

Original Research Article

Mutagenesis Development of Microalgae *Nannochloropsis* sp. To Produce Biofuel using EMS (Ethyl Methanesulfonate)

ABSTRACT (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

Microalgae ability to grow substantially in a short period of time has become a starting point in rapid biofuel production. To date, only certain microalgae species that produce large biomass and has high lipid acid content can be turned into raw material in producing biofuel. One of the methods used in increasing microalgae biomass is the mutagenesis method. Mutagenesis can be conducted chemically by adding certain compound, ethyl methanesulfonate (EMS) into microalgae individual cells. This research showed that the highest density among three treatments (control, 0.1 M and 0.5 M) occurred at 0.5 M EMS treatment, with a number of $60,7 \pm 11,93 \times 10^6$ individual cells/mL on day 6 (stationery phase). Among the three treatments, highest specific growth occurred at 0.1 M EMS treatment (0.52/day) on day 5 (logarithmic phase), and treatment on 0.5 M EMS has highest RNA/DNA ratio (0.55 ± 0.46), while control has the lowest ratio (0.12 ± 0.04). In dry biomass, the highest number stationery and death phase occurred in *Nannochloropsis* sp. cultivation with EMS concentration of 0.1 M (1.08 ± 0.33 dan 1.11 ± 0.07 gr/L). Fat content percentage in both phase occurred in ~~*Nannochloropsis* sp. cultivation with~~ EMS concentration of 0.5 M (12.17 ± 0.30 dan 18.14 ± 0.35). Chromatography test revealed 22 compounds of saturated fatty acid and 3 compounds of unsaturated fatty acid. Methyl palmitate (C16:0) was the saturated fatty acid with the highest concentration in three cultivation treatments. Mono unsaturated fatty acid (MUFA) with a relatively high concentration is methyl palmitoleate in two cultivation treatments. The only poly unsaturated fatty acid detected was methyl linoleate (C18:2).

Keywords: *Nannochloropsis* sp., mutagenesis, ethyl methanesulphonate, fatty acid, biomass.

1. INTRODUCTION (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

Microalgae is known as one of the oldest life forms. It is a primitive vegetation (thallophyta) that has no root, stock nor leaf. It also doesn't have sterile enclosure cells surrounding its reproductive cell and has chlorophyll as its primer photosynthesis pigment (Brennan and Owende, 2009). With chlorophyll, microalgae can conduct photosynthesis process much like macro vegetation and can produce food compound needed for its growth process, such as glucose. Microalgae synthesize fatty acid as building blocks to form various lipid compounds, such as polarized structural lipid namely phospholipid and glycolipid together with neutral lipid such as monoglycerine, diglyceride (DAGs) and triglyceride (TAG), free fatty acid, hydrocarbon and pigment. Limited polarized lipid is located in organelle membrane within the cell, such as tilacoide in chloroplast. TAG is a neutral lipid stored in vacuola inside cell (Olofsson et al., 2012).

High C18:0 and C18:1 content inside microalgae is the precondition in biodiesel production for their oxidative stability and high adaptation ability potential for industrial scale production (Olofsson et al., 2012). C16:0 is also proposed as suitable fatty acid in renewable biodiesel production. Both are rarely found in *Nannochloropsis* sp. cells. But with other

30 MUFA besides stearate acid, palmitate acid, and oleate acid, this microalgae species can
31 become a potential biofuel material species.

32 Researches on microalgae mutated by ethyl methanesulfonate are still rare. A few
33 notable studies in this subjects are by Ong et al. (2010) about microalga species *Chlorella*
34 sp. characterization towards high temperature, Chaturvedi and Fujita (2006) about EPA fatty
35 acid compound of mutated *Nannochloropsis* sp. species, also Doan and Obbard (2012) who
36 explored lipid compound increase of of mutated *Nannochloropsis* sp. species.

37 From the research conducted by Ong et al. (2010), it is known that *Chlorella* sp. that
38 has been mutated and cultivated in 40°C temperature has higher specific growth compared
39 to its original form. Also, higher lipid productivity of the mutated species cultivated with high
40 temperature is higher compared to its original form. From another research conducted by
41 Chaturvedi and Fujita (2006), it is proven that EPA type fatty acid can be boosted by
42 applying mutagenesis on *Nannochloropsis oculata*. Research by Doan and Obbard (2012),
43 that tested lipid content increase of *Nannochloropsis* sp., it is known that, mutated
44 microalgae species can produce more fatty acid compared to its original state, both in
45 exponential and stationery phase.

