

## Effect of ethyl methane sulfonate (EMS) on cell size, fatty acid content, growth rate, and antioxidant activities of microalgae *Dunaliella* sp.

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**Abstract.** In this study, two treatments (0.1 M and 0.5 M) of Ethyl Methane Sulfonate (EMS) were injected to increase *Dunaliella* sp. biomass. EMS concentration of 0.1 M and 0.5 M were added in the early stages of microalgae cultivation. Microalgae cell size at control treatment was 4.08 µm, while at EMS 0.1 M were 10.09 µm, and at EMS 0.5 M were 3.89 µm. These results show that the concentration of 0.1 M EMS had a cell size three times larger than the control cell size and had a higher percentage of fatty acids. Each treatment had different growth rate. EMS treatment had lower growth rate and antioxidant activity compared to the control treatment.

**Key Words:** early stages, induction, microalgae, cultivations.

**Introduction.** Microalgae are microscopic living organisms that can grow well in freshwater and seawater. They are better known as phytoplankton. The chlorophyll of pigment in microalgae is able to perform photosynthesis like other higher plants, so microalgae acts as producers in the waters. In addition, a marine microalgae is marine organic matter that can be used as one component of the formation of petroleum on the seabed (Kwaroe et al 2010).

*Dunaliella* sp. is microalgae which belongs to class of green algae, its movement is motile with two flagella of the same length. Generally, the cell shape of *Dunaliella* sp. is rounded to the width of 4-15 µm and length of 6-25 µm, depending on the phase of growth or environmental conditions. In general, cell shape of *Dunaliella* sp. is radially bilateral symmetrical and partly shaped, flat, curved top or slightly asymmetrical (Gonzalez et al 2009). *Dunaliella* sp. can be found in fresh water and sea water and some high-salinity lakes throughout the world. Temperature, salinity and nutrients are limiting factors for the growth and development of *Dunaliella* sp. (Gonzalez et al 2009).

Most species of microalgae produce distinctive products such as fatty acids, antioxidants and carotenoids (Hossain et al 2008). According to De Fretes et al (2012), *Dunaliella* sp. is one of the microalgae are quite widely studied because of its carotenoid content and high glycerol. In addition to the feed and food, microalgae can be used as a producer of alternative fuels or biofuels.

Until now, researches are still conducted to get the type of microalgae that are able to produce high biomass to support the next stage of the process. According to Aranez (1981), one technique that is widely used to increase the biomass of microalgae is by mutagenesis techniques. Mutagenesis techniques are divided into two, namely physical and chemical mutagens. Physical mutagen used is the x-rays and gamma rays,

while the chemical compound is colchicine, ethyl methane sulfonate (EMS), and ethylene oxide (Ridhwan 2012). EMS is a mutagen compound most commonly used in chemical mutagenesis because it has a mutation effectiveness and mutagenic properties can be lost after a hydrolysis reaction with water (Van Harten 2007). Therefore, this study was conducted in order to analyze the effect of the provision of EMS on microalgae species *Dunaliella* sp.

## Material and Method

**Microalgae cultivation.** Cultivation was done in Microalgae Laboratory, Surfactant and Bioenergy Research Center-LPPM, Bogor Agricultural University, Indonesia from March 2014 to January 2015. Microalgae were cultivated at laboratory scale with a ratio of 1:3 as many as 100 mL of *Dunaliella* sp. inoculant added to 300 mL of sterile sea water, and walne fertilizer. *Dunaliella* sp. cells were cultivated in 500 mL Erlenmeyer and placed in culture shelves and aerated and lighting of 1000 lux light for 24 hours.

