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Efect of Gamma ^{60}Co Irradiation on The Growth, Lipid Content and Fatty Acid Composition of *Botryococcus* sp. Microalgae

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Abstract

Botryococcus sp. is microalgae species that has high lipid content. Mutagenesis induced by Gamma ^{60}Co irradiation can be utilized to alter *Botryococcus* sp. characteristics to get microalgae mutant strain that have better characteristics than the wild strain. The aim of this research was to know the effect of gamma ^{60}Co irradiation to the growth, biomass, total lipid content and fatty acid compotition characteristics of *Botryococcus* sp. *Botryococcus* sp. was irradiated with different doses of gamma ray of ^{60}Co (0, 2, 4, 6, and 10 Gy). Biomass and lipid content was analysed by quantitative analysis. Fatty acid composition was analyzed by Gas Chromatography-Mass Spectrometry. Results showed that Gamma irradiated gave an effect on growth, biomass and lipid content of *Botryococcus* sp. There was significantly different only between control (0 Gy) and 10 Gy irradiated microalgae. The highest biomass and lipid content are found in 10 Gy irradiated microalgae are 0.833 gram biomass and 41 % lipid content. Fatty acid profile of *Botryococcus* sp. control has 6 fatty acids while 10 Gy irradiated microalgae has 12 fatty acids, with the long-chain fatty acids increased, where as short-chain fatty acids decreased. This research could be the basis for engineering of microalgae for biodiesel production.

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INTRODUCTION

Microalgae is an unicelular photosynthetic organism that has great potential in the biotechnology industry, especially as a source of renewable energy (Chisti, 2007; Derner, 2006). Microalgae can provide several types of renewable energy sources, such as methane produced by anaerobic metabolic process of algae biomass, biodiesel derived from lipid of microalgae and biohidrogen production (Spolaore et al., 2006; Chisti, 2007; Fedorov et al., 2001; Kapdan & Kargi, 2006).

Microalgae as a source of biodiesel has the potential to completely replace fossil diesel, because compared to other oil-producing crops, microalgae grows very rapidly and contains high lipid (Spolaore et al., 2006). Microalgae can double its biomass within 24 hours, duplication of microalgae biomass during the exponential phase of growth can occur within 3.5 hours, and microalgae lipid content can exceed 80% of dry biomass weight in certain environmental conditions (Metting, 1996;. Spolaore et al., 2006).

Botryococcus sp. is one of microalgae type included in the Chlorophyceae class with the highest lipid content compared to other types of microalgae, which amounted to 75% of the dry biomass weight (Sawayama et al., 1995; Chisti, 2007). *Botryococcus* has a main fatty acid profile such as oleic acid (C18: 1, 54.9%), palmitic acid (C16: 0, 12.2%), linolenic acid (C18: 3, 5.5%), stearic acid (C18: 0, 3.9%) and linoleic acid (C18: 2, 5.5%), these fatty acids can be used for biodiesel feedstock (Knothe, 2008). However, the production of biodiesel from microalgae is still lacking industrial applicability because it requires a higher cost compared with fossil diesel (Yang et al., 2012).

The use of microalgae strains with optimum lipid content is one of solutions to minimize the cost of microalgae production (Hannon et al., 2010; Pal et al., 2011). Mutagenesis technology is a fast and efficient method to obtain microalgae strains with high lipid content (Hu et al., 2013). Mutagenesis by radiation ^{60}Co Gamma rays has time and intensity controlled, has greater energy than other radiations such as Ultraviolet so that it can affect the atoms and molecules in the cell to induce the genetic alteration of the cell (Kovacs and Keresztes, 2002; Hwang et al., 2014). Microalgae mutant has higher capacity to produce lipid because some of the genes that correlated with lipid biosynthesis mutated to produce a positive expression, such as the genes expression of acetyl-CoA carboxylase (ACCase) that increased five times after mutagenesis (Cheng et al., 2014). The similar research showed that *Scenedesmus dimorphus* gamma ray mutant can improve lipid accumulation by 71.3%, because some proteins correlated with lipid biosynthesis and energy metabolism are over expressed (Han et al., 2014). Based on it, this study conducted ^{60}Co Gamma ray irradiation on *Botryococcus* sp. to induce the alteration of lipid metabolism to produce higher lipid content as a reference of biodiesel production development. The benefits of this research are as reference research on the effect of ^{60}Co gamma ray radiation to growth, the production of lipids and fatty acid composition of *Botryococcus* sp. microalgae. Additionally, this research could be the basis for engineering of microalgae for biodiesel production.

