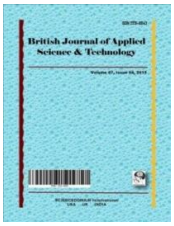

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Chemical Mutagenesis of Microalgae *Nannochloropsis* sp. Using EMS (Ethyl Methanesulfonate)

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Authors' contributions

This work was carried out in collaboration between all authors. Author MK designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Authors AOS, JH and DA managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

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 fatty acid content can be turned into raw material in producing biofuel. One of the methods used in increasing microalgae biomass is the mutagenesis method. This mutagenesis research were conducted chemically by adding ethyl methanesulfonate (EMS) into microalgae individual cells. The results showed the highest density among three treatments (control, 0.1 M and 0.5 M)

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occurred at 0.5 M EMS treatment, with a number of 60.7±11.93 x 10^6 cells/mL on day 6 (stationery phase). Among the three treatments, the highest specific growth occurred at 0.1 M EMS treatment (μ= 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 and 1.11±0.07 g/l). Fat content percentage in both phase occured in EMS concentration of 0.5 M (12.17±0.30% and 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

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 [1]. 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 [2].

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 [2]. C16:0 is also proposed as suitable fatty acid in renewable biodiesel production. Both are rarely found in *Nannochloropsis* sp. cells. But with other MUFA besides stearate acid, palmitate acid, and oleate acid, this microalgae species can become a potential biofuel material species.

Researches on microalgae mutated by ethyl methanesulfonate are still rare. A few notable studies in this subjects such as Ong et al. [3] about microalgae species *Chlorella* sp. characterization towards high temperature, Chaturvedi and Fujita [4] about EPA fatty acid compound of mutated *Nannochloropsis* sp., also Doan and Obbard [5] who explored lipid compound increase of of mutated *Nannochloropsis* sp.

From the research conducted by Ong et al. [3], it is known that *Chlorella* sp. that has been mutated and cultivated in 40°C temperature has higher specific growth compared to its original form. Also, higher lipid productivity of the mutated species cultivated with high temperature is higher compared to its original form. From another research conducted by Chaturvedi and Fujita [4], it is proven that EPA type fatty acid can be boosted by applying mutagenesis on *Nannochloropsis oculata*. Research by Doan and Obbard [5], that tested lipid content increase of *Nannochloropsis* sp., it is known that, mutated microalgae species can produce more fatty acid compared to its original state, both in exponential and stationery phase.

Chemical mutagenesis method [ethyl methanesulfonate (EMS)] was used in this research. This mutagenesis method is relatively new and complicated. Thus, the goal of this this research is to study the role of ethyl methanesulfonate in mutation induction, especially in *Nannochloropsis* sp. and to identify proximate cell composition (carbohydrate, protein, fat) and fatty acid in *Nannochloropsis* sp.

2. MATERIALS AND METHODS

2.1 Microalgae Cultivation

In laboratory scale cultivation, one third of the innoculant was submerged inside sterilized seawater that has been added by culture medium. Then, this culture pot is put on the cultivation rack, under lamp light and aerated. The medium used is Walne [6]. In the logarithmic

phase, 500 ml of the original cultivation species was placed inside an erlenmeyer to be mutated with 0,1 M and 0,5 M of ethyl methanesulfonate (Sigma, USA) for 60 minutes with medium rate of shakes, under microalgae normal growth condition. In such condition, as many as 55-60% of the individual cells would die. Then, the microalgae cells were washed three times using sterilized seawater to remove all EMS excess in the mixed microalgae. The microalgae and EMS mixture were re-suspended using 10 ml Walne medium and left to grow for further selection. The mutated microalgae cells (2×10^6 individual cells/ ml) was evenly dispersed on a plate (1.2% agar). After three weeks incubation at a temperature of 25°C, the remaining cell colonies within the plate were counted and each colony was inoculated with 10 ml seawater medium. For twelve days, microalgae density was measured using Haemacytometer and hand counter when observed through microscope.

