values were considered significantly different at $p < 0.05$. The optimal extraction condition was determined using regression analysis.

Results and discussion

Effect of high pressure homogenization on *in situ* transesterfication process

Most of of marine micaroalgae, are known to have the ultrastructure of cell wall referred to as ribs and a trilaminar structure and especially, *Scenedesmus* sp., the subfamily *Scotiellocystoideae*, has very solid three-layered (cellulosic layer→middle layer→cell organelles) cell walls,[28,29] which is commonly described feature for these subfamily.[30] Thus, various pretreatment processes such as homogenization, ultrasonication and microwave, etc. have been applied to improve the biodiesel yield, and high pressure homogenization was found to be most effective in terms of yield and process time.[20] Fig. 2 also clearly demonstrated the effect of high pressure homogenization pretreamtment process by showing that complete destruction of the cell walls after the pretreatment, which can definitely enhance the biodiesel produciton yield from *in situ* transesterficiation as also reported elsewhere.[28,29,31] As shown in Fig. 2 (B), the starch droplets (white vacuole), and the widespread crushed cytosol and stroma of chloroplasts (grey amorphous materials) were observed while for non-treated biomass the cell wall maintained their own circular shape. Therefore, to increase the crude biodiesel yield, high pressure homogenized biomass was used in this study as its advantage was also reported elsewhere.[21,22]

Generally, methanol plays the roles of both reactant and extractant in *in situ* transesterification, but it was proved to be a poor solvent for lipid extraction. This would probably lead to low conversion efficiency from algal biomass to biodiesel by *in situ* transesterification. To overcome this bottleneck, *n*-hexane or chloroform could be used for the higher biodiesel yield,[6] and in this work, hexane was used as co-solvent in *in situ* transesterification. The formation of FAME (biodiesel) from this process was confirmed by TLC analysis by comparing with a standard biodiesel as shown in Fig. 3. It could tell that most of fatty acids in the biomass were changed to crude

