



## Effect of Electron-Beam Irradiation on the Growth Profile and Fatty Acid Composition of *Botryococcus* sp.

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### Abstract

*Botryococcus* sp. is an important microalgae with high content of saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) for biofuel production sources. Induction of mutagenesis using irradiation can potentially alter the characteristic of mutant strain. The aim of this study was to know the effect of electron-beam irradiation on the growth profile and fatty acid composition of *Botryococcus* sp. In this study, *Botryococcus* sp. adjusted an optical density 1.0 after exposed to different doses of electron-beam irradiation (160 kGy, 240 kGy, and 320 kGy) and induced random mutagenesis for strain improvement was observed based on high energy (1.5 MeV and 2 mA). Several mutants obtained were designated as strains of B160, B240, and B320 respectively. The profile growth was significantly different between the control (0 kGy) and irradiated microalgae strain. The highest growth was found in B320 mutant. Fatty acid of *Botryococcus* sp. control produced 7 fatty acids, B160 produced 7 fatty acids, B240 produced 6 fatty acids, whereas B320 produced 9 fatty acids. B320 produced hydrocarbon and phthalic acid as well as fatty acids. Total SFAs and MUFAs of B240 was increased 1.6 times compared to the wild type. The results would give some implications to improve the quality of biodiesel from *Botryococcus* sp.

### How to Cite

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## INTRODUCTION

Microalgae contain metabolite that can be applied in food biotechnology and a source of renewable energy (Ermavitalini *et al.*, 2017). High lipid content in microalgae has potential as a renewable energy source for replacing diesel fossil fuels. Opportunities for the development of biodiesel energy from microalgae sources—are greatly improved to overcome the energy crisis of oil globally (Brutyan, 2017). Selection of the species for producing biodiesel is highly dependent on fatty acid composition (Tasić *et al.*, 2016). *Botryococcus* sp. has a lipid content with SFA and MUFA composition of up to 85.1% per dry weight and PUFA content of 14.90%. The microalgae with high composition of SFA and MUFA can be developed as a potential producer of lipids (Ermavitalini *et al.*, 2017). High composition of SFA and MUFA in cell of organism can be recommended processed into biodiesel (Singh & Mallick, 2014). Some studies reported that the *Botryococcus* sp is a type of microalga that has a slow growth compared to other microalgae.

Several studies have been reported to work for the improvement of cultivation system. Research by optimizing variation of nutrition and light in cultivation of *Botryococcus* sp. showed influences on the growth and accumulation of lipid (Ruangsomboon, 2012). Mutagenic engineering using gamma irradiation to *Botryococcus* sp. has been increased biomass and lipid production up to 1.5 times (Ermavitalini *et al.*, 2017). A similar study showed that *Arthrospira platensis* that irradiated with high-dose electron beam may increase lipid accumulation by about 30 - 34 times higher than the control (Kim *et al.*, 2014). Apparently, mutagenesis technology is a fast and efficient method to improve strain performance by increasing the lipid and accelerating the growth of microalgae (Deng *et al.*, 2011).

Irradiation with electron beam does not use radioactive compounds, so it is safer when compared to other irradiations (Zhang *et al.*, 2017). The advantage of using electron beam irradiation method in improving the performance of microalgae strains is to accumulate more lipids. This electron beam irradiation has been widely practiced in high-level plants (Luo *et al.*, 2012), microorganisms (Liu *et al.*, 2014), and microalgae (Kim *et al.*, 2014). There is no previous study on the application of electron beam irradiation in isolates of *Botryococcus* sp. This research was conducted to obtain changes in the growth profile of the microalgae and fatty acid composition.

## METHODS

### Sample Preparation

The isolate of *Botryococcus* sp. was supplied from laboratory of natural Feed BBPAP Jepara, Central of Java, Indonesia. The isolate was cultivated at room temperature on the modified Johnson medium (Table 1) in volume of 1 L for two weeks under 2500 lux light intensity with bright and dark light 16:8 cycles of white spectrum lights (Gouveia, 2010). The samples for irradiation were prepared into bottle containing 15 mL of *Botryococcus* sp. at optical density reached 1.0 using UV-VIS spectrophotometer at 680 nm of wavelength.