2.2 DNA and RNA Extraction

The method used in extracting DNA and RNA from *Nannochloropsis* sp. was using Qiagen Dneasy Plant Mini Kit for DNA extraction and Qiagen Rneasy Plant Mini Kit for RNA extraction. Especially for RNA extraction, ethanol 96% and beta mercaptoethanolare needed as primer solvent. Method for RNA extraction is similar to DNA extraction. But, RNA molecules are relatively shorter and harder to break with shearing thus cell disruption could be done more aggressively. For RNA extraction, glove was a requirement and medium used for isolation must contain strong detergent to denaturate remaining RNase. After the extraction process has done, sample results were analyzed using Nanophotometer in order to calculate its DNA and RNA concentration. The replication in measuring DNA and RNA concentration was conducted three times.

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. A known volume of harvested biomass was dried by sun exposure or using oven in 80°C temperature. 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 (modified from Bligh and Dyer [7]). 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 ^{0}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 H_2SO_4. The ammonium sulphate was then degraded into NaOH. Released ammonia was then bonded with boric acid and titrated with standard acid liquid (*SNI* 01-2891-1992). Carbohydrate content was measured using phenol titration [8]. 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 BF_3 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 Na_2SO_4 to omit its content. This esterification product was then placed inside a vial to be analyzed using GC-MS [9].

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 80^0C 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.

2.8 Fatty acid Identification

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

$$Cx = \frac{m-74}{14} + 2$$

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

2.9 Data Analysis

2.9.1 Growth rate analysis

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

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

where:

N = microalgae density (ind/mL);
n = amount of microalgae observed

2.9.2 RNA/DNA ratio analysis

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

Ratio = RNA / DNA

where:

RNA : amount of RNA extracted (ng/µl)
DNA : amount of DNA extracted (ng/µl)

2.9.3 Lipid content analysis

Lipid content can be calculated with formula:

$$\% \text{ lipid} = \frac{W2-W1}{W} x\ 100\%$$

where:

W2 = sample weight after extraction (gram)
W1 = sample weight before extraction (gram)
W = sample weight (gram)

2.9.4 Protein content analysis

Protein content can be calculated with formula:

$$\text{Protein content} = \frac{(V1 - V2) \text{ x } N \text{x } 0{,}014 \text{ x f.k x fp}}{w}$$

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

2.9.5 Carbohydrate content analysis

Carbohydrate content can be calculated with formula:

Total sugar(%) = ((GxFP)/W) x 100

Whereas:

G = sugar concentration from standard curve (gram)

FP = dilution factor
W = sample weight (gram)

2.9.6 Statistical analysis

Results was stated as average value ± standard deviation. Each method was replicated three times. This research uses single factor complete random sampling with EMS concentration as treatment. Treatment effect toward response factor was analyzed using analysis of variance. Treatment that gave significant effect was tested further using F-test with MS Excel 2013.

3. RESULTS AND DISCUSSION

3.1 Microalgae Growth Rate

In 11 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 ± 11.93 x 10^6 cells/ml. The 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).

Fig. 1 shows that growth phase occurrence in EMS treatment cultivation is different from control cultivation. Lag or initial phase in control was occurred in day 0 and straight to logarithmic phase to day 2. Stationary phase in control was occurred in day 4, and then death phase was occurred from day 6 to day 11. In 0.1 EMS treatment, lag phase was started in day 0 and continued to day 3, and then logarithmic phase was occurred in day 4. Stationary phase in control was occurred in day 6, and then death phase was occurred from day 7 to day 11. The phases in 0.5 EMS treatment are similar with 0.1 EMS treatment.

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

Specific growth rate in this research show similar value with the research conducted by Doan and Obbard [5], 0.56/day for original species cultivation and 0.58/day for mutant species cultivation with certain EMS concentration. That research stated that, cultivation experiment that use higher concentration of EMS would produce lower density, when compared to its original species. It is proven by this research, where specific growth rate went lower from 0.1 to 0.5 M EMS concentration.