METHODS

This research was conducted in September 2015 until January 2016 at the Laboratory of Plant Bioscience and Technology, Biology Department, Faculty of Mathematics and Natural Sciences, Institut Teknologi Sepuluh Nopember Surabaya. The research was conducted with the following methods :

Sterilization of Equipment and Media Culture

All glassware and aerator plastic hoses were washed with soap, rinsed with water and dried. The sea water is conditioned with 25 ppt salinity and 7.2 pH (Susilowati and Amini, 2010). Furthermore, the tool and the culture medium was sterilized by autoclave at 121° C and 1 atm pressure for 30 minutes.

Fertilizers and Media Culture Preparation

Walne Fertilizers used in this study was obtained from Natural Feed Laboratory, Balai Budidaya Air Payau (BBAP) Situbondo accordance with the composition shown in Table 1. Walne fertilizer was dissolved in 1 L of distilled water.

Determining of the Age Starter

Botryococcus sp. obtained from Situbondo BBAP with cell density of 17 million cells / mL was taken about 60 mL and put into culture bottles containing 240 mL of sea water and 0.3 mL of Walne fertilizer. *Botryococcus* sp. cell density was measured every 24 hours until it reached the death phase using GENESYS 10S UV-Vis spectrophotometer at 680 nm wavelength (Andersen, 2005).

Table 1. Walne Fertilizer Compotition (Andersen, 2005)

Ingredients	Total (g/L)
Nutrient Components	
NaH_2PO4	20
Na_2EDTA	45
$FeCl_3$	1.30
$NaNO_3$	100
$MnCl_2$	0.36
H_3BO_3	33.60
Vitamin Stock Solution	
Vitamin B12 (Cyanocobalamin)	5.00
Vitamin B1 (Thiamine.HCl)	100.00

Making Starter

Botryococcus sp. was taken 60 mL and put into culture bottles containing 240 mL of sea water and 0.3 mL of Walne fertilizer. Having reached the half exponential phase with Optical Density (OD) ± 0.45, the microalgae are taken and prepared for irradiated (Cheng et al., 2014).

Figure 1. *Botryococcus* sp. microalgae culture

Microalgae Irradiation

Botryococcus sp. was taken 60 mL (OD: ± 0.45 at 680 nm wavelength) or half-exponential phase, then was put in a culture bottle (Cheng et al., 2014). Microalgae was irradiated with ^{60}Co gamma rays at a dose of 2, 4, 6, and 10 Gy using Gamma irradiators Chamber 4000 A in Badan Tenaga Nuklir Nasional (BATAN), Jakarta.

Determination of Harvesting Time

The harvesting time was done by making the *Botryococcus* sp. growth curve on each microalgae irradiated ^{60}Co gamma rays to determine the end of the exponential growth phase for harvesting time (BBAP, 2013). The method was used similarly to the method of determining the age of starter in section 3.

Research Design

This research was conducted by descriptive quantitative based on Gas Chromatography (GC) analysis, and also quantitatively based on the analysis by Anova (Analysis of Variance). The research design used was completely randomized design. This research consisted of 5 treatments, with three repetitions. Microalgae *Botryococcus* sp. was irradiated with doses of 0, 2, 4, 6, and 10 Gy, then the microalgae biomass and lipid content were analyzed.