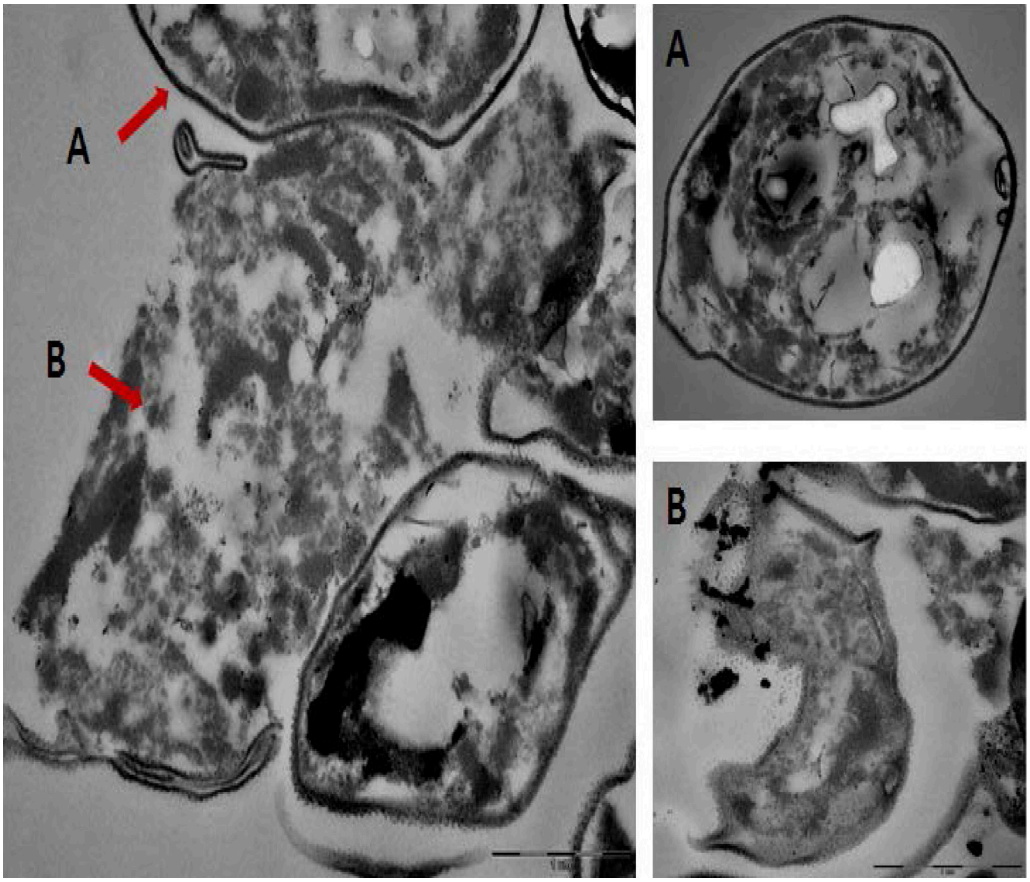

Fig. 2 – *Transmission electron microscopic graphs of Scenedesmus sp. by different treatments (scale bar: 1 μm); (A) Lyophilized algae without high pressure homogenization; (B) Lyophilized algae with high pressure homogenization*

Fig. 3 – *Comparison of FAME from in situ transesterification with acid catalyst process and a standard FAME by TLC analysis*

FAME by showing the same retention time with a standard even though there were some residuals as mono, di or tri glycerides at the bottom of the plate in the sample lane in Fig. 3. The highest crude FAME production yield was estimated as 69.11 % (w/w) based on the lipids in the biomass and its transesterification yield was increased up to 96.7 % in purifying the FAME (data not shown). Therefore, this result confirmed that *in situ* transesterfication via acid catalyst associated with high pressure homogenization would be an efficient process for biodiesel production from microalga.

Optimization of biodiesel production by Response Surface Methodology (RSM)

To find the optimum condition for biodiesel production, a five-level-two-factor Central Composite Design (CCD) was employed in this study by using 13 experiments. Two of main factors, biomass to solvent ratio and catalyst amount, were selected for the study based on previous results by using our work (not shown data), and whose results were that amounts of solvent and catalysts were more important than process time and temperature, etc.[32,33] For *in situ* transesterification of algal biomass containing rigid cell walls, acidic catalyst was found to be more effective; however, it reguired more longer process than that of conventional transesterificiation due to slow process of acidic transesterification and much less homogenous reaction for biomass, compared to simple oil samples even though it woud not be practical yet. For other reported, transesterification time would take more than 20 hours.[32,33] Table 1 shows the independent factors, levels and treatment conditions in terms of coded and uncoded values by CCD. Depending on the combination of treatments, the crude biodiesel yield ranged from minimum of

Table 1 – *Values of independent variables and treatment conditions by the central composite experimental design (% w/w, base on lipid weight)*

Variables	Level				
	–1.414	–1	0	1	1.414
Catalyst amount X_1 (%)	2.17	3	5	7	7.83
Biomass to solvent ratio X_2 (w/v, g mL^{-1})	1:7.93	1:10	1:15	1:20	1:22.07

Exp No.	Code value		Experimental value		Crude FAME yield Y (%, w/w)
	X_1	X_2	X_1	X_2	
1	–1	1	3	1:20	59.31±2.62
2	1	1	7	1:20	61.56±1.59
3	1	–1	7	1:10	45.14±4.59
4	–1	–1	3	1:10	53.60±1.91
5	0	0	5	1:15	60.77±0.84
6	–1.414	0	2.17	1:15	55.14±3.83
7	0	1.414	5	1:22.07	70.86±4.78
8	1.414	0	7.83	1:15	49.16±3.15
9	0	–1.414	5	1:7.93	52.19±2.77
10	0	0	5	1:15	60.02±3.69
11	0	0	5	1:15	57.25±2.00
12	0	0	5	1:20	59.94±2.52
13	0	0	5	1:20	60.77±0.84

Table 2 – *Values of regression coefficient for biodiesel yield*

Factor	Regression coefficient	Standard error	*T*-value	*P*
Beta0	59.7497	0.7339	81.417	0**
Beta1	–1.8338	0.5802	–3.16	0.016*
Beta2	6.0675	0.5802	10.457	0**
Beta11	–4.2851	0.6223	–6.886	0**
Beta12	2.6781	0.8205	3.264	0.014*
Beta22	0.4039	0.6223	0.649	0.537