46 
47 ~~Mutagenesis method used in this research is a chemical one, by adding certain~~
48 ~~compound known as ethyl methanesulfonate (EMS) into microalgae individual cell.~~ This
49 mutagenesis method is relatively new and complicated. Thus, the goal of this this research is
50 to study the role of ethyl methanesulfonate in mutation induction, especially in
51 *Nannochloropsis* sp. and to identify proximate cell composition (carbohydrate, protein, fat)
52 and fatty acid in microalgae *Nannochloropsis* sp., that has been injected by ethyl
53 methanesulfonate (EMS).

54 55 **2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY** 56 **(ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)**

57 58 **2.1 Microalgae Cultivation**

59 In laboratory scale cultivation, one third of the innoculant was submerged inside sterilized
60 seawater that has been added by culture medium. Then, this culture pot is put on the
61 cultivation rack, under lamp light and aerated. The medium used is Guillard (f2). In the
62 logarithmic phase, 500 mL of the original cultivation species was placed inside an
63 erlenmeyer to be mutated with 0,1 M and 0,5 M of ethyl methanesulfonate (Sigma, USA) for
64 60 minutes with medium rate of shakes, under microalgae normal growth condition. In such
65 condition, as many as 55-60% of the individual cells would die. Then, the microalgae cells
66 were washed three times using sterilized seawater to remove all EMS excess in the mixed
67 microalgae. The microalgae and EMS mixture were re-suspended using 10 mL f/2 medium
68 and left to grow for further selection. The mutated microalgae cells (2×10^7 individual cells/
69 mL) was evenly dispersed on a plate (1.2% agar). After three weeks incubation at a
70 temperature of 25°C, the remaining cell colonies within the plate **are** counted and each
71 colony was inoculated with 10 mL seawater medium (Chaturvedi and Fujita, 2006). For
72 twelve days, microalgae density was measured using Haemacytometer and hand counter
73 when observed through microscope.

74 **2.2 DNA and RNA Extraction**

75 The method **use** in extracting DNA and RNA from *Nannochloropsis* sp. **is** by using Qiagen
76 Dneasy Plant Mini Kit for DNA extraction and Qiagen Rneasy Plant Mini Kit for RNA
77 extraction. Especially for RNA extraction, ethanol 96% and beta mercaptoethanolare needed
78 as primer solvent. Method for RNA extraction is similar to DNA extraction. But, RNA
79 molecules are relatively shorter and harder to break with shearing thus cell disruption could

be done more aggressively. For RNA extraction, glove **is** a requirement and medium used for isolation must contain strong detergent to denature remaining RNase. After the extraction process **is** done, sample results were analyzed using Nanophotometer in order to calculate its DNA and RNA concentration.

2.3 Microalgae harvesting

Harvesting the microalgae was conducted by filtration using vacuum pump and filter paper. Harvested microalgae biomass was measured during its exponential and death phase to compare the fat content in each phase. The harvested biomass was dried by sun exposure or using oven. Its powder product, was the analyzed further, including weighing the biomass. The result of the weighing was noted and compared per each treatment.

2.4 Lipid content

Extraction of microalgae to produce oil was conducted using liquid hexane (Bligh and Dyer, 1959). The harvested microalgae samples was weighed and wrapped using filter paper and sealed using fat-free cotton. The samples was the extracted using 200 ml n-hexane for 6-7 hours inside soxhlet tube. The lipid extract was the vaporized using distillation equipment and then dried inside an oven with 50-60 °C for an hour and the weighed to obtain lipid content value.

2.5 Protein and carbohydrate content

Crude protein content was measured with titration method using kjehldahl tube. The crude protein analysis principal is nitrogen was transformed into ammonium sulphate by strong H₂SO₄. The ammonium sulphate was then degraded into NaOH. Released ammonia was then bonded with boric acid and titrated with standard acid liquid (SN/ 01-2891-1992). Carbohydrate content was measured using phenol titration (Dubois et al., 1956). This method is also known as TS (total sugar) method used to measured total sugar content. This method can measure two reducing sugar molecules. Simple sugar, oligosaccharide and it derivatives can be detected with phenol in strong sulfuric acid that would produce a stable yellowish orange color.

2.6 Esterification

Esterification is intended to lower vaporizing point of fatty acid by changing the fatty structure into ester thus relatively easier to be analyzed using GC-MS. 0.5 – 1 gram of extracted fat sample was saponified using 4.5 ml NaOH 0.5 N, and poured into reaction tube and mixed with BF₃ in methanol. The mixture was then shaken and heated for 15 minutes. When the mixtures settled two layers were formed. The upper layer was separated by centrifuge and purified by adding Na₂SO₄ to omit its content. This esterification product was then placed inside a vial to be analyzed using GC-MS (Hermanto et al. 2008).