**Table 1.** Modified Jhonson medium composition (Borowitzka *et al.*, 1988).

Ingredients	Total (g/L)
MgSO <sub>4</sub>	0.5
MgCl	0.2
KNO <sub>3</sub>	0.1
KCl	0.2
NaCl	27
Trace element	1 mL
FeCl <sub>3</sub> -EDTA	1 mL

### E-beam Irradiation

The Samples were transported in a dry ice cooler to an e-beam facility at the National Nuclear Energy Agency (BATAN), Jakarta. The suspension of samples at a volume of 15 mL was put in the petri dish and was subjected to different irradiation doses of 0 kGy (control), 160 kGy, 240 kGy, and 320 kGy of electron linear accelerators (1.5 MeV and 2 mA). A day later, the irradiated sample was cultured on Johnson medium in the volume 1 L and the optical density was 1.0. The irradiation dose was calculated according to the equation below (Kim *et al.*, 2013).

$$\text{Irradiation doses (kGy)} = \frac{1 \text{ MeV} \times 1 \text{ mA} \times 1 \text{ s}}{\text{kg}}$$

### Growth Profile Determination

Harvesting of biomass by centrifugation at 3000 rpm for 15 minutes. The cell density was determined using UV-VIS spectrophotometer at 680 nm wavelength. The specific biomass profile of each *Botryococcus* strain was calculated from the slope of the linear regression of biomass and

optical density in exponential growth phase. The data obtained were analyzed using the Ms.Excell 2007 program (Song *et al.*, 2013).

### Fatty Acid Composition Analysis

Preparation of sample maceration was done following Choi *et al.*, (2014) method, with a composition of 0.5 % of the cells was mixed with 95% of methanol solvent. The mixture was stirred for 6 h at room temperature to extract the lipids. The organic solvent layer and cell debris were separated by centrifugation (HERMLE Z300) at 5000 rpm for 10 minutes, then it was evaporated using a vacuum evaporator (EYELA N-1110). Analysis of fatty acid composition was done by Gas Chromatography-Mass Spectrometry (GC-MS SHIMADZU) based on the method of Song *et al.*, (2013). GC-MS analysis was conducted at the Laboratory of Biochemistry, Department of Chemistry, Indonesia University of Education (UPI).

### Data Analysis

Biomass of *Botryococcus* sp. (g/L) was analyzed with statistical analysis by Anova (Analysis of Variance), with variation of doses 0, 160, 240, 320 kGy, at 95% level accumulated for culturing 30 days.

## RESULTS AND DISCUSSION

### E-Beam Irradiation Effect on the Growth Profile of *Botryococcus* sp.

Growth profile of *Botryococcus* sp. without and with the E-beam irradiation treatment is presented in Figure 1. Non-irradiated *Botryococcus* (0 kGy) as a wild type (BW) showed a trend of growth pattern similar to E-beam irradiated strain of B160 (160 kGy) and B320 (320 kGy). The growth profile of these three isolates showed no adaptation phase at the beginning of growth with a slow and a long growth period of 21 days, after which, they began to show a log phase until at the end of the observation for 30 days. On the other hand, the strain of B240 (240 kGy) during cultivation showed a stagnant growth during observation. In this study, after 30 days there has been no sign to the phase of death, so the possibility of this growth will still occur with cultivation more than 30 days. Research conducted by Al-Hothaly *et al.* (2015) showed that *Botryococcus* sp. had the log growth phase until 40 days.

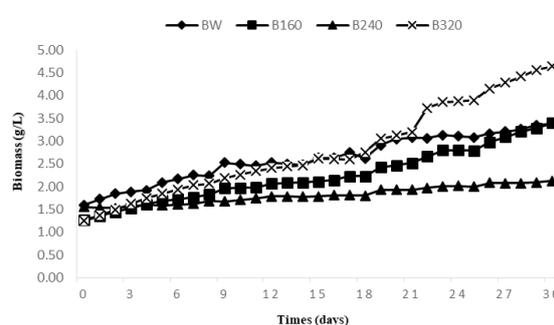
Irradiation E-beam showed an effect for increasing of the biomass of the B320 strain, with highest accumulated of the biomass (dry weight) of 4.645 g/L, while the strain B240 showed lowest dry weight of 2.13 g/L with significant differences of biomass among different irradiation doses ( $p < 0.05$ ). This result was considered to

be significantly different to confirm that the dose of E-beam irradiation effects on biomass production.