*$p < 0.001$, **$p < 0.01$

Table 3 – *ANOVA table for in situ transesterification via acid catalysis*

Variance source	Degree of freedom	Square sum	Square mean	F_0
Regression	5	484.281	96.856	35.97**
Error	7	18.85	0.09	
Total	12	503.131		

**$p < 0.01$

45.14±4.59 % (w/w) to maximum of 70.86±4.78 % (w/w) representing a significance at $P < 0.05$ level. The results of RSM analysis were summarized in Table 2 and Table 3 by showing the result of analysis of variance (ANOVA) for quadratic models of the crude biodiesel yield. The regression sum of squares was 484.281, sum of square of error was 0.09 with 0.9625 of regression coefficient (R^2) and 35.97 of F value that was below a preset statistically significant level, p <0.01, which tells that the regression analysis indicated that all the two parameters had a significant influence on the crude biodiesel yield.

Using the coefficient determined, the predicted model for crude biodiesel yield was determined as follows:

$$Y = 59.7497 - 1.8338X_1 + 6.0675X_2 - \\ - 4.2851X_{12} + 2.6781X_1X_2 + 0.4039X_{22} \quad (3)$$
$$(R^2 = 0.9625, p < 0.01)$$

However, one variable, X_{22} was deleted from the above equation (3) since it did not have the significance of the interaction. Therefore, simplified equation for this process would be as follows:

$$Y = 60.0306 - 1.8337X_1 + 6.0675X_2 - \\ - 4.3377X_{12} + 2.6781\ X_1X_2 \quad (4)$$
$$(R^2 = 0.9603, p < 0.01)$$

From these results, it can tell that biomass to solvent ratio was larger than that of catalyst amount, and biomass to solvent ratio-catalyst amount interaction effect was found to be positive. The interaction of two factors was also illustrated as dimensional surfaces and contour plots in Fig. 4. It was also found that maximum crude biodiesel yield were obtained at high biomass to solvent ratio. However, at the high catalyst amount of 7 %, crude biodiesel yield increased initially, reaching maximum at intermediate catalyst amount around 5 %, and then decreased at level of high catalyst amount. This is due to the negative effect of catalyst amount, probably caused by polymerization of unsaturated fatty acids.[34]

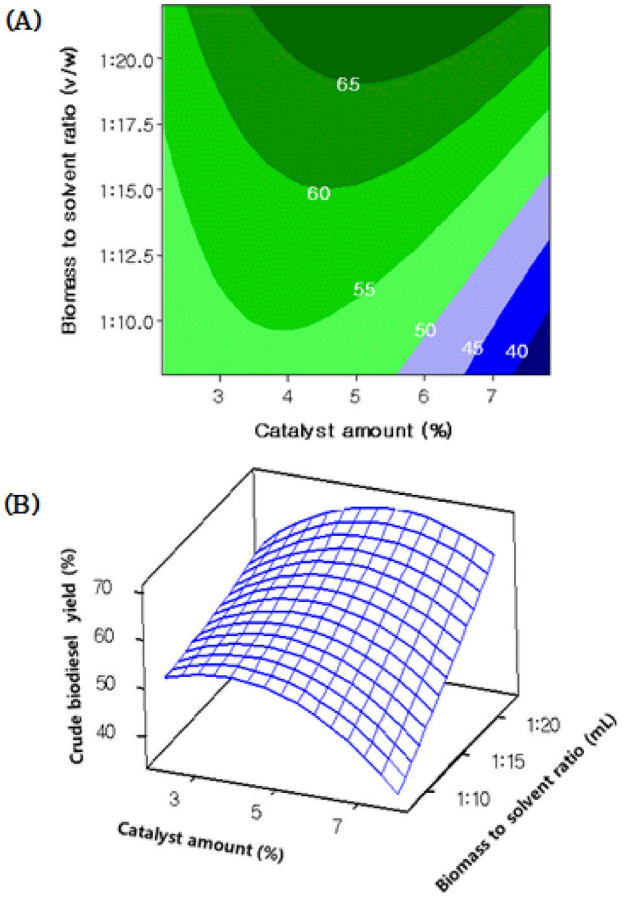

Fig. 4 – *2-D contour (A) and 3-D surface (B) plot for the effects of catalyst amount and biomass to solvent ratio*

