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**Research Article**

# Growth Kinetic Modelling of Efficient *Anabaena* sp. Bioflocculation

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**ABSTRACT**
Bioflocculation is a harvesting technique that employs flocculant agents such as bacteria and microalgae. The benefit is the absence of a chemical-added flocculant. Because bacteria need a particular medium, microalgae flocculant agents are more effective. This study used *Anabaena* sp. to collect fat, protein, and carbohydrates from the Glagah consortium. Three replications of those microalgae were grown in 300 ml of Bold Basal Medium culture for eight days. On the day of harvest, flocculant microalgae (*Anabaena* sp.) and non-flocculant microalgae (Glagah) were combined to accomplish flocculation. On the day of harvest, parameters were observed by combining *Anabaena* sp. with the Glagah consortium in the ratios 1: 1, 0.5: 1, and 0.25: 1. There were three times of each parameter test. Utilizing a wavelength of 750 nm, the proportion of precipitation was calculated spectrophotometrically. Bligh and Dyer were used to measure the lipids. The phenol sulfate technique was used to calculate the amount of carbohydrates. By employing the Bradford method, proteins were quantified. Biofocculation percentages and carbohydrate content were optimum on a ratio of 0.25:1. Lipid and protein content were optimum on a ratio of 1:1.

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## INTRODUCTION

The high-energy input for harvesting biomass makes current commercial microalgal biodiesel production economically unfeasible. In order to minimize energy consumption, it needed more efficient harvesting technique (Benemann 1997). Microalgae flocculation is a more effective technique than other microalgae harvesting techniques. They are considered safer and more environmentally friendly than synthetic flocculants. Furthermore, using microalgae as bioflocculants does not require special media, so they are more cost-effective than bacteria (Salim et al. 2011).

Global warming is driving the development of renewable energy. One of the renewable energy is from the Glagah consortium (Pradana et al. 2018). The Glagah consortium is a microalgae taken from the coast of Glagah Beach in Kulon Progo Regency, Yogyakarta. The Glagah consortium consists of 6 species, namely *Cyclotella polymorpha*, *Cylindrospermosis raciborskii*, *Golenkinia radiata*, *Corethron criophilum*, *Chlamydomonas* sp. and *Syracosphaera turquoise* (Suyono et al. 2016b). Glagah consortium is potential for biodiesel and lipid production (Suyono et al. 2016a). However, it

needs help in harvesting the Glagah consortium. To harvest the Glagah consortium using microalgae, *Anabaena* sp. is a potential biofloccluant. *Anabaena* sp. produces EPS that plays a role in bioflocculation. The increase in EPS production directly influences bioflocculant activity (Tiwari et al. 2015).

Bioflocculation is a spontaneous flocculation of microalgae cells due to the secretion of the EPS (Sathe 2010). This EPS causes the formation of clumps of cells, which will become biomass so that it is deposited. Stress conditions trigger EPS secretion in limited nutrients (Lee et al. 2009).

The harvesting Glagah consortium using *Anabaena* sp. has never been done before. Thus, it is important to study the harvesting Glagah consortium using *Anabaena* sp. to determine the percentage of flocculation, lipids, and protein of the Glagah consortium.

## MATERIALS AND METHODS

### Materials

The materials needed include Culture of *Anabaena* sp., medium cultivation, Glagah consortium, methanol, chloroform, phenol, sulfuric acid, Bradford solution, and Bovine Serum Albumin (BSA).

### Methods

The culture of *Anabaena* sp. was harvested on the 4$^{th}$ day, while the Glagah consortium was on the 3rd day (based on preliminary tests). Samples were inserted in 15 ml conicles with a ratio of flocculant microalgae (*Anabaena* sp.), and non-flocculant (Glagah consortium) was 1: 1, 0.5: 1, and 0.25: 1. Treatment measurements were carried out three times with triplicate repetitions of control *Anabaena* sp. and the Glagah consortium. The samples parameters of lipids, carbohydrates, and proteins were left to stand for 24 h, aiming to mix the Glagah consortium and *Anabaena* sp. The parameter measurement method is as follows:

#### Growth Calculation of *Anabaena* sp. and Glagah consortium

Cell of *Anabaena* sp. and Glagah consortium density was counted every 24 h using hemocytometer (Neubauer, Germany). Cell density was determined on cells larger than 8 μ in size.

