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 composition 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, whereas short-chain fatty acids decreased. This research could be the basis for engineering of microalgae for biodiesel production.

How to Cite

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INTRODUCTION

Microalgae is an unicellular 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 biohydrogen 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 60Co 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 60Co 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 60Co 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 Composition (Andersen, 2005)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Total (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient Components</td>
<td></td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>20</td>
</tr>
<tr>
<td>Na₂EDTA</td>
<td>45</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>1.30</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>100</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>0.36</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>33.60</td>
</tr>
<tr>
<td>Vitamin Stock Solution</td>
<td></td>
</tr>
<tr>
<td>Vitamin B12 (Cyanocobalamin)</td>
<td>5.00</td>
</tr>
<tr>
<td>Vitamin B1 (Thiamine.HCl)</td>
<td>100.00</td>
</tr>
</tbody>
</table>

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](image)

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 ⁶⁰Co 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.

![Figure 1. Botryococcus sp. microalgae culture](image)

Determination of Harvesting Time

The harvesting time was done by making the *Botryococcus* sp. growth curve on each microalgae irradiated ⁶⁰Co 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.

![Figure 2. Harvesting microalgae with Whatman filter paper No 40](image)

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)

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

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 ⁶⁰Co Rays Irradiation on the Growth of Botryococcus sp.
Botryococcus sp. microalgae growth profile without and with treatment of ⁶⁰Co Gamma ray irradiation is presented in Figure 4.

Results showed that ⁶⁰Co 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 occurred for 11 days starting on 1st to 11th day of growth. Then the stationary phase occurred 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 occurred 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 occurred in the 1st day growth. Then the exponential phase, where the 2 Gy and 4 Gy dose irradiated microalgae had until the 15th day, whereas in 6 Gy and 10 Gy dose irradiated microalgae occurred 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](image-url)
of gamma radiation on Nitschia 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 40Co 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>
<thead>
<tr>
<th>Radiation Dose</th>
<th>Biomass (g)</th>
<th>Lipid Content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Gy</td>
<td>0.130±0.02a</td>
<td>27.173±1.6a</td>
</tr>
<tr>
<td>2 Gy</td>
<td>0.183±0.05b</td>
<td>23.772±6.7b</td>
</tr>
<tr>
<td>4 Gy</td>
<td>0.133±0.03a</td>
<td>29.764±5.0ab</td>
</tr>
<tr>
<td>6 Gy</td>
<td>0.177±0.03a</td>
<td>31.544±3.1ab</td>
</tr>
<tr>
<td>10 Gy</td>
<td>0.333±0.11b</td>
<td>41.044±4.0b</td>
</tr>
</tbody>
</table>

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 effect 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 results of the irradiation dose 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 occurred 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 occurred because gamma rays mutagenesis irradiation induce genetic alteration that depend on irradiation dose (Ahowowalia and Maluszynski, 2001). This is supported by Tamam 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.

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. 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. 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 occurred 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 occurred because gamma rays mutagenesis irradiation induce genetic alteration that depend on irradiation dose (Ahowowalia and Maluszynski, 2001). This is supported by Tamam 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 ⁶⁰Co Rays Irradiation on Fatty Acid Profile of Botryococcus sp.

Fatty acid composition analysis in this study was conducted by Gas Chromatography-Mass Spectrophotometry (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 chromatograph (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 hexadekadienoic acid, linolenic acid, arachidonic 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, linoleic, oleic, linolenic, arachidonic and linolenic acid. The higher percentage of fatty acid were oleic (36.53%) and palmitic (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, whereas 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

<table>
<thead>
<tr>
<th>Fatty Acid Compositions</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Capric acid (C10: 0)</td>
<td>3.15</td>
</tr>
<tr>
<td>Myristic acid (C14: 0)</td>
<td>2.32</td>
</tr>
<tr>
<td>Palmitate acid (C16: 0)</td>
<td>33.44</td>
</tr>
<tr>
<td>Palmitoleic acid (C16: 1)</td>
<td>-</td>
</tr>
<tr>
<td>7,10 Hexadecadienoic acid (C16: 2)</td>
<td>-</td>
</tr>
<tr>
<td>Stearic acid (C18: 0)</td>
<td>9.66</td>
</tr>
<tr>
<td>Oleic acid (C18: 1)</td>
<td>36.53</td>
</tr>
<tr>
<td>Linoleic acid (C18: 2)</td>
<td>14.90</td>
</tr>
<tr>
<td>Linolenic acid (C18: 3)</td>
<td>-</td>
</tr>
<tr>
<td>Arachidonic acid (C20: 0)</td>
<td>-</td>
</tr>
<tr>
<td>Arachidonic acid (C20: 4)</td>
<td>-</td>
</tr>
<tr>
<td>Lignoseric acid (C24: 0)</td>
<td>-</td>
</tr>
</tbody>
</table>

Fatty acid analysis in 10 Gy (Figure 6)⁶⁰Co Gamma irradiated Botryococcus sp. results showed...
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 composition of fatty acids between control and 10 Gy irradiated microalgae was allegedly due to the induction of enzyme that correlated 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 60Co 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, whereas short-chain fatty acids decreased.

ACKNOWLEDGEMENT

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