2.7 Gas Chromatography - Mass Spectrometry (GC-MS) analysis

Gas Chromatography-Mass Spectrometry (GC-MS) analysis used Shimadzu QP2010 chromatography gas equipped with DB-5 ms silica column (length 30 m; inner diameter 0.25 mm ; dan 0.25 µm film layer thickness) and helium as boosting gas. Gas chromatography has detection limit of 0.001 ppb. Gas chromatography uses split injection method with a ration of 1 : 200. The oven temperature of gas chromatography was set at 80°C and constant for 2 minutes, then raised to with the rate of 100/minute and then constant for the next minute, and then raised again into 280°C with a rate of 60/minute and then constant for the next five minutes. GC-MS condition are potential ionization/ electron energy 70eV, ion source temperature 250°C and interface temperature 280°C. Full mass data was measured between 50-400 Dalton per second. The retention period is 0 to 32.67 minutes. Data was recorded and analyzed using GC-MS Real Time Analysis and GCMS Postrun Analysis.

127 **2.8 Fatty acid identification**

128 Fatty acid identification was conducted using gas chromatography and gas chromatography-
129 mass spectrometry. Fatty acid methyl ester was identified by comparing *mass spectra* with
130 literature data. Carbon number in methyl esters compounds was determined by calculating
131 molecule weight that appeared in mass spectra. Characteristic of fatty acid methyl ester is a
132 powerful peak at $m/z = 74$, which is straight structure methyl ester basic peak. Widest peak
133 in each group represented fragment of $C_nH_{2n-1}O_2$ and $m/z = 14(n-2) + 74$. It can be
134 simply formulated as:

$$135 \quad C_x = \frac{m-74}{14} + 2$$

136 where: x = carbon number (FAME)
137 m = molecule weight appeared in mass spectra peak
138 14 = molecule weight of CH_2
139

140 **2.9 Data Analysis**

141 **2.9.1 Growth rate analysis**

142 The observation of cell amount was conducted each day with each sampling was
143 repeated three times where one drop was taken from each sample, and then observed
144 under a microscope with 100 or 400 magnification, with 5 field view. Microalgae density can
145 be calculated with formula:

$$146 \quad N = \left(\frac{n}{4} \right) \times 10^6$$

147 where: N = microalgae density (ind/mL);
148 n = amount of microalgae observed
149

150 **2.9.2 Microalgae RNA/DNA ratio analysis**

151 RNA/DNA ratio can be calculated after the amount of RNA and DNA are obtained.
152 The formula for calculating the ratio is as follow:

$$153 \quad \text{Ratio} = \text{RNA} / \text{DNA}$$

154 where: RNA : amount of RNA extracted
155 DNA : amount of DNA extracted
156

157 **2.9.3 Lipid content analysis**

158 Lipid content can be calculated with formula:

$$159 \quad \% \text{ lipid} = \frac{W_2 - W_1}{W} \times 100\%$$

160 where:
161 W_2 = sample weight after extraction (gram)
162 W_1 = sample weight before extraction (gram)
163 W = sample weight (gram)
164

165 **2.9.4 Protein content analysis**

166 Protein content can be calculated with formula:

$$\text{Protein content} = \frac{(V1 - V2) \times N \times 0.014 \times f.k \times f.p}{w}$$

where:

W = sample weight

V1 = volume of HCl 0,01 N that was used for sample titration

V2 = volume of HCl that was used for blanco titration

N = normality FICI

f.k = conversion factor for protein and food as a general: 6,25 for milk & its products:

638 peanut butter: 5,46

fp = dilution factor

Carbohydrate content analysis

Carbohydrate content can be calculated with formula:

$$\text{Total sugar(\%)} = ((G \times FP) / W) \times 100$$

Whereas :

G = sugar concentration form standard curve (gram)

FP = dilution factor

W = sample weight (gram)

3. RESULTS AND DISCUSSION

3.1 Microalgae Growth Rate

In the 12 days cultivation period, *Nannochloropsis* sp. experienced fluctuating growth rate. Highest density occurred in day 4 for control, and in two other EMS treatments highest density occurred in day 6. The highest density in all three treatment occurred in EMS 0.5 M treatment with a number of $60,7 \pm 11,93 (x 10^6)$ individual cells/mL. Highest specific growth rate in control occurred in day 1, while in the other two treatments it occurred in day 5. Among those three treatment, highest specific growth occurred in EMS 0.1 M treatment (Table 1).