The maximum crude biodiesel yield of 69.36 % (w/w, base on lipid weight) could be calculated under the condition at 70 ºC for 10 hrs reaction time with biomass to methanol ratio of 1:22.07 with catalyst amount of 5.46 % using MINITAB® 16 software (Minitab Inc., Pennsylvania, USA). 69.11±1.16 % of actual experimental yield from the process was showed good agreement with 69.36 % of the theoretical yield from the proposed model. This production yield was ca. 1.43-fold higher than 48.41±0.21 % (w/w) from conventional Taguchi method. Besides this result, significant differences of FAME yield from various micralgae were obtained such as 36 % (w/w, base on biomass) from *Chlorella gracilis* and 7.1 % from *Synechocystis elongatus* under the con-

dition of 1.8 % H_2SO_4 at 80 ºC for 20 min from the chloroform extraction.[35] It indicated that the different species microalgae had different production yields even under the same transesterification conditions. For other case, 68 % of biodiesel yield (base on lipid weight) from *Chlorella protothecoides* was also reported from the condition of 100 % acid catalyst quantity (on oil basis) with 56:1 molar ratio of methanol to oil at 30 ºC.[36] Therefore, it can tell that there was possible to achieve a high yield biodiesel from algae, using acid catalyzed *in situ* transesterification, but it is denfinitely necessary that the optimaization process should always be applied for the production of biodiesel in utilizing various microalgae.

Characteristics of the FAME from *Scenedesmus sp*.

To understand the quality of the FAME from this process, Fig. 5 showed the fatty acid profiling of the FAME, by comparing known standard FMAE retention times,[37] among the fatty acids in FAME C16:2 and C18:2 did not belong to fatty acids of the standards. It was found that most predominat FAMEs were C16 and C18 methyl esters such as methyl palmitate (C16:0), methyl palmitolate (C16:1), methyl hexadecadienoate (C16:2), methyl oleate (C18:1), methyl linolate (C18:2) and methyl linolenate (C18:3). The order of the most abundant fatty acids contents were C18:2> C16:2> C16:0. These results showed good agreement with the lipid compostions from *Scenedesmus* sp. that contained higher amounts of unsaturated fatty acids than other microalgae in general even though their fatty acid compositions and amounts were related to culture conditions such as light cycle, carbon and nitrogen sources, etc.[38–40] These results tell that most of fatty acids in the biomass was transesterfied to make FAME as a biodiesel thorugh this process. Moreovere, this result implied that FAME from this mciroalga had better quality of biodiesel since higher saturated fatty acids such as palmitic acid or stearic acid in biodiesel enhances the oxidative stability, but worsen the cold flow property. As the biodiesel is oxidized, the biodiesel becomes more viscous, and results in gumming of the fuel. However, higher unsaturated fatty acid such as linoleic acid or linolenic acid reveals the opposite properties of saturated fatty acid.[39–42] Therefore, algal biodiesel from this process could predict having a good cold flow properties and a relatively poor oxidation stability.

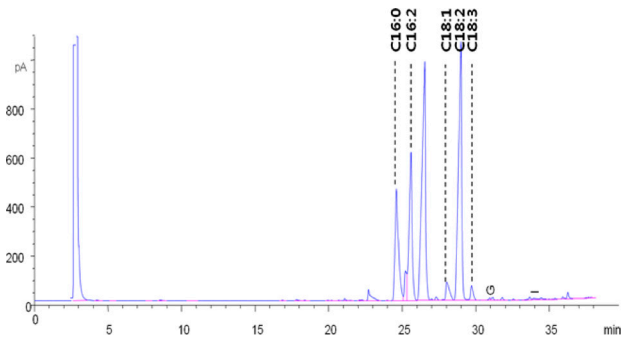

Fig. 5 – *GC chromatogram of FAMEs from Scenedesmus sp*

Conclusion

To increase the yield of crude biodiesel, two most important parameters for *in situ* acidic transesterification process was optimized by RSM, showing 5.46 % of H_2SO_4 and % and 1:22.07 of biomass to methanol ratio at 70 ºC for 10 hours. It was also found that this process would also be pretreated with high pressure homogenization to easily break down the rigid cell walls of *Scenedesmus* sp. From the quadratic response surface model with R^2 of 0.9625, theoretical yield of 69.36 % was expected, and 69.11±1.16 % (base on lipid weight) of maximum experimental yield under the optimaed conditions was well matched. From GC analysis of biodiesel, ten species of C16 ~ C22 with saturated and unsaturated fatty acid were identified as C18:2> C16:2> C16:0 of the order of main fatty acids. As relatively high unsaturated fatty acid content, the biodiesel from this process seemed to have the characteristic of good flow property at low temperature.