#### Bioflocculation Harvesting

Absorbance was measured at the wavelength of 750 nm, with $t_0$ 0 hour and $t_n$ 24 h. The percentage of precipitation was calculated using the following formula (Salim et al. 2011):

$$\% \text{ Flocculation} = \frac{OD_{750}(t_0) - OD_{750}(t_n)}{OD_{750}(t_0)} \times 100\%$$

#### Lipid content

The Bligh and Dyer (1959) method measured lipid content. The 15 ml sample was centrifuged at 4000 rpm for 15 minutes at 4° C. Supernatant was removed, and 2 ml of methanol and 1 ml of chloroform were added to pellets. Then the sample was vortexed for 1 minute. Next, 1 ml distilled water and 1 ml chloroform were added to the models. The sample was vortexed for 1 minute and centrifuged for 15 minutes at 1800 rpm at 4° C. The sample was divided into three layers. Lipids are at the bottom. Lipids were taken and placed on a petri dish; then the petri dish was put in the incubator. The chloroform evaporation process is carried out with an open petri in an incubator, for 12 h at 33° C. The empty weight of a petri dish, the weight of a petri dish with lipids, and the weight after the

oven is weighed, then calculated using the formula (Novaryatiin et al. 2015):

$$\text{Lipid content (mg/ ml)} = \frac{\text{filled weight} - \text{empty weight of petri dish}}{\text{dry weight x sample volume}}$$

### Carbohydrate content

The method of Dubois et al. (1956) was used to measure carbohydrate content. A 15 ml was centrifuged at 3300 rpm for 10 minutes at room temperature. 0.5 ml of 5% phenol was added to the pellet. The sample was vortexed and allowed to stand for 10 minutes. 1 ml of sulfuric acid was added. The sample was vortexed and allowed to stand for 20 minutes. A spectrophotometer measured the absorbance of the sample at 490 nm. Carbohydrate concentration was measured using a glucose standard curve obtained from the FALITMA Laboratory, Faculty of Biology, Universitas Gadjah Mada. The glucose standard curve formula was:

$$Y = 0.0884x + 0.0095$$

### Protein content

Protein was estimated by the (Bradford 1976) method. A total of 15 ml of the sample was centrifuged at 1800 rpm at room temperature for 10 minutes. The supernatant was removed. Pellets were added with 45 µl of 10% SDS to damage the cell wall. Samples were put on waterbath and heated at 95°C for 5 minutes. The sample was cooled for 5 minutes in an ice cupboard. An 8 µl of supernatant was taken and put into a microplate, and 200 µl of Bradford solution was added. Absorbance was measured using the ELX bioTek ELISA at 595 nm. Protein concentration was estimated based on the Bovine Serum Albumin standard curve obtained from the FALITMA Laboratory, Faculty of Biology, Universitas Gadjah Mada. The Bovine serum albumin standard curve formula was:

$$Y = 0{,}0005x + 0{,}011$$

## RESULTS AND DISCUSSION

Microalgae have eight-day cycles. Increasing the number of cells showed that microalgae absorbed nutrients and used them for their metabolism. Based on Figure 1, the $4^{th}$ day showed the highest number of *Anabaena* sp., indicating the harvest day. The death phase of *Anabaena* sp. occurred from the $5^{th}$ day to the $8^{th}$ day. The number of cells marked the occurrence significantly. The death phase of microalgae cells is caused by a decrease in nutrients directly proportional to the reduction in metabolic activity. Another possibility is the accumulation of organic substances ($NO_2^-$ and $NH_4^+$) derived from dead microalgae cells. So, they can poison microalgae and can interfere with the absorption of dissolved oxygen and nutrients (Nugroho 2006; Suantika et al. 2009).

Glagah consortium occurred indicated that the lag phase is microalgae adaptation to the new environment (Figure 1). It was followed by an exponential phase and characterized by a high rate of cell division. The $3^{rd}$ day was the harvest day of the Glagah consortium. The death phase of the Glagah consortium was from the $4^{th}$ day to the $8^{th}$ day and marked by a significant decrease in the number of cells.