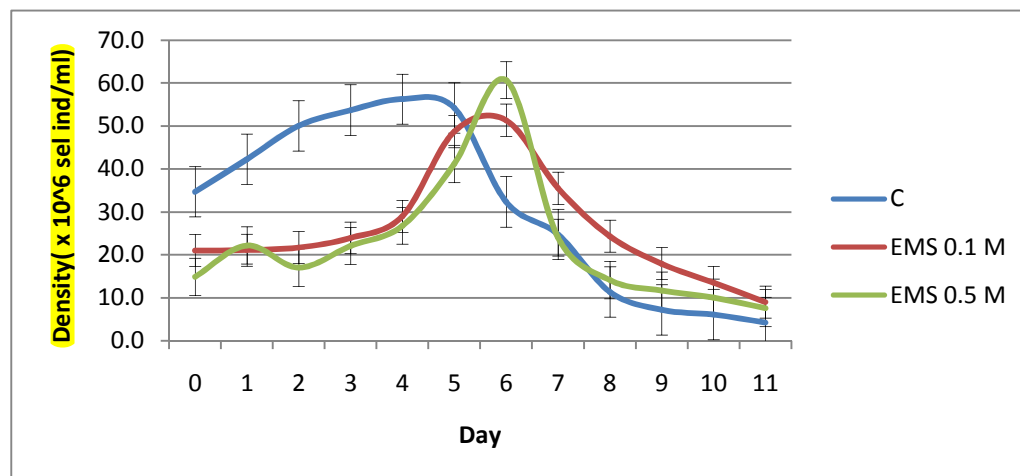
Table 1. Density and specific growth rate of *Nannochloropsis* sp. cultivation with three treatments

Day-	Control (K) (x 10 ⁶ cells/ml)	μ (day)	0.1 M (x 10 ⁶ cells/ml)	μ(day)	0.5 M (x 10 ⁶ cells/ml)	μ(day)
0	34.7±10.73	-	21±2.30	-	14.8±2.63	-
1	42.3±14.64	0.20	21.1±7.74	0.003	22.2±3.18	0.40
2	50±19.47	0.17	21.7±8.78	0.03	17±8.68	-0.27
3	53.7±19.35	0.07	23.9±7.95	0.10	22.1±7.18	0.26
4	56.3±18.90	0.05	29±6.01	0.19	26.8±5.15	0.19
5	54.2±13.39	-0.04	48.7±7.81	0.52	41.2±9.15	0.43
6	32.3±3.85	-0.52	51.3±8.18	0.05	60.7±11.93	0.39
7	24.8±9.55	-0.27	35.5±4.36	-0.37	24±3.61	-0.93
8	11.3±4.94	-0.78	24.3±3.14	-0.38	14.1±4.75	-0.53
9	7.2±5.57	-0.46	17.9±1.23	-0.31	11.7±5.03	-0.19
10	6.1±4.21	-0.16	13.5±1.95	-0.28	10±5.30	-0.15

11	4.3±2.47	-0.36	9±2.48	-0.41	7.6±5.78	-0.28
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198

199 Figure 1 shows that growth phase occurrence in EMS treatment cultivation is
 200 different to one in control cultivation. Lag or initial phase in control occurred in day 0 and
 201 straight to logarithmic phase to day 2. Stationary phase in control occurred in day 4, and
 202 then death phase occurred from day 6 to day 11. In 0.1 EMS treatment, lag phase started in
 203 day 0 and continued to day 3, and then logarithmic phase occurred in day 4. Stationary
 204 phase in control occurred in day 6, and then death phase occurred from day 7 to day 11.
 205 The phases in 0.5 EMS treatment are similar to the ones in 0.1 EMS treatment.



206

207 **Figure 1. *Nannochloropsis* sp. growth rate with three cultivation treatment for 12 days**
 208 (Axis Y: Density (x 10⁶ cells/ml); axis x: day-;)
 209 Mean ± S.E.M = Mean values ± Standard error of means of six experiments
 210

211 Among three treatments, cultivation with 0.5 M EMS concentration has the highest
 212 density at day 6 (stationary phase). For specific growth rate, cultivation with 0.1 EMS
 213 concentration has the highest value (0.52/day) in day 5 (logarithmic phase). This value is
 214 higher than specific growth rate of highest density with 0.5 EMS concentration (0.39/day).
 215 When compared to control cultivation, specific growth rate in both EMS treatment has higher
 216 value, 0.52/day and 0.43/day in day 5. While at control, highest specific growth occurred in
 217 day 2 with a value of 0.20/day.

218 Specific growth rate in this research show similar value with the research conducted
 219 by Doan and Obbard (2012), 0.56/day for original species cultivation and 0.58/day for mutant
 220 species cultivation with certain EMS concentration. That research stated that, cultivation
 221 experiment that use higher concentration of EMS would produce lower density, when
 222 compared to its original species. It is proven by this research, where specific growth rate
 223 went lower from 0.1 to 0.5 M EMS concentration.