**Table 2.** Effect of Electron beam irradiation on the Biomass of *Botryococcus* sp.

Treatment	Biomass (g/L)
B240	1.8145 a
B160	2.2433 b
BW (control)	2.6149 c
B320	2.8026 d

Description: Duncan test at 95% significance level. The error term is Mean Square (Error)= 0.010.



**Figure 1** *Botryococcus* sp. growth at various doses of electron beam irradiation

### E-beam Irradiation on Fatty Acid Composition of *Botryococcus* sp.

The fatty acid (FA) composition of *Botryococcus* strains is presented in Table 2. A comparison of non-irradiated (BW) and E-beam irradiation strains (B160, B240, and B320) revealed that there were radiation-induced fatty acids in the forms of PUFA, SFA, and MUFA. There was alteration in percentage of the major fatty acid content and some compounds in *Botryococcus* strains. Their composition and percentage of these compounds did not show a linear relationship with the irradiation dose. Changes in fatty acid composition of strains are due to the irradiation, of which it also affects some other chemical components in the cell. The GC analysis results also showed the presence of phthalic acid, hydrocarbons and a small fraction of alcohols in non-irradiated strains.

The BW strain had a fatty acid composition of PUFA, SFA, and MUFA which was almost balanced (Table.2). This strain had a main fatty acid profile such saturated fatty acid (SFA) as stearic acid (C18: 0) and pentadecanoic acid (C15:0), MUFA as oleic acid (C18:1) and 14-Octadecenoic acid (C18: 1n-4); whereas PUFA included linolenic acid (C18: 3), linoleic acid (C18:2),

Acid 7.10 Hexadecadienoate (C16:2); and it was also found phthalic acid, three types of alcohols, and two types of hydrocarbons. The highest percentage of fatty acid from PUFA group was indicated as linoleic acid (27.96%). Accumulation of the SFA and MUFA reached 50%. From this data, which made clear that the *Botryococcus* sp. has potential as a producer of fatty acids which can be processed to be a biodiesel.

The E-beam induced composition and fatty acid profile are presented in Fig.2. The strain B160 had high accumulated amount of PUFA (58%). Mutagenic effects showed that PUFA levels increased about 1.5 times in both of B160 and B320 strains, however the fatty acid lowest in B240 strain. Fig 2 showed the result of GC-MS analysis and presence of the fatty acid composition of *Botryococcus* sp. Profile of fatty acids of the B160 strain, which had 7 types of fatty acids, comprising 25% SFA as stearic acid (C18:0) and with a high percentage as 22.6% palmitate acid (C16:0). B160 strain also presented PUFA as linolenic acid (C18:3), linoleic acid (C18:2), and  $\alpha$ -linolenic acid (C18:3), MUFA as palmitoleic acid (C16:1) and 11-Octadecenoic acid (C18:1n-7). Percentage of MUFA in B160 strain only 12% and 5% as phthalic acid. The instability of the double bond in the chain of PUFA structure may have an effect on structure, so with different doses could cause change content of the fatty acid.

Profile of fatty acid of the B240 strain presence of 44% SFA, 34% MUFA, only 18% PUFA, and 4% hydrocarbons. The strain dominated by five fatty acid such as types: SFA stearate acid (18:0), aracidic acid (C20:0), and palmitic acid (16:0); MUFA included oleic acid (C18:1) and 11-octadecenoic acid (C18:1n7-4); PUFA linolenic acid (C18:3), and 1 type of hydrocarbon that was 6.9 -cis-3, 4-epoxy-nonadecadiene. SFA was accumulated in large numbers on strains of the B240 strain. Interestingly, E-beam irradiation shifted the PUFA composition to the lowest level by 18% by substituting the SFA level, so that it increased almost 2- times compared to the B160 and B320 strains. This study was the first to show that it is feasible to use electron-beam mutagenesis as a means to produce *Botryococcus* sp. mutants that can be screened for high productivity of fatty acid for the use in industrial processes.

The strain of B320 had accumulation of 50% PUFA and 21% hydrocarbons based on Table.2. Profile of fatty acid composition of the B320 strain was dominated by nine types of fatty acids that consisted of SFA (stearic acid, arakidic acid, and palmitate acid), MUFA (palmitoleic acid and 11-octadecenoic acid), and PUFA (linolenic acid, linoleic acid, 7, 10 hexadecadienoic acid, and acid 8, 11, 14-docosatrienoic), on this profile can also determined eight types of hydrocarbons, iron, and phthalic acid.