References

1. *Bajpai, D.,Tyagi, V. K.,* J. Olo. Sci. **55** (2006) 487.
2. *Meng, X., Yang, J., Xu, X., Zhang, L., Nie, A., Xian, M.,* Renew. Energy **34** (2009) 1. http://dx.doi.org/10.1016/j.renene.2008.04.014
3. *Stiefel, S., Dassori, G.,* Ind. Eng. Chem. Res. **48** (2009) 1068. http://dx.doi.org/10.1021/ie8005512
4. *Yusuf, C.,* Biotechnol. Adv. **25** (2007) 294. http://dx.doi.org/10.1016/j.biotechadv.2007.02.001
5. *Gouveia, L., Oliveira, A. C.,* J. Ind. Microbiol. Biotechnol. **36** (2009) 269. http://dx.doi.org/10.1007/s10295–008–0495–6
6. *Li, P., Miao, X., Li, R., Zhong, J.,* J. Biomed. Biotechnol. **2011** (2011) 1. http://dx.doi.org/10.1155/2011/141207
7. *Makkar, H. P. S., Klaus, B.,* Eur. J. Lipid Sci. Technol. **111** (2009) 773. http://dx.doi.org/10.1002/ejlt.200800244
8. *Mata, T. M., Martins, A. A., Caetano, N. S.,* Renew. Sustainable Energy Rev.**14** (2010) 217. http://dx.doi.org/10.1016/j.rser.2009.07.020
9. *Richimond, A.,* CRC Handbook of Microalgal Mass Culture, Vol. 1, Taylor & Francis Group., Florida, 1986, pp 199–243.
10. *Haag, A. L.,* Nature **447** (2007) 520. http://dx.doi.org/10.1038/447520a