224 In a research by Chaturvedi and Fujita (2006), *Nannochloropsis* sp. was given 0,1 M
 225 EMS for certain herbicide substance resistance has higher specific growth rate than original
 226 species. This suits with recent research result that indicated genetic transformation within
 227 the cell of *Nannochloropsis* sp. without losing its morphology and genome form.

Ong et al. (2010) stated that *Chlorella* sp. mutated with 0.1 M EMS and cultivated in higher temperature from the original species has higher specific growth rate. This is in accordance with this research where *Nannochloropsis* sp. growth rate in the same EMS concentration gave higher value than its original species.

However, when compared to Anandarajah et al. (2012) research, growth rate in this research is lower. That Research which uses EMS resulted in *Nannochloropsis* sp. specific growth rate value of 0.72/day from its original species and 0.9/day from mutant species, with cultivation with bright lighting for 6 days. It is probably because in that research carbon dioxide had been added as much as 1% during cultivation.

3.2 Microalgae DNA and RNA Ratio

Highest DNA concentration produced occurred in control microalgae cultivation, and the lowest in 0.5 EMS treatment. For RNA concentration, highest value occurred in 0.5 EMS treatment, while the lowest occurred in 0.1 EMS treatment (Table 2).

Table 2. Concentration and ratio of nucleic acid (DNA and RNA) in ng/uL with 3 cultivation treatment of *Nannochloropsis* sp.

Treatment	DNA (ng/uL)	RNA (ng/uL)	RNA/DNA Ratio
K	28.17±8.73	3±0.75	0.12±0.04
0.1	13.73±3.91	2.67±0.09	0.21±0.07
0.5	10.63±6.21	3.6±1.42	0.55±0.46

Control cultivation has highest DNA concentration among other treatments. And for RNA concentration, cultivation with 0.5 EMS treatment has the highest value (3.60±1.42 ng/uL). For RNA/DNA ratio in all three *Nannochloropsis* sp. cultivation, it was concluded that 0.5 EMS treatment has the highest value, while control has the lowest. From that calculation, it can be concluded that *Nannochloropsis* sp. cultivation using 0.5 M EMS concentration enable more increase of DNA and RNA concentration compared to normal cultivation (control).

DNA concentration of *Nannochloropsis* sp. from this research is lower when compared to the research conducted by Nicklisch and Seiberg (2009). That research obtained DNA extraction value from Chlorophyceae (species unknown) of 147 (unit was stated as relative). As with RNA concentration, result from this research is also lower to the one from Nicklisch and Seiberg (2009) research. The result from that research is 18.4. This is probably caused by different method of extraction used, using *flow cytometry* and special coloring.

Compared to the research conducted by Kim et al. (2012) that used *Botryococcus braunii* with Dneasy from Qiagen method, DNA concentration from this research is lower. The previous research achieved 38.75 ng/uL of DNA concentration. This is probably because the differing species used as object, the previous research used freshwater species. Kim et al (2012) did not extract RNA concentration because the result from Qiagen kit was very weak.

According to Adiputra et al. (2012), method for RNA extraction using commercial kit rely on silica membrane to tie the vegetation total RNA, and wash all inhibitors through the membrane that gave very pure RNA. However, commercial kit does not always provide good

268 amplified result for all type of vegetation. In that research, total RNA concentration produced
269 are 2.92 – 7.52 µg/mL.

270 According to Nicklisch and Seinberg (2009), DNA/RNA ratio is predicted to have a
271 connection with microalgae and depends to microalgae pigment. However, from that
272 research, connection between RNA/DNA ratio and specific growth rate for Chlorophyceae
273 algae are not significantly correlated.

274 In this research, RNA/DNA ratio of 0.5 M EMS cultivated samples are higher than
275 EMS 0.1 M EMS samples. This result is not in line with specific growth rate regression from
276 EMS 0.1 M to MES 0.5 M. Thus, Nicklisch and Seinberg (2009) statement that there is no
277 correlation between RNA/DNA ratio and specific growth rate is proven, especially to species
278 that belong to Chlorophyceae class.

279 3. Biomass and proximate content of microalgae

280 Result of biomass gravimetry from *Nannochloropsis* sp. microalgae cultivation was
281 measured in 2 days, shows different growth phase, stationary phase (day 6) and death
282 phase (day 7) (Table 3).