Table 1. Density and specific growth rate of *Nannochloropsis* sp. cultivation with three treatments (± means Standard Deviation)

Day-	Control (C) (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)	M (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

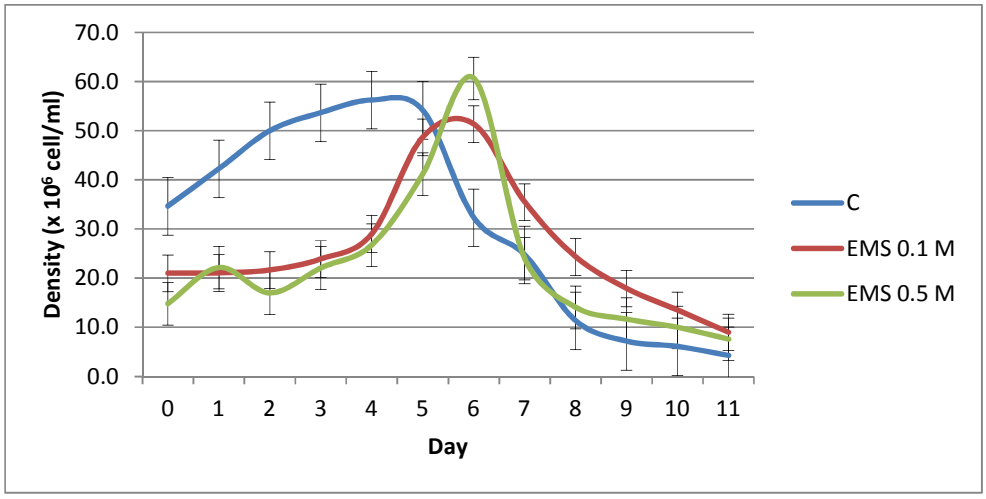

Fig. 1. *Nannochloropsis* sp. growth rate with three cultivation treatment for 11 days
(Axis Y: Density (x 10^6 cells/ml); axis x: day-;)
Mean±S.E.M = Mean values ± Standard deviation of means of 3 experiments

In Chaturvedi and Fujita [4], *Nannochloropsis* sp. was given 0.1 M EMS for certain herbicide substance resistance has higher specific growth rate than original species. This suits with recent research result that indicated genetic transformation within the cell of *Nannochloropsis* sp. without losing its morphology and gnome form.

Ong et al. [3] 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 that its original species.

However, when compared to Anandarajah et al. [10] 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 many as 1% during cultivation.

After the F-test, the result showed that EMS treatment had no significant effect on growth rate of *Nannochloropsis* sp. whereas the value of P was bigger than 0.05 and F count was lower than F table.

3.2 Microalgae DNA and RNA Ratio

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

Table 2. Concentration and ratio of nucleic acid (DNA and RNA) in ng/µl with 3 cultivation treatment of *Nannochloropsis* sp. (± means Standard Deviation)

Treatment	DNA (ng/µl)	RNA (ng/µl)	RNA/DNA ratio
Control	28.17±8.73	3.00±0.75	0.12±0.04
0.1 M	13.73±3.91	2.67±0.09	0.21±0.07
0.5 M	10.63±6.21	3.60±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/µl). 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 was lower when compared to the research conducted by Nicklisch and Steinberg [11]. 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 was also lower to the one from Nicklisch and Steinberg [11]. The result form that research was 18.4. This was probably caused by different method of extraction used, using *flow cytometry* and special coloring.

Compared to the research conducted by Kim et al. [12] that used *Botryococcus braunii* with Dneasy from Qiagen method, DNA concentration from this research was lower. The previous research achieved 38.75 ng/µl of DNA concentration. This was probably because the differing species used as object, the previous research used freshwater species. Kim et al. [12] did not extract RNA concentration because the result from Qiagen kit was very weak.

According to Adiputra et al. [13], 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 amplified result for all type of vegetation. In that research, total RNA concentration produced are 2.92 – 7.52 µg/ml.