**EMS mutagenesis.** After the age of cultivation reached logarithmic phase, microalgae samples were taken to be mutated using 0.1 M and 0.5 M ethyl methane sulfonate (Sigma, USA) and stably shaken with a magnetic stirrer for 60 minutes. After the sample has been given EMS, microalgae rinsed 3 times replications with sterile sea water to remove the remaining EMS on microalgae. Furthermore, the sample was added with the walne fertilizer and separated for each treatment (EMS 0.1 M, 0.5 M and controls EMS) into 10 mL test tube. Microalgae are maintained for 7 days and cell density was calculated before selected. Selected tubes were then cultivated on a larger scale (scaling up) at 400 mL and 2.000 mL.

**Harvesting the microalgae.** Harvesting was done when the age of microalgae reached stationary and death phase by filtration using a vacuum pump and a 90 mm Whatman paper. Filtered microalgae biomass was then dried using oven at temperatures below 105°C for  $\pm 1$  hour.

**Fat extraction.** Dry samples were weighed as much as 1-2 grams, wrapped in a sleeve which previously included fat-free cotton. Then the samples were inserted into the Soxhlet tube and hexane was used as the extraction solvent for 6 hours (Bligh & Dyer 1959). The fat extract was distilled using a Soxhlet extractor. After that the extract was dried in the oven with a temperature of 105°C for approximately 1 hour and then weighed.

**Growth rate based on cell density.** *Dunaliella* sp. cell density in each treatment was counted every day by using Neubaur haemocytometer under microscope type BM180 Boeco Germany. Each calculation was done by counting the 5 visual field with 4 repetitions. Cells that have been counted subsequently calculated using the following formula:

Cell density (cell mL<sup>-1</sup>) =  $n \times \frac{25}{5} \times 10^4$  Where: n = amount of observed cells.

**Determination of moisture content.** Weigh moisture sample immediately and record as "wet weight of sample". Dry the wet sample to a constant weight, at a temperature not exceeding 115°C using the suitable drying equipment. Allow the sample to cool. Weigh the cooled sample again, and record as the "dry weight of sample"

**Determination of ash content.** The sample was weighed as much as 2-3 grams and put in a cup of known weight. After that, the cup was inserted into the electric furnace at a temperature of 600°C for 4-6 hours until perfect ashing was achieved. Then the sample was cooled in a desiccator and weighed.

**Determination of crude protein content.** The sample was weighed as much as 0.51 grams of sample and placed in a 100 mL Kjeldahl flask. Two (2) grams of selain and 25 mL of H<sub>2</sub>SO<sub>4</sub> concentrated was added and heated in an electric heater or burner flame for

about 2 hours until boiling point is reached and the solution color become clear greenish. And then the sample was cooled down, then diluted and inserted into 100 mL volumetric flask. Five (5) mL of solution was pipetted and incorporated into the refiner and 5 mL of 30% NaOH and some PP indicators also were added. Then it was distilled for 10 minutes, 10 mL of 2% boric acid solution was used as the container that has been mixed with the indicator. Then it was titrated with 0.001 N HCl.

**Esterification.** A total of 2 grams of fat extracted using hexane that has been saponified with 25 mL of 0.5 M NaOH methanol. Then, 300 mg of saponified fat samples were reacted with 8 mL of BF<sub>3</sub>-methanol and heated for 3-4 minutes. After that, 2-3 mL petroleum ether (60°C) was added to separate the ester. Samples was added with NaCl to float fatty acid compound to the top of the flask and then analyzed by means of GC-MS (Prommuak et al 2012).

**Gas chromatography–mass spectrometry (GC-MS).** Gas chromatography–mass spectrometry (GC-MS) analysis was conducted using chromatography gas Shimadzu QP2010 gas chromatography that has been equipped with DB-5 ms silica column (30 mm in length; 0.25 mm in inner diameter; and 0.25 µm film layer thickness) and helium as booster gas. Gas chromatography has a detection limit of 0.001 ppb. Gas chromatography use split injection mode with a ratio of 1:200. Oven temperature was programmed to 80°C are held constant for 2 minutes, then raised to 210°C at a speed of 10°C min<sup>-1</sup>, held constant for 1 min, then increased again to 280°C with a speed of 6°C min<sup>-1</sup>, held constant for 5 minutes. GC-MS condition is the ionization potential/electron energy 70eV, ion source temperature of 250°C and 280°C temperature interface. Full mass of data recorded between 50-400 Dalton every second. Retention time is from 0 to 32.67 minutes. Then the data were recorded and analyzed by GC-MS Real Time Analysis and GCMS Postrun Analysis softwares.