283 **Table 3. Wet and dry biomass of *Nannochloropsis* sp. In three cultivation treatment**
284

Treatment	Wet biomass (gr/L)	Dry biomass (gr/L)
	H-6 (stationery phase)	
K	6.60±0.28	0.79±0.22
0.1 M	2.85±0.10	1.08±0.33
0.5 M	1.55±0.07	0.81±0.21
H-7 (death phase)		
K	7.99±0.02	0.91±0.06
0.1 M	10.63±0.15	1.11±0.07
0.5 M	6.75±0.05	0.82±0.03

285
286 In stationary phase, highest wet biomass of *Nannochloropsis* sp. value occurred in control
287 cultivation (6.60±0.28 gr/L). Different from the death phase, wet biomass is highest in
288 cultivation with 0.1 M concentration EMS (10.63±0.15 gr/L). For dry biomass, the highest
289 values in both phases occurred in cultivation with 0.1 M concentration EMS (1.08±0.33 and
290 1.11±0.07 gr/L). This is made possible because water content during harvest is higher
291 compared to the amount of harvested individual, thus add to the wet biomass.

292 Biomass obtained in this research is slightly higher compared to the Doan and
293 Obbard (2012) research. That research stated that biomass of *Nannochloropsis* sp. from
294 original species and mutant cultivation were 0.20±0.02 and 0.22±0.08 gr/L at the last day.
295 From that result, Doan and Obbard (2012) stated that experiment using high concentration
296 of EMS (1 M) did not have significant effect on *Nannochloropsis* sp. biomass during the
297 experiment.

298 Compared to the research by Ong et al. (2010), this research have similar biomass
299 values. Previous research results range are 0.6 - 1.2 g/L for mutated *Chlorella* sp. with 0.1 M
300 EMS and 0.4 - 0.9 g/L for its original form with 40° C cultivation temperature. Thus, this
301 research produced mutant species of *Nannochloropsis* sp. that has high biomass potential.

Proximate content consists of three parameters: lipid, carbohydrate and protein. As with biomass, lipid content was also measured during two phases, stationary and death phase (Table 4).

Table 4. Proximate content of *Nannochloropsis* sp. In three cultivation treatments

Treatment	Lipid (%)		Karbohidrat (%)	Protein (%)
	H-6	H-7		
K	8.32±0.44	5.53±0.04	1.42±0.02	4.93±0.61
0.1 M	11.30±0.01	16.14±0.47	1.11±0.18	6.08±0.42
0.5 M	12.17±0.30	18.14±0.35	1.18±0.16	5.89±0.57

Highest lipid content in both phases occurred in *Nannochloropsis* sp. cultivation with 0.5 M EMS treatment. Highest carbohydrate content occurred in control cultivation treatment. Highest protein content in both phases occurred in *Nannochloropsis* sp. cultivation with 0.1 M EMS treatment. Highest lipid content, shown in table 4, occurred in 0.5 M EMS cultivation treatment, inversely compared to biomass value (table 3).

Highest biomass occurred in *Nannochloropsis* sp. cultivation with 0,1 M EMS. Lipid content in 0.1 M EMS concentration is also considerably high, but this research also discovered that high biomass does not always resulted in high lipid content. High amount of biomass in *Nannochloropsis* sp. with 0,1 M EMS, gave more protein content compared to lipid content in the cells. While biomass in *Nannochloropsis* sp. with 0,5 M EMS produced more lipid content than protein content the cells. However, when compared, lipid content in both EMS treatment are not significantly different. Carbohydrate value in both EMS treatment are also not significantly different.

Lipid content in this research is higher than the result from Doan and Obbard (2012). That research stated that lipid content from mutant species cultivation was 1.5 to 2 time as much as original species cultivation at both growth phases (exponential and stationary). While in this research, lipid content from 0.5 M mutant cultivation increased as much as 1.5 to 3.3 time the content from control cultivation at stationary and death phase. This shows that there is a similarity in harvest period for *Nannochloropsis* sp., that is best to conducted at the end of stationary phase to get more lipid content.

Another microalgae species that has been tested using EMS method is *Chlamydomonas reinhardtii*. Lee et al. (2014) stated that after randomly mutated by EMS, that species produced more lipid content than its original form. At exponential phase, mutant species lipid content is twice as much as the original species. And at the stationary phase, lipid content in mutant species did not show any significant rise. This fact in line with this research's result where *Nannochloropsis* sp. that had been given certain amount of EMS produced more lipid than the original species, at both exponential and stationary phase. Thus, this research and the one previously mentioned showed that a possibility where EMS could produce more lipid content in microalgae generation that has been mutated by random mutagenesis method.