Harvesting and Biomass Analysis

The biomass production of microalgae was harvested and measured by 100 mL filtered with Whattman filter paper No. 40 and rinsed with distilled water. Filter paper and yields are dried in oven for 24 hours at 60° C, and weighed. Dry weight of harvested microalgae is derived from the difference between the dry weight of the filter paper and the dry weight of microalgae with filter paper.

Figure 2. Harvesting microalgae with Whatman filter paper No 40

Measurement of Lipid Content

Total lipid content was measured by Bligh and Dyer method, (1959) that has been modified. Taken 10 mL cell culture and centrifuged at 6000 rpm for 15 minutes. For the lipid extraction was added 3 mL of methanol, then sonicated 4 x 1 minute. Next it was added 6 mL of chloroform and shaked for 1 hour. Then added 10 mL of distilled water and shaked again for 15 minutes. Microalgae lipid is still mixed with chloroform were taken using a pasteur pipette into a test tube which has been weighed. The reaction tubes are stored so that the chloroform evaporated and left only lipid. Calculation % of the total lipid using the following formula:

$$\% \text{ lipid} = \frac{(A \times B)}{C}$$

Description:
A: weight of lipid (g)
B: concentration of solution (mL)

C: weight of biomass (g)
(Lestari & Amrullah, 2013).

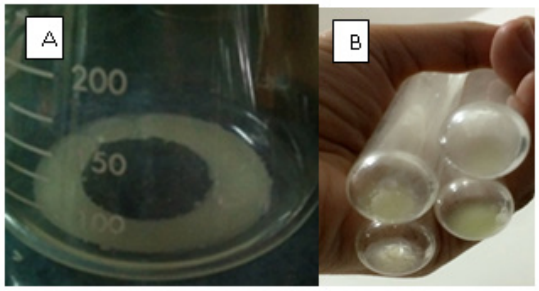

Figure 3. Mixture of microalagae lipid with methanol, chloroform and aquades (A) . Microalgae lipid after chloroform have been evaporated (B)

Data Analysis

Data from the observations of biomass and lipid content (%) were analyzed with statistical analysis by Anova (Analysis of Variance) one factor at 95% level. Analysis of fatty acid composition was done by Gas Chromatography-Mass Spectrometry (GC-MS) based methods of Song et al., (2013). GC-MS analysis was conducted at the Laboratory Testing Services Unit, Faculty of Pharmacy, Airlangga University. Lipid samples were dissolved in n-hexane and inserted into the derivatization tube. Sample solution was evaporated to dryness and add 2 mL of NaOH-methanolic, sealed, mixed by vortex then heated at 90°C for 5 minutes and then cooled to room temperature. Then sample was derivatized by adding 2 mL of BF3, sealed, mixed by vortex then heated at 90°C for 30 minutes then cooled to room temperature. Then added 4 mL of n-hexane, mixed by vortex for 2 minutes and then allowed to stand to separate into two phases. N-hexane phase (top layer) was taken up volume of 500 µL and incorporated into GC vial for GC-MS analysis.

RESULT AND DISCUSSION

Gamma ^{60}Co Rays Irradiation on the Growth of *Botryococcus* sp.

Botryococcus sp. microalgae growth profile without and with treatment of ^{60}Co Gamma ray irradiation is presented in Figure 4.

Results showed that ^{60}Co Gamma ray irradiation effected on microalgae growth profile by 4-5 days longer of exponential growth phase than the microalgae without irradiation (0 Gy / control). In Figure 1 showed that *Botryococcus* sp. with 0 Gy (control) treatment had a lag phase to 1st day of growth, then the log/exponential phase occured for 11 days starting on 1st to 11th day of growth. Then the stationary phase occured until **12th** day, while the next day its growth was decreased. This is similar with research conducted by Sari et al., (2013) that showed *Botryococcus* sp. had the log growth phase until **1st** day of growth, following an exponential phase which in this study occured up to **10th** day, then a stationary phase for 1 day followed by the decreased in growth.