11. *Mark, E. H., Donald., G. R.,* Mitigation Adapt. Strateg. Global Change. **12** (2007) 573.
12. *Carrapiso, A. I.,Timón, M. L., Petrón, M. J., Tejeda, J. F., García, C.,* Meat. Sci. **56** (2000) 159. http://dx.doi.org/10.1016/S0309–1740(00)00035–8
13. *Sahena, F., Zaidul, I. S. M., Jinap, S., Karim, A. A., Abbas, K. A., Norulain, N. A. N., Omar, A. K. M.,* J. Food. Eng. **95** (2009) 210. http://dx.doi.org/10.1016/j.jfoodeng.2009.06.026
14. *Georgogianni, K. G., Kontominas, M. G., Pomonis, P. J., Avlonitis, D., Gergis, V.,* Fuel Proc. Technol. **89** (2008) 503. http://dx.doi.org/10.1016/j.fuproc.2007.10.004
15. *Qian, J., Wang, F., Liu, S., Yun, Z.,* Bioresour. Technol. **99** (2008) 9009. http://dx.doi.org/10.1016/j.biortech.2008.04.059
16. *Johnson, M. B., Zhiyou, W.,* Energy Fuels **23** (2009) 5179. http://dx.doi.org/10.1021/ef900704h
17. *Fernandez, C. G., Sialve, B., Bernet, N., Steyer, J. P.,* Biomass Bioenergy **40** (2012) 105. http://dx.doi.org/10.1016/j.biombioe.2012.02.008
18. *Matsunaga, T., Matsumoto, M., Maeda, Y., Sugiyama, H., Sato, R., Tanaka, T.,* Biotechnol. Lett. **31** (2009) 1367. http://dx.doi.org/10.1007/s10529–009–0029-y
19. *Kim, N. Y., Oh, S. H., Lee, H. Y., Lee, S. Y.,* KSBB J. **25** (2010a) 363.
20. *Kim, N. Y., Oh, S. H., Lee, H. Y., Lee, S. Y.,* KSBB J. **25** (2010b) 371.
21. *Cho, S. C., Choi, W. Y., Oh, S. H., Lee, C. G., Seo, Y. C., Kim, J. S., Song, C. H., Kim, G. V., Lee, S. Y., Kang, D. H., Lee, H. Y.,* J. Biomed. Biotechnol. **2012** (2012) 1. http://dx.doi.org/10.1155/2012/359432
22. *Choi, W. Y., Lee, C. G., Ahn, J. H., Seo, Y. C., Lee, S. E., Jung, K. H., Kang, D. H., Cho, J. S., Choi, G. P., Lee, H. Y.,* JSBB. J. **26** (2011) 400.
23. *Obibuzor, J. U., Abigor, R. D., Okiy, D. A.,* JAOCS. **80** (2003) 77. http://dx.doi.org/10.1007/s11746–003–0654–8
24. *Ichihara, K., Shibahara, A., Yamamoto, K., Nakayama, T.,* Lipids **31** (1996) 535. http://dx.doi.org/10.1007/BF02522648
25. *Liu, B., Zhao, Z. J.,* Chem. Technol. Biotechnol. **82** (2007) 775. http://dx.doi.org/10.1002/jctb.1744
26. *Yang, W. H., Tarng, Y. S.,* J. Mater. Process. Technol. **84** (1998) 122. http://dx.doi.org/10.1016/S0924–0136(98)00079-X
27. *Lu, X., Chlang, H. H., Fong, L., Zhao, J., Chen, S. C. S.,* Polym. Eng. Sci. **39** (1999) 62. http://dx.doi.org/10.1002/pen.11397
28. *Bisalputra, T., Weier, T. E.,* Am. J. Bot. **50** (1963) 1011. http://dx.doi.org/10.2307/2439909
29. *Tukaj, Z.*, Protoplasma **151** (1989) 47. http://dx.doi.org/10.1007/BF01403300
30. *Lesser, M. P., Barry, T. M., Banaszak,A. T.,* J. Phycol. **38** (2002) 473. http://dx.doi.org/10.1046/j.1529–8817.2002.01171.x
31. *Kim, M. K., Park, J. W., Park, C. S., Kim, S. J., Jeune, K. H., Chand, M. U., Acreman, J.,* Bioresour. Technol. **98** (2007) 2220. http://dx.doi.org/10.1016/j.biortech.2006.08.031
32. *Shimi, H. I. E., Attia, N. K., Sheltawy, S. T. E., Diwani, G. I. E.,* J. Sustain. Bioenergy Syst. **3** (2013) 224. http://dx.doi.org/10.4236/jsbs.2013.33031
33. *Orta, S. B. V., Lee, J. G. M., Harvey, A. P.,* Biochem. Eng. J. **76** (2013) 83. http://dx.doi.org/10.1016/j.bej.2013.04.003
34. *Revellame, E., Hernandez, R., French Holmes, W., Alley, E.,* J. Chem. Technol. Biotechnol. **85** (2010) 614. http://dx.doi.org/10.1002/jctb.2317
35. *Ehimen, E. A., Sun, Z. F., Carrington, C. G.,* Fuel **89** (2010) 677. http://dx.doi.org/10.1016/j.fuel.2009.10.011
36. *Ramadhas, A. S., Jayaraj, S., Muraleedharan, C.,* Fuel **84** (2005) 335. http://dx.doi.org/10.1016/j.fuel.2004.09.016
37. *Watanabe, K., Oura, T., Sakai, H., Kajiwara, S.,* Biosci. Biotechnol. Biochem. **68** (2004) 721. http://dx.doi.org/10.1271/bbb.68.721
38. *Kim, N. Y., Oh, S. H., Lee, H. Y., Lee, S. Y.,* KSBB J. **25** (2010) 371.
39. *Knothe, G., Matheaus, A. C., Ryan, T. W.,* Fuel **82** (2003) 971. http://dx.doi.org/10.1016/S0016–2361(02)00382–4
40. *Kinney, A. J., Clemente, T. E.,* Fuel Proc. Technol. **86** (2005) 1137. http://dx.doi.org/10.1016/j.fuproc.2004.11.008
41. *Knothe, G.,* Fuel Proc. Tech. **86** (2005) 1059. http://dx.doi.org/10.1016/j.fuproc.2004.11.002
42. *Park, J. I., Woo, H. C., Lee, J. H.,* Korean Chem. Eng. Res. **46** (2008) 833.