Suitable kinetic modeling needs to be developed to learn the dynamics of biomass growth of microalgae; suitable kinetic modeling can be used for predicting the performance and optimization of photobioreactor operating conditions (Galvao et al. 2013).

The two non-linear models Logistic and Gompertz, were chosen for the *Anabaena* sp. and Glagah consortium. For rapid population

growth of organisms, Logistic and Gompertz models are commonly used (Lam et al. 2017). Logistic and Gompertz model was the simplest models in microbial growth because it is not limited by substrate type and consumption.

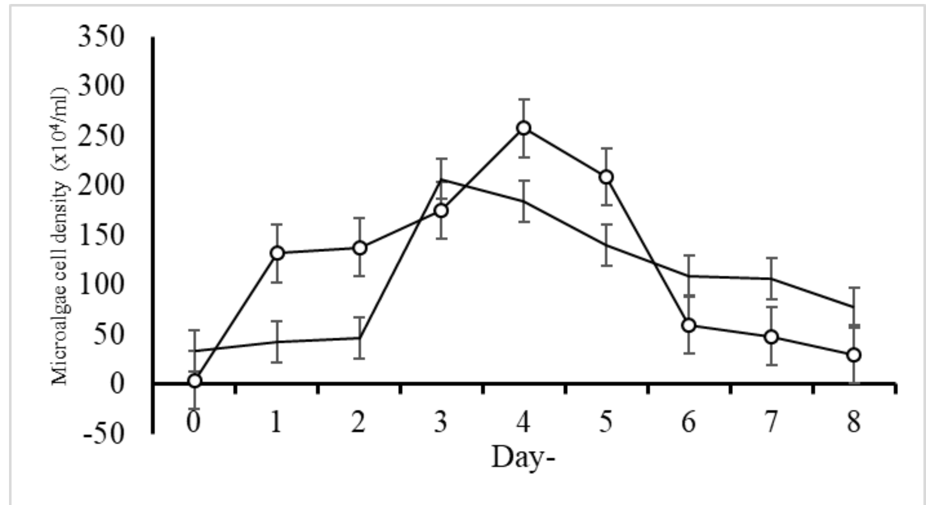


**Figure 1.** Microalgae growth curves *Anabaena* sp. and Glagah consortium with-BBM (Rahmawati 2020).

The Logistic model predicts the number of stable populations using the maximum growth rate per day as its parameter. The Logistic model was calculated using the following formula. X is cell density, $X_0$ is the initial cell density, $X_{max}$ is the maximum cell density, and $\mu_{max}$ is the maximum specific growth rate (Phukoetphim et al. 2017; Hanief et al. 2020).

Based on logistic modeling (Figure 2), the maximum specific growth rate ($\mu_{max}$) of *Anabaena* sp. and Glagah consortium were 1.6265/day and 0.8827/day, respectively. The $R^2$ error were 0.70 and 0.74 for the *Anabaena* sp. and Glagah consortium. For the Gompertz modeling, the maximum cell production rate (rm) of *Anabaena* sp. was 0.6370 x $10^6$ cells/mL. The top cell production rate (rm) of the Glagah consortium was 1.6792 x $10^6$ cells/mL.

Table 1 shows the results of the Logistic and Table 2 shows growth rate parameter of Gompertz Model of the *Anabaena* sp. and Glagah consortium experimental growth data. The Gompertz model (Table 2) fits the microalgae growth curves better than the Logistic model (Table 1) for two microalgae species. The coefficient of determination $R^2$ values established the goodness of fit of the Gompertz model over the Logistic model in the study.

**Table 1.** Growth Rate Parameter of Logistic Model.

| Parameter | *Anabaena* sp. | Glagah consortium |
|---|---|---|
| $\mu_{max}$ | 1.6265 | 0.8827 |
| $R^2$ | 0.70 | 0.74 |

**Table 2.** Growth Rate Parameter of Gompertz Model.

| Parameter | *Anabaena* sp. | Glagah consortium |
|---|---|---|
| rm | 0.6370 | 1.6792 |
| tl | -0. 3 | 1.9 |
| $R^2$ | 0.88 | 0.90 |