Carbohydrate and protein content in this research did not show higher values compared to other research that did not use EMS mutagen. In the research conducted by Hu and Gao (2003), carbohydrate and protein content in *Nannochloropsis* sp. after cultivated with mixotrophia using carbon dioxide-photoautotrophic gas are 7–13% and 34–41%. Those

values are much higher compared to this research's result. The same result also occurred in the research by Xiao et al (2013), where protein and carbohydrate content of *Nannochloropsis oceanica* that has been cultivated by reducing nitrogen compounds are 4,26-7,3% and 4,72-6,62%. It can be concluded that EMS mutagens does not give significant difference compared to other standard cultivation treatments, like carbon dioxide gas injection and nitrogen compound reduction on media culture.

4. Microalgae fatty acid compounds characterization

Identification process using gas chromatography in this research showed that there are 25 fatty acid compounds, in the form of saturated fatty acid and mono unsaturated fatty acid (MUFA) (Table 5). There is only 1 poly unsaturated fatty acid (PUFA) detected in this research.

Table 5. Fatty acid compound from chromatography gas identification

No	Compound	K h-7	K h-8	0.1 M h-7	0.1 M h-8	0.5 M h-7	0.5 M h-8
SFA							
1	methyl caprylate				0.230±0.040		
2	methyl laurate	0.785±0.045	1.025±0.055	2.965±0.165	0.790±0.250	0.335±0.005	0.56
3	methyl dodecanoate			0.37			
4	methyl myristate	2.740±0.250	3.685±0.025	8.645±0.555	13.140±4.780	6.705±0.125	8.92
5	methyl tridecanoate	0.385±0.025	0.61	1.365±0.375			
6	methyl pentadecanoate	0.680±0.090	0.770±0.070	1.180±0.070	1.515±0.525	0.61	0.97
7	methyl tetradecanoate	0.315±0.015	1.04	1.900±0.120			
8	methyl palmitate	20.360±2.070	51.405±0.285	18.58	56.085±15.325	67.70±0.05	57.325±0.735
9	methyl margarate	0.900±0.040	0.83	1.765±0.015	1.320±0.470		1.01±0.05
10	methyl octadecanoate			3.815±0.505	8.52		
11	methyl stearate	10.200±0.910	13.360±0.350	9.510±0.590	3.90	3.605±0.055	4.90±0.09
12	methyl tricosanoate			1.36			
13	methyl erucate	1.115±0.145	1.135±0.075	1.620±0.110	0.475±0.145	0.175±0.015	0.275±0.005
14	methyl heneicosanoic			1.155±0.065	0.25		
15	methyl behenate	9.380±5.930	2.815±0.025	3.020±0.170	0.425±0.155	0.215±0.035	0.19±0.04
16	methyl tricosanoate			0.765±0.045			
17	methyl lignocerate	4.700±2.620	1.880±0.140	1.265±0.105			
18	methyl azelate				0.32		
No	Compound	K h-7	K h-8	0.1 M h-7	0.1 M h-8	0.5 M h-7	0.5 M h-8
19	methyl hexadecadienoic				1.34		
20	methyl nonanoate				0.24		
21	dimethyl azelate				0.57		
22	methyl hexadecanoate					0.61±0.02	
TOTAL		51.560±12.140	78.555±1.030	59.280±2.890	89.120±21.690	79.955±0.305	74.150±0.920
MUFA & PUFA							
1	methyl oleate	4.99±0.70	3.775±0.035		2.70	5.46±0.01	4.775±0.105
2	methyl palmitoleate			6.83±0.44	10.26±3.76	12.625±0.055	17.59±0.42
3	methyl linoleate (PUFA)	2.13±0.23	1.31	2.41		0.57	
TOTAL		7.12±0.93	5.085±0.035	9.24±0.44	12.96±3.76	18.655±0.065	22.365±0.525

In the table above, there are 22 type of compounds of saturated fatty acid and 2 type of mono unsaturated fatty acid compounds. The only poly unsaturated fatty acid detected is

358 methyl linoleat with low concentration. Saturated fatty acid with the highest concentration **is**
 359 methyl palmitat in all three treatments. This compound has highest area percentage among
 360 all detected saturated fatty acids. There are also 2 saturated fatty acid with high values in all
 361 three treatments: methyl stearat and methyl miristate.

362 Among three treatments, highest percentage mostly found at 0.1 M EMS treatment,
 363 such as methyl palmitate and methyl miristate in 0.5 M EMS treatment, those two
 364 compounds of saturated fatty acid have low percentage area. Highest total percentage area
 365 of saturated fatty acid (SFA) occurred in 0.1 M EMS concentration (day 8). While lowest total
 366 percentage area occurred in control cultivation (day 7). Thus, *Nannochloropsis* sp. cultivation
 367 treatment with 0.1 M EMS produced more saturated fatty acid than 0.5 M EMS treatment
 368 and control.