In this research, RNA/DNA ratio of 0.5 M EMS cultivated samples were higher than 0.1 M EMS samples. This result was not in line with specific growth rate regression from EMS 0.1 M to EMS 0.5 M. Thus, Nicklisch and Seinberg [11] statement that there was no correlation between RNA/DNA ratio and specific growth rate was proven, especially to species that belong to Chlorophyceae class.

An increase in the concentration of RNA in 0.5 M EMS treatment makes the ratio of RNA / DNA in 0.5 M EMS treatment is higher. This indicated EMS effect on DNA transcription process into RNA. According to Warwick [14], alkylating agents such as EMS enough potential to inhibit DNA synthesis but not for RNA synthesis process.

3.3 Biomass and Proximate Content of Microalgae

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

Table 3. Wet and dry biomass of *Nannochloropsis* sp. In three cultivation treatment (± means Standard Deviation)

Treatment	Wet biomass (gr/l)	Dry biomass (gr/l)
	H-6 (stationery phase)	
Control	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)	
Control	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

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

Biomass obtained in this research was slightly higher compared to the Doan and Obbard [5]. That research stated that biomass of *Nannochloropsis* sp. from original species and mutant cultivation were 0.20±0.02 and 0.22±0.08 gr/L at the last day. Doan and Obbard [5] stated that experiment using high concentration of EMS (1 M) did not have significant effect on *Nannochloropsis* sp. biomass during the experiment.

Compared to Ong et al. [3], this research have similar biomass values. Ong et al. [3] results range were 0.6 - 1.2 g/l for mutated *Chlorella* sp. with 0.1 M EMS and 0.4 - 0.9 g/l for its original form with 40°C cultivation temperature. Thus, this 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 (± means Standard Deviation)

Treatment	Lipid (%)		Carbohydrate (%)	Protein (%)
	H-6	H-7		
Control	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).

The highest biomass occurred in *Nannochloropsis* sp. cultivation with 0.1 M EMS. Lipid content in 0.1 M EMS concentration was 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 in the cells.

Lipid content in this research was higher than the result from Doan and Obbard [5] (11.3% and 12.17%). Doan and Obbard [5] stated that lipid content from mutant species cultivation was 1.5 to 2 fold 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 fold from control cultivation at stationary and death phase. This shows that there was a similarity in harvest period for *Nannochloropsis* sp., which was best conducted at the end of stationary phase to get more lipid content.

Another microalgae species that has been tested using EMS method is *Chlamydmonas reinhardtii*. Lee et al. [15] stated that after randomly mutated by EMS, that species produced more lipid content than its original form. At exponential phase, mutant species lipid content was twice as much as the original species. And at the stationary phase, lipid content in mutant species did not show any significant rise [15]. 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.

In the research conducted by Hu and Gao [16], carbohydrate and protein content in *Nannochloropsis* sp. after cultivated with mixotropia using carbon dioxide-photoautotropic 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. [17], where protein and carbohydrate content of *Nannochloropis oceanica* that has been cultivated by reducing nitrogen compounds are 4,26-7,35% and 4,72-6,62%. Carbohydrate and protein content in this research did not show higher values compared to some research that did not use EMS mutagen [17,18]. 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 [17].

3.4 Microalgae Fatty Acid Compounds Characterization

Fatty acid identification process using gas chromatography in this research showed that there are 25 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. Following the lipid and biomass, fatty acid compound was also measured in different growth phase which are stationary phase (day 6) and death phase (day 7).

Table 5. Fatty acid compound (% area) from chromatography gas identification (± means Standard Deviation)