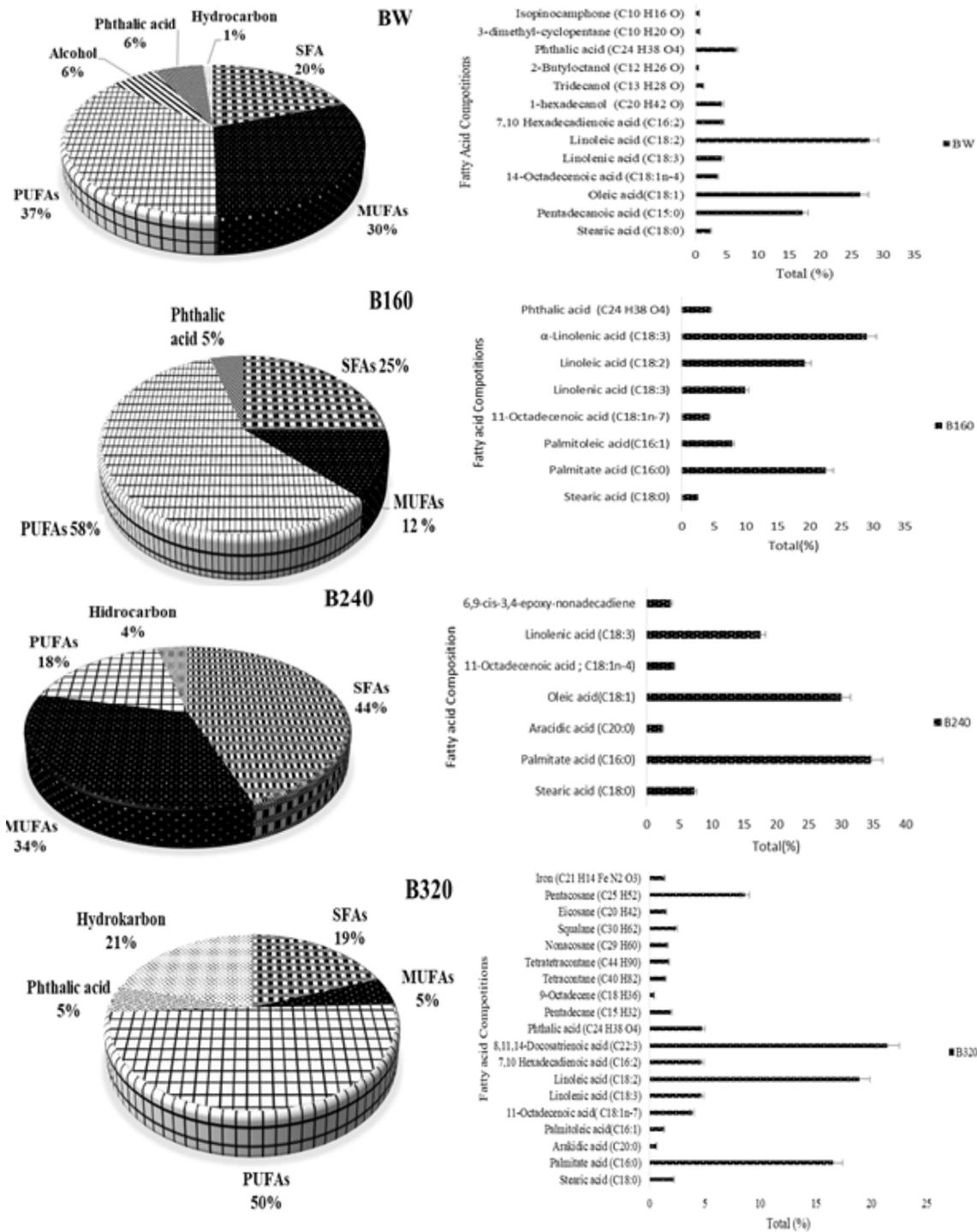
The different composition of fatty acid from different strains can be used as a starting point to determine the potentially application of each strain. The *Botryococcus* B240 strain has prospect as biofuel feedstock with high percentage of SFA and MUFA. Monounsaturated fatty acids (MUFAs) are the best components for biodiesel when considering the low temperature fluidity and oxidative stability, therefore the fatty acid composition should be modified to increase MUFA contents as well as to enhance oil and lipid production.