Irradiated Microalgae growth profile showed a lag phase occured in the 1st day growth. Then the exponential phase, where the 2 Gy and 4 Gy dose irradiated microalgae had until the 15th day, where as in 6 Gy and 10 Gy dose irradiated microalgae occured until the 16th day. After that microalgae growth started to decrease. Differences in the duration of this growth was microalgae specific response of environmental conditions (Hu and Gao, 2006). Research on the effect

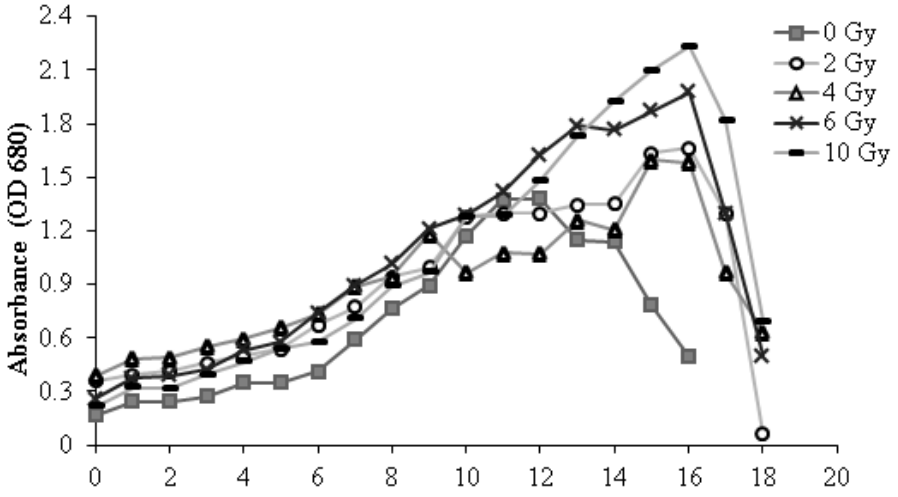

Figure 4. *Botryococcus* sp. growth curve at times after irradiated with gamma rays at various doses

of gamma radiation on *Nitzschia* sp. also showed that the irradiated microalgae had increased growth with increasing radiation dose (Cheng et al., 2014). The extension of exponential phase was allegedly due to the irradiated microalgae synthesized energy savings in its cell for survival. Radiation causes oxidative stress cells that triggers cell to make defense process to maintain its growth and reduce the effects of stress (Bellou et al., 2013).

Gamma ^{60}Co Rays Irradiation on the Biomass and Lipid Content of *Botryococcus* sp.

Harvesting biomass and calculation of total lipid of *Botryococcus* sp. microalgae was done at the end of the exponential growth phase according to the growth profile. Ma et al., (2013) stated that the greatest biomass accumulation microalgae was in exponential phase which has the highest growth rate. The average biomass and total lipid of *Botryococcus* sp. can be seen in Table 2.

Table 2. Effect of Gamma ^{60}Co Rays Irradiation on the Biomass and Lipid Content of *Botryococcus* sp.

Radiation Dose	Biomass (g)	Lipid Content (%)
0 Gy	0.130±0.02^{a}	27.173±1.6^{a}
2 Gy	0.183±0.05^{ab}	23.772±6.7^{a}
4 Gy	0.133±0.03^{a}	29.764±5.0^{ab}
6 Gy	0.177±0.03^{a}	31.544±3.1^{ab}
10 Gy	0.333±0.11^{b}	41.044±4.0^{b}

Description: *number followed by the same letter show no significant results by ANOVA and Tukey test continued at 95% significance level. Standard deviation, n = 3.

Table 2 showed that the irradiation effected on microalgae biomass and total lipids. The highest biomass of *Botryococcus* sp. was achieved in the treatment of 10 Gy irradiation dose. Anova test results showed that the irradiation dose effected on biomass of *Botryococcus* sp. with P = 0.009 (P <0.05). So the advanced test conducted by Tukey's test. Tukey's test result to the *Botryococcus* sp. biomass showed between 0 Gy dose treatment (control) and 2 Gy, 4 Gy and 6 Gy irradiation dose were not significantly different. Result of *Botryococcus* sp. biomass between 2 Gy and 10 Gy was also not significantly different. While the control treatment with 10 Gy microalgae irradiated dose showed significant difference.