**Antioxidant activity test.** Solution of DPPH (2,2-diphenyl-1-picrylhydrazyl hydrate) was prepared by dissolving 0.004 grams of DPPH in 100 mL of methanol (Romeilah et al 2012; Das et al 2011). One mL of microalgae were centrifuged at a speed of 10000 rpm at 4°C for 15 minutes, then the precipitate was extracted with 1 mL of ethanol and then vortex at a steady pace. Extract was settled at 4°C for 4 hours and add 2 mL of DPPH solution. Then the mixture was incubated for 30 min at room temperature in the dark room. Blank sample (ethanol solution) was the control. Absorbance was calculated at a wavelength of 517 nm using a spectrophotometer.

**Results and Discussion.** In this study, cultivation of microalgae combined with the addition of EMS was successful. EMS concentrations given at each treatment affects the size of the cell and the chemical content of microalgae. Effect of each treatment of EMS to microalgae *Dunaliella* sp. can be identified from different cell sizes (Figure 1).

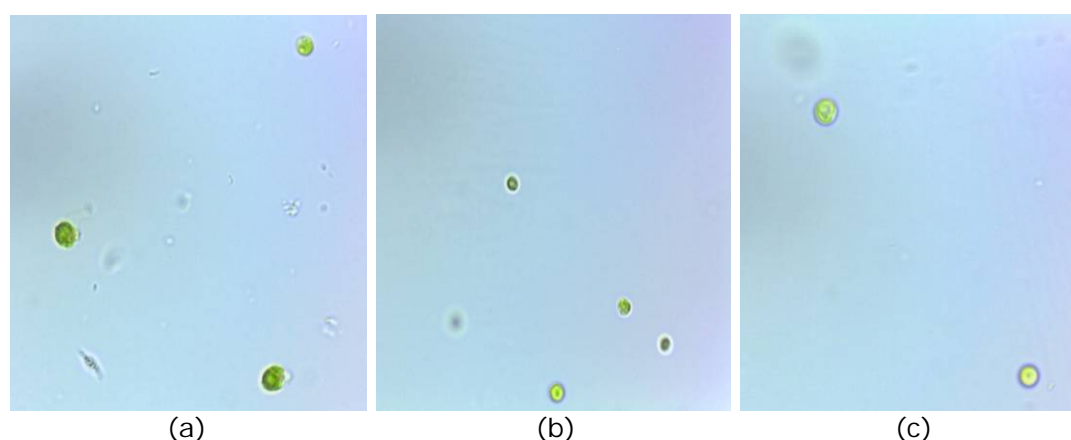


Figure 1. Size cell *Dunaliella* sp. (1000x) on cultivation (a) EMS 0.1, (b) EMS 0.5 M, (c) Control.

Figure 1 showed microalgae cell size, which at control treatment was 4.08 µm, while at EMS 0.1 M was 10.09 µm, and at EMS 0.5 M was 3.89 µm. Addition of 0.1 M EMS to the

cultivation medium can increase cell size of microalga compared to the other two treatments (0.5 M and control). According to Aranez (1981), microalgae species *Scenedesmus* sp. that was given EMS treatment have cell size twice and three times larger then that of normal cells. EMS additions to the microalgae can produce a larger cell size twice or three times the size of round or oval cells more than normal cells (Aranez 1981). Each treatment has different growth rate. EMS treatment had a lower growth rate compared to the control treatment. *Dunaliella* sp. growth rate base on cell density is presented in Figure 2.