369 Mono unsaturated fatty acid (MUFA) compounds detected in two treatments were in
 370 high area percentage, especially methyl palmitoleate. In control, methyl palmitoleate was not
 371 detected. This compound has higher area percentage in 0.5 M EMS treatment compared to
 372 0.1 M EMS treatment. Similar condition also applied to methyl oleate, where its area
 373 percentage in 0.5 M EMS treatment was higher compared to it percentage on control or 0.1
 374 M EMS cultivation treatment. Total area percentage of both fatty acid compounds is higher
 375 compared to other cultivation treatments (control and 0.5 M EMS concentration). From
 376 MUFA analysis, it can be concluded that *Nannochloropsis* sp. cultivation with 0.5 M EMS
 377 concentration was more effective in increasing MUFA concentration compared to control and
 378 0.1 M EMS cultivation.

379 The only poly unsaturated fatty acid detected is methyl linoleate with a very small
 380 area percentage in all cultivation treatments. Among all three, cultivation with 0.1 M EMS
 381 has higher area percentage. It can be concluded that, 0.1 M EMS treatment can more
 382 effectively increase PUFA level compared to *Nannochloropsis* sp. control cultivation.

383 When compared to the research conducted by Doan and Oboard (2012), total
 384 saturated fatty acid (SFA) in this research is higher. The total saturated fatty acid in previous
 385 research are 43.8 and 49.1 in original species cultivation  42.5 and 45.9 in EMS mutated
 386 species cultivation. Total MUFA and PUFA in the **previous research** are higher compared to
 387 this research. Total MUFA of original species cultivation from the previous research are 34.8
 388 and 36.6, and 42.8 and 45.8 in EMS mutated species cultivation. Total PUFA of original
 389 species cultivation from the previous research are 21.4 and 14.3, and 14.6 and 8.3 in EMS
 390 mutated species cultivation.

391 Result from Ma et al. (2013) research, SFA total from the original species and the
 392 mutated one are 55.4 ± 1.7 and 58.1 ± 1.7 , respectively. This result is lower compared to this
 393 research; where cultivation of original species and mutated one have 78.56 ± 1.03 and 89.12
 394 ± 21.69 , respectively. For MUA and PUFA total, Ma et al. (2013) obtained higher value than
 395 this research, with values of 31.8 ± 1.9 and 12.8 ± 0.6 for original species and 29.7 ± 1.5 and
 396 12.2 ± 1.3 for mutant species.

397 Anandarajah et al. (2012) stated that methyl miristate and methyl palmitate are SFA
 398 that increased 63% in his research. Chaturvedi and Fujita (2006) reported that miristate acid
 399 and palmitate acid are dominant compounds in their research which used EMS for herbicide
 400 and antibiotic resistance. Myristic acid experienced an increased as many as 3.7 to 4.6 times
 401 to cerulenin and eritromicin. While palmitate acid increased as many as 26% and 15%. The
 402 same thing also occurred in this research where both compounds have the highest
 403 concentration compared to other fatty acid, and also higher compared to the original species

404 cultivation. While Doan and Obbard (2012), stated that miristate acid dan palmitate acid
405 content are lower compared to its original species.

406 For MUFA group, Ma et al. (2013) research revealed that methyl oleate as one of
407 the dominant compounds. Meanwhile, this research gives different result, where methyl
408 oleate in EMS concentrated cultivation give lower value when compared to the original
409 species cultivation. Methyl palmitoleate is the dominant MUFA compound in this research
410 form two EMS concentration (10% and 18%). Palmitoleate acid increase also occurred in
411 Chaturvedi and Fujita (2006) research with similar value. The increase was 11% and 8% for
412 cerulenin and eritomicin resistance. Doan and Obbard (2012) research also resulted in an
413 increase of palmitoleic acid as much as 30% compared to its original species. That value is
414 significantly higher compared to the one obtained in this research.

415

416 4. CONCLUSION

417

418 The use of EMS mutagen does not significantly affect carbohydrate and protein content but it
419 increased the lipid content in *Nannochloropsis* sp. Three fatty acid compounds: oleate acid,
420 stearate acid and palmitate acid detected in this research showed that a percentage area
421 suitable for biodiesel raw material. Methyl palmitate is the fatty acid with high percentage
422 area in SFA group. The existence of those three fatty acid compounds and with low PUFA
423 content, *Nannochloropsis* sp. That received cultivation treatment with EMS in certain
424 concentration can produce potential value as biodiesel raw material.

425

426 CONSENT (WHERE EVER APPLICABLE)

427

428 Authors may use the following wordings for this section: "All authors declare that 'written
429 informed consent was obtained from the patient (or other approved parties) for publication of
430 this case report and accompanying images. A copy of the written consent is available for
431 review by the Editorial office/Chief Editor/Editorial Board members of this journal."

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