Result of 10 Gy radiation dose treatment which has the highest biomass was alleged that enzyme activity in the photosynthesis process *Botryococcus* sp. increased so the cell growth also increased. Gamma ray irradiation at low doses can increase enzyme activity, cell proliferation, and cell growth (Chakravarty and Sen, 2001). Similar research on Spirogyra irradiated with Gamma ray also showed an increased activity of the Rubisco enzyme so that the rate of photosynthesis and growth increased compared to wild-type of Spirogyra (Yoon et al., 2013).

Gamma irradiation also affected the total lipid of *Botryococcus* sp. as shown in Table 2. The results of the ANOVA test showed that the irradiation dose effected on total lipid content with P = 0.008 (P <0.05). So the advanced test conducted by Tukey's test. Tukey's test results of the total lipid content showed that between the control dose treatment and 2 Gy, 4 Gy and 6 Gy dose were not significantly different. The same result also found in total lipid of 4 Gy, 6 Gy and 10 Gy were not significantly different. But between the control treatment and 10 Gy showed significant difference. This was allegedly due to the increased enzyme activity correlated with lipid biosynthesis in 10 Gy irradiated microalgae. Gamma irradiation at certain doses can cause changes in the structure and cell metabolism (Wi et al., 2005).

Gamma rays mutagenesis induced random mutation, causing various response of cells (Acquaah, 2007). The results of biomass and total lipid analysis in this study showed a different response on *Botryococcus* sp. irradiated with 2 Gy dose that had higher biomass but lower lipid content than control. While on 4 Gy, 6 Gy and 10 Gy irradiated *Botryococcus* sp. biomass and lipid content increased with increasing irradiation doses. The reasons may explain the above results because at 2 Gy irradiation dose microalgae was conducted by increased enzyme activity that correlated the metabolism of carbohydrates, where as at 10 Gy irradiated dose *Botryococcus* sp. had increased enzyme activity occured more towards lipid metabolism. Lipid and carbohydrate are energy reserves product that is produced by cells during stress as a defense of its life and also for the production of neutral lipids (Li et al., 2011). These different responses occured because gamma rays mutagenesis irradiation induce genetic alteration that depend on irradiation dose (Ahlowowalia and Maluszynski, 2001). This is supported by Tammam et al., (2005) finding on microalgae *Dunaliella salina* that showed four mutants with different nucleotide variation as a results of Gamma ray irradiation at different doses.

C.Gamma ^{60}Co Rays Irradiation on Fatty Acid Profile of *Botryococcus* sp.

Fatty acid composition analysis in this study was conducted by Gas Chromatography-Mass Spectophotometry (GC-MS). The analysis was performed on *Botryococcus* sp. control treatment and the results of 10 Gy radiation because it has a significant difference in the biomass and lipid content. Determination of fatty acid composition is based on microalgae lipid fractionation conformity with the standard of GC-MS. Total percentage of fatty acids obtained based on the area under the curve on cromatograph (Figure 5 and Figure 6). Results of analysis of fatty acid profiles can be seen in Table 3.

Based on the table 3 above there are differences in the fatty acid species composition and their content in the control microalgae and irradiated microalgae at a dose of 10 Gy. Several new fatty acids such as palmitoleic acid , 7,10 hexadekadienoat acid, linolenic acid, arakidic acid, arachidonic acid and lignoseric acid appear on microalgae are irradiated at a dose of 10 Gy. There is a decrease in the percentage content of some fatty acids such as capric acid, myristic acid, palmitate acid, stearic acid and oleic acid in the 10 Gy irradiated microalgae when compared to the control microalgae. However, there is only one fatty acid (linoleic acid) that increase in the percentage content in 10 Gy irradiated microalgae.