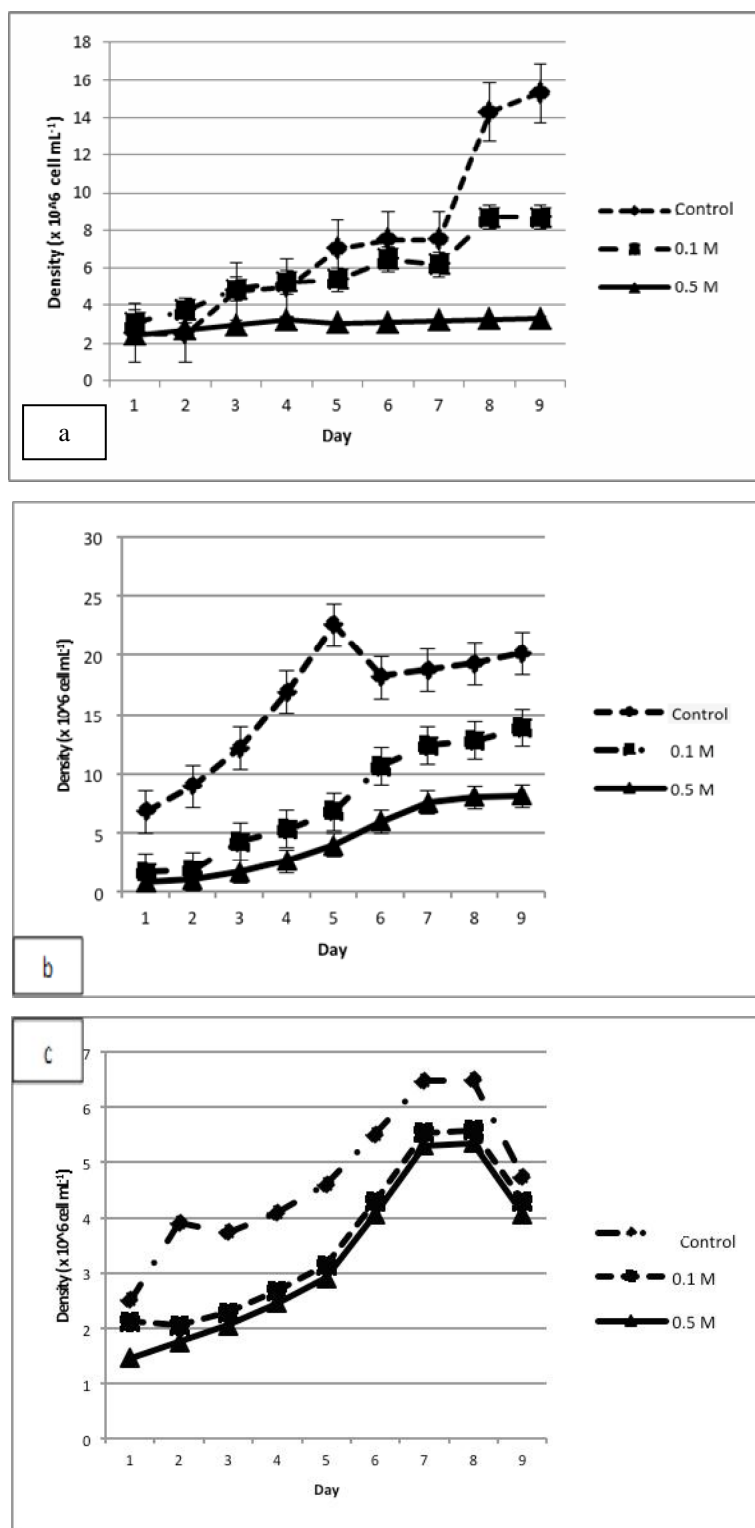


Figure 2. *Dunaliella* sp. growth rate in the culture medium volume of (a) 10 mL, (b) 400 mL, (c) 2000 mL (20L).