No	Compound	C h-6	C h-7	0.1 M h-6	0.1 M h-7	0.5 M h-6	0.5 M h-7
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	0.560
3	methyl dodecanoate			0.370			
4	methyl myristate	2.740±0.250	3.685±0.025	8.645±0.555	13.140±4.780	6.705±0.125	8.920
5	methyl tridecanoate	0.385±0.025	0.610	1.365±0.375			
6	methyl pentadecanoate	0.680±0.090	0.770±0.070	1.180±0.070	1.515±0.525	0.610	0.970
7	methyl tetradecanoate	0.315±0.015	1.040	1.900±0.120			
8	methyl palmitate	20.360±2.070	51.405±0.285	18.580	56.085±15.325	67.700±0.050	57.325±0.735
9	methyl margarate	0.900±0.040	0.830	1.765±0.015	1.320±0.470		1.010±0.050
10	methyl octadecanoate			3.815±0.505	8.520		
11	methyl stearate	10.200±0.910	13.360±0.350	9.510±0.590	3.900	3.605±0.055	4.900±0.090
12	methyl tricosanoate			1.360			
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	0.250		
15	methyl behenate	9.380±5.930	2.815±0.025	3.020±0.170	0.425±0.155	0.215±0.035	0.190±0.040
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				0.320		
19	methyl hexadecadienoic				1.340		
20	methyl nonanoate				0.240		
21	dimethyl azelate				0.570		
22	methyl hexadecanoate					0.610±0.020	
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	4.990±0.700	3.775±0.035		2.700	5.460±0.010	4.775±0.105
2	methyl palmitoleate			6.830±0.440	10.260±3.760	12.625±0.055	17.590±0.420
3	methyl linoleate (PUFA)	2.130±0.230	1.310	2.410		0.570	
Total		**7.120±0.930**	**5.085±0.035**	**9.240±0.440**	**12.960±3.760**	**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 was methyl linoleat with low concentration. Saturated fatty acid with the highest concentration was methyl palmitat in all three treatments. This compound has highest area percentage among all detected saturated fatty acids. There are also 2 saturated fatty acid with high values in all three treatments: methyl stearat and methyl miristate.

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

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

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

When compared to the research conducted by Doan and Obbard [5], total saturated fatty acid (SFA) in this research is higher. The total saturated fatty acid in Doan and Obbard [5] were 43.8 and 49.1 in original species cultivation and 42.5 and 45.9 in EMS mutated species cultivation. While total MUFA and PUFA in Doan and Obbard [5] were higher compared to this research. Total MUFA of original species cultivation were 34.8 and 36.6, and 42.8 and 45.8 in EMS mutated species cultivation. Total PUFA of original species cultivation were 21.4 and 14.3, and 14.6 and 8.3 in EMS mutated species cultivation [5].

Result from Ma et al. [18] research, SFA total from the original species and the mutated one are 55.4±1.7 and 58.1±1.7, respectively. This result is lower compared to this research; where cultivation of original species and mutated one have 78.56±1.03 and 89.12±21.69, respectively. For MUA and PUFA total, Ma et al. [18] obtained higher value than this research, with values of 31.8±1.9 and 12.8±0.6 for original species and 29.7±1.5 and 12.2±1.3 for mutant species.

Anandarajah et al. [10] stated that methyl miristate and methyl palmitate are SFA that increased 63% in his research. Chaturvedi and Fujita [4] reported that miristate acid and palmitate acid are dominant compounds in their research which used EMS for herbicide and antibiotic resistance. Myristic acid experienced an increased as many as 3.7 to 4.6 times to cerulenin and eritromicin. While palmitate acid increased as many as 26% and 15%. The same thing also occurred in this research where both compounds have the highest concentration compared to other fatty acid, and also higher compared to the original species cultivation. While Doan and Obbard [4] stated that miristate acid dan palmitate acid content are lower compared to its original species.

For MUFA group, Ma et al. [18] revealed that methyl oleate as one of the dominant compounds. Meanwhile, this research gives different result, where methyl oleate in EMS concentrated cultivation give lower value when compared to the original species cultivation. Methyl palmitoleate is the dominant MUFA compound in this research form two EMS concentration (10% and 18%). Palmitoleate acid increase also occurred in Chaturvedi and Fujita [4] with similar value. The increase was 11% and 8% for cerulenin and eritomicin resistance. Doan and Obbard [4] also resulted in an increase of palmitoleic acid as much as 30% compared to its original species. That value is significantly higher compared to the one obtained in this research.

4. CONCLUSION

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

CONSENT

All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this case report and accompanying images.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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