GC-MS analysis (Figure 5) showed the presence of 6 types of fatty acids contained in *Botryococcus* sp. control (0 Gy) lipid as shown in Table 3. Profile of fatty acids was dominated by fatty acids with 16 and 18 carbon chain, such as palmitic acid, linoleic acid, oleic acid and stearic acid. The higher percentage of fatty acid were oleic acid (36.53%) and palmitic acid (33.44%). Botryococcus lipid has a main fatty acid profile such as oleic acid (C18: 1), palmitic acid (C16: 0), linolenic acid (C18: 3), stearic acid (C18: 0) and linoleic acid (C18: 2) (Knothe, 2008). Stearic acid and palmitic acid, include in saturated fatty acid, where as oleic acid, linoleic acid, linolenic acid include in unsaturated fatty acids (Canakci and Van Gepen, 2003).

Table 3. Fatty Acid Profile of *Botryococcus* sp. Control and 10 Gy irradiated dose

Fatty Acid Compotiotions	Total (%)	
	Control	10 Gy
Capric acid (C10: 0)	3.15	0.49
Myristic acid (C14: 0)	2.32	1.42
Palmitate acid (C16: 0)	33.44	26.72
Palmitoleic acid (C16: 1)	-	2.20
7,10 Hexadekadienoat acid (C16: 2)	-	2.37
Stearic acid (C18: 0)	9.66	9.38
Oleic acid (C18: 1)	36.53	33.84
Linoleic acid (18: 2)	14.90	16.57
Linolenic acid (C18: 3)	-	1.40
Arakidic acid (C20: 0)	-	0.82
Arachidonic acid (C20: 4)	-	3.44
Lignoseric acid (C24: 0)	-	1.39

Fatty acid analysis in 10 Gy (Figure 6) ^{60}Co Gamma irradiated *Botryococcus* sp. results showed

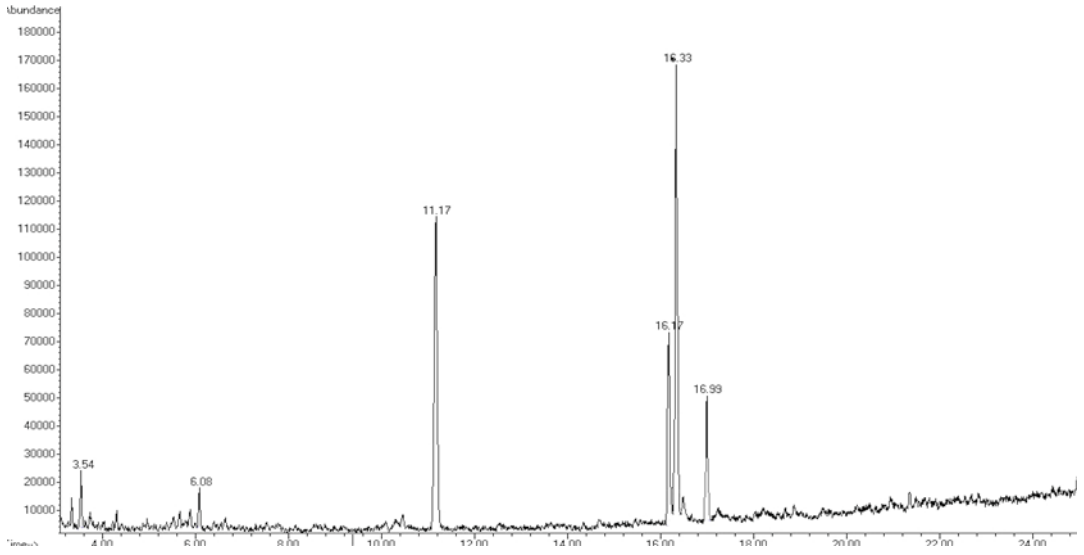

Figure 5. GC-MS analysis of *Botryococcus* sp. Microalgae Fatty Acid Composition that do not irradiated (Control)