Cultivation at 10 mL in this study was an early stage of the culture process of microalgae *Dunaliella* sp. Figure 2a showed that treatment of 0.5 M has the lowest density compared to the treatment of 0.1 M and control. It is thought to be caused due to the EMS concentration of 0.5 M which was dense enough to damage or kill the microalgae cells up to 60%, so that its density decreases. Other things are EMS addition can decrease acidity (pH) of cultivation media and it can be seen from bleaching of media (water) color. If the pH decreases then the rate of photosynthesis will be limited by carbon reduction and pH.

Cultivation was scale up to 400 mL in 8 day culture. The result showed that the control treatment had a higher cell density compared to the of EMS addition treatment. The lowest cell density treatment was at treatment of EMS 0.5 M. The cell density peak of control treatment was on day 4 with  $22.6 \times 10^6$  cells mL<sup>-1</sup>, whereas the cell density of 0.1 M EMS treatment continued to increase with the peak density on day 5 of  $10.68 \times 10^6$  cells mL<sup>-1</sup>. Chaturvedi & Fujita (2006) states that mutant microalgae species has a growth rate that is lower than the density of native species (wild type). In this study prior to scale up, initial pH control treatment and 0.1 M EMS was 7 while pH of 0.5 M EMS was 2.

In the scale 20 L microalgae cultivation, cells density was decreased. This was presumably because of at this stage, seed of microalgae *Dunaliella* sp. used was in the death phase since they were from previous cultivation. One factor determines that the length of adaptation phase was the age of the culture used as an inoculum (Prihantini et al 2005). Research results from Doan & Obbard (2012) showed that the number of dead cell at control culture by 0.8% and would grow along with the addition of EMS concentration. The amount of cell death in EMS 0.5 M and 1 M are for 67.3% and 92%, respectively, while the EMS 1.2 M concentration will cause death in microalgae cells *Nannochloropsis* sp.

Characteristics of microalgae can be seen from the content of protein, carbohydrates, fats, vitamins and minerals. Each species of microalgae have different characteristics and influenced by environmental factors (light intensity, temperature, salinity, pH, aeration, and nutrients). Results of proximate analysis in this study were in stationary and death phase (Table 1).

Table 1

Characteristics (%) of microalgae species *Dunaliella* sp.

Characteristics (%)	Control		EMS 0.1 M		EMS 0.5 M	
	D7	D8	D7	D8	D7	D8
Water content	82.05	88.01	85.51	90.22	84.15	85.64
Ash content	8.27	5.70	7.83	4.26	7.82	6.45
Fat	1.95	9.85	1.19	15.80	1.45	1.17
Protein	14.52	19.15	14.24	22.92	13.99	16.46

D7: harvest on Day-7 (stationary phase), D8: harvest on Day-8 (death phase).

Table 1 showed that, the highest water content found in death phase of 0.1 M EMS (90.22%). This result was different from Yudha (2008) that was 65.22%. Ash content indicates mineral content of *Dunaliella* sp. Ash content either in control or EMS on the stationary phase were higher than the death phase. The highest ash content were in stationary phase of control treatment (8.27%). High and low ash content influenced by the condition of the environment so that the intake of minerals that are absorbed by each individual will vary. The highest fat content in the death phase of 0.1 M EMS treatment (15.80%). This was consistent with Doan & Obbard (2012) which showed that total fat of mutant *Nannochloropsis* sp. in exponential phase and stationary phase will be higher 1-2 times compared with original species. Differences in the levels of fat in each treatment are caused by nutrients contained in each cultivation. Microalgae can produce more fat when experiencing stress (Kawaroe et al 2012). Protein plays an important role in the formation of microalgae cells. From these results, *Dunaliella* sp. has protein content of 13-14% (dry weight) on the stationary phase and 16-30% (dry weight) on the death

phase. However, when compared to Yudha (2008), the percentage of protein in *Dunaliella* sp. does not differ greatly in the amount of 18.12% (dry weight).

Table 2 shows that in all treatments the highest concentration of unsaturated fatty acids was for hexadecanoic acid (methyl palmitate). Different cultivation treatments and conditions can result in some different compounds of fatty acids during cultivation (Prartono et al 2010).