In this study, the E-beam irradiation was related to the decreasing of MUFAs of the B160 and B320 strains. A higher amount of SFAs would lead to a biodiesel with elevated Cetane number (CN), which would present a higher combustion quality. Saturated fatty acids (SFA) and MUFA play a significant role in fuel properties. Modifying the fatty acid compositions of *Botryococcus* is critical to promote its practical application in the biodiesel industry. CN is related to the ignition readiness and is affected by the carbon chain length of the fatty acid present in the oil. The higher the carbon number, the higher is the viscosity of the biodiesel (Cabanelas *et al.*, 2015).

B160 and B320 strains that contain high PUFA are more suitable in the field of food. B-160 contains linolenic acid and ALA of 9.98% and 27% respectively. These fatty acids are omega 3 fatty acids, and there is linoleic acid which is 19.34% omega 6. When compared to the previous study (Ermavitalini *et al.*, (2017), the linoleic and linolenic acids produced by B160 were 1.2 and 7-times higher respectively. At a high dose, B320 has several types of hydrocarbons. Hydrocarbons in *Botryococcus* are known as botryococcene and squalene which can be used as biofuel

**Table 3.** The fatty acid (FA) composition of strains of *Botryococcus* sp. after irradiation

Strain <i>Botryococcus</i>	PUFA (%)	SFA (%)	MUFA (%)	SFA & MUFA (%)	Hydrocarbon (%)	Phthalic acid (%)	Alcohol (%)
BW	37	20	30	50	1	6	6
B160	58	25	12	37	0	5	0
B240	18	44	34	78	4	0	0
B320	50	19	5	24	21	5	0



**Figure 2.** Fatty acid Composition of *Botryococcus* sp. : (a) BW, (b) B160, (c) B240, and (d) B320 after irradiation with electron beam.

feedstock. In this study, squalene was found to be 2.42%. Squalene can also be used as biofuel feedstock through methylation that can also be converted to sterol membrane through epoxidation process (Uchida *et al.*, 2015). The most common hydrocarbon found in B320 is pentacosane (8.61%) that is a saturated aliphatic hydrocarbon generally found in beeswax.

The ability of green microalgae to produce hydrogen ( $H_2$ ) is very interesting, but it should be noted that additional metabolites, including organic acids and alcohols, are also secreted by microalgae species during anoxia (Radakovits *et al.*, 2010). Interestingly, this study found alcohol in wild type of *Botryococcus* (BW), however it was not found in the strains with effect irradiation tre-

atment. Becker (1994) also mentioned that the lipid components present in the microalgae can be classified by their polarity such as non-polar (lipophilic) lipids, carbon chains (fatty acids), polar (hydrophilic) and other lipids (carboxylic, alcohol and sugar groups).

Phthalic acid, which is an aromatic dicarboxylic acid, is also found in B160 and B320 strains. Phthalic acid (benzene dicarboxylic acid) commonly used in industry as raw material for synthesis. The discovery of phthalic acid was also reported by Wayan *et al.* (2017) in *Lingbya* sp. and consider that microalgae with content of this metabolite is potential as antibacterial and antifungal. The phthalic acid found in this study was Bis-2-ethylhexyl phthalate (DEHP). DEHP is the most popular phthalate in plasticizer. Studies show that DEHP and other phthalates can also be present in food such as milk, butter, meat and fatty tissue. However, DEHP is a toxic plasticizer that can affect the liver and kidneys and even cause cancer if eaten by humans. Therefore, phthalic acid ester is not allowed to be added as a food additive by government regulations (Wang *et al.*, 2014). Meanwhile, phthalic acid was found in strains of BW, B160, and B320, so that for food development this should be studied further.

## CONCLUSION

E-beam irradiation had significant effects on the growth profile and fatty acid composition of *Botryococcus* sp. High dose irradiation at 320 kGy increased the relative growth profile from biomass accumulation for 30 days. Considering a major proportion of PUFA in B160 and B320 strain, so that this strain would be more suitable for nutritional supply. However, induction of e-beam irradiation in B240 strain had a significant effect resulted in higher composition of total SFA and MUFA of 1.6 times compared to the wild type. The results would give some implications to improve the quality of *Botryococcus* sp. for production of biodiesel.

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