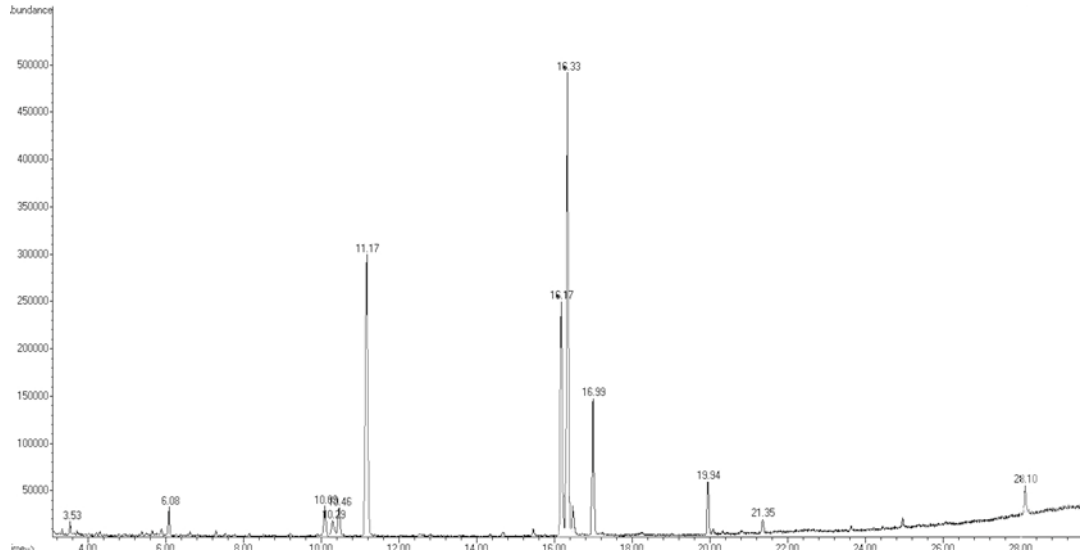

Figure 6. GC-MS analysis of *Botryococcus* sp. Microalgae Fatty Acid Composition that have been Irradiated with 10 Gy of gamma rays

the presence of 12 types of fatty acids as shown in Table 3. The main fatty acids still dominated by oleic acid (32.17%) and palmitic acid (26.72%).

The different compotition of fatty acids between control and 10 Gy irradiated microalgae was allegedly due to the induction of enzyme that corelated to fatty acid elongation process, so the fatty acid elongated into arachidonic acid, acid and acid aracidic lignoserat. Therefore the percentage of C16 and C18 decreased due to the elongation process. Gamma ray radiation can cause oxidative stress in the cell affect the enzyme activity in the cell (Agarwal et al., 2008). These results were supported by the results on *Desmodesmus* sp., which the C16 and C18 fatty acids were decreased due to the formation of long chain fatty acids (Hu et al., 2013).

An important finding in this research that irradiation using gamma rays at a dose of 10 Gy to the cell *Botryococcus* sp. microalgae has changed the characteristics of their growth, biomass, percentage of total lipids cell and fatty acid profile. The findings in this research can be the basis for the engineering production of biodiesel using *Botryococcus* sp. microalgae as raw material.

CONCLUSION

Gamma irradiation gave an effect on the growth and lipid content of *Botryococcus* sp. Results showed that ^{60}Co Gamma irradiated gave an effect on biomass, growth and lipid content of *Botryococcus* sp. There was significantly different only between control (0 Gy) with 10 Gy irradiated microalgae. The highest biomass and lipid content are found in 10 Gy irradiated microalgae are 0.833 gram biomass and 41 % lipid content. Fatty acid profile of control *Botryococcus* sp. microalgae has 6 fatty acids while 10 Gy irradiated microalgae has 12 fatty acids, with the long-chain fatty acids increased, where as short-chain fatty acids decreased.

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