Table 2

Fatty acid compounds of *Dunaliella* sp.

Fatty acid compounds	Stationary phase (%)			Death phase (%)		
	C	E1	E5	C	E1	E5
Hexadecanoic acid methyl ester	22.81	23.87		16.57	11.41	19.36
Hexadecenoic acid methyl ester	4.4	2.79		1.67	1.81	
Octadecanoic acid methyl ester	5.80	5.13	6.88	4.62	2.47	8.57
Octadecadienoic acid methyl ester		15.65	11.36	6.73	8.67	6.63

C: Control, E1: EMS 0.1 M, E2: EMS 0.5M.

The results showed that the highest percentage of fatty acid compound was at treatment of 0.1 M EMS in Hexadecanoic acid methyl ester. This was consistent with Augustine et al (2014) that stated 0.1 M EMS treatment on *Nannochloropsis* sp. has higher percentage of fatty acids compared to the EMS concentration of 0.5 M. Culture conditions, growth phases, and environmental factors are factors that affect the fat content and fatty acid nature (Li et al 2011)

Fatty acid calculation of algae biomass is one procedure to indicate which fat is suitable to be converted into biodiesel. An effective selecting phase of high oil content and harvesting methods are needed to produce bioediesel from microalgae (Parmar et al 2011) Hexadecanoic or more commonly known as palmitic acid is a fatty acid contained in many biodiesel and have a percentage of 23.7% of the total fatty acids in microalgae *Dunaliella salina* (Rasoul-Amini et al 2014). This result showed that hexadecanoic at stationary phase has a value more than the death phase. The high content of saturated fatty acids have good value and oxidative stability for biodiesel. Octadecanoic acid (stearic acid) in *Dunaliella salina* is higher than *Botryococcus braunii*, *Chlorella vulgaris* and *Scenedesmus* sp. (Yoo et al 2010)

Antioxidants serve as an antidote to free radicals that can damage body tissues of living creatures (Agustini 2012). Currently, many marine microbiology organisms have been used for their antioxidant content. *Dunaliella* sp. has high carotenoid content; beta carotene reached 4% dry weight (Barrow & Shahidi 2007).

Based on the results of this study, treatment control has a green color and 0.1 M EMS treatment has a yellow color. This proves that qualitatively microalgae *Dunaliella* sp. in control and EMS 0.1 M treatment have the free radicals reduction power against DPPH. In the treatment of EMS 0.5 M, when the solution was added DPPH, the color becomes purple. Qualitatively *Dunaliella* sp. 0.5 M EMS treatment does not have the power to reduce free radicals against DPPH. Figure 3 shows the color change that occurs when testing the antioxidant activity of *Dunaliella* sp. Observation on free radical reducing using DPPH method used spectrophotometric method with the reaction time of 30 minutes and a wavelength of 517 nm.

Table 3 shows the control treatment had high absorbance values compared to the 0.1 M and 0.5 M EMS treatments. Absorbance value determines the level of reducing free radicals. According to Amrun et al (2007) a decrease of absorbance showed increased DPPH free radical, which means that the higher the concentration gives high of the antioxidant activity.

Table 3

Antioxidant activity (%) of microalgae *Dunaliella* sp.

Phase	Control	EMS 0.1 M	EMS 0.5 M
Stationery	84.33	54.78	24.63
Death	77.76	66.72	59.70

Figure 3. Antioxidant activity test on *Dunaliella* sp. using DPPH method.

**Conclusions.** Addition of Ethyl Methane Sulfonate (EMS) with certain concentration can affect the microalgae species *Dunaliella* sp. In this study, 0.1 M EMS concentrations was the concentration that can affect microalgae species *Dunaliella* sp. EMS with concentrate 0.1 M has higher cell size and percentage of fatty acid than the control and 0.5 M treatment. EMS treatment has the growth rate and lower antioxidant activity compared to control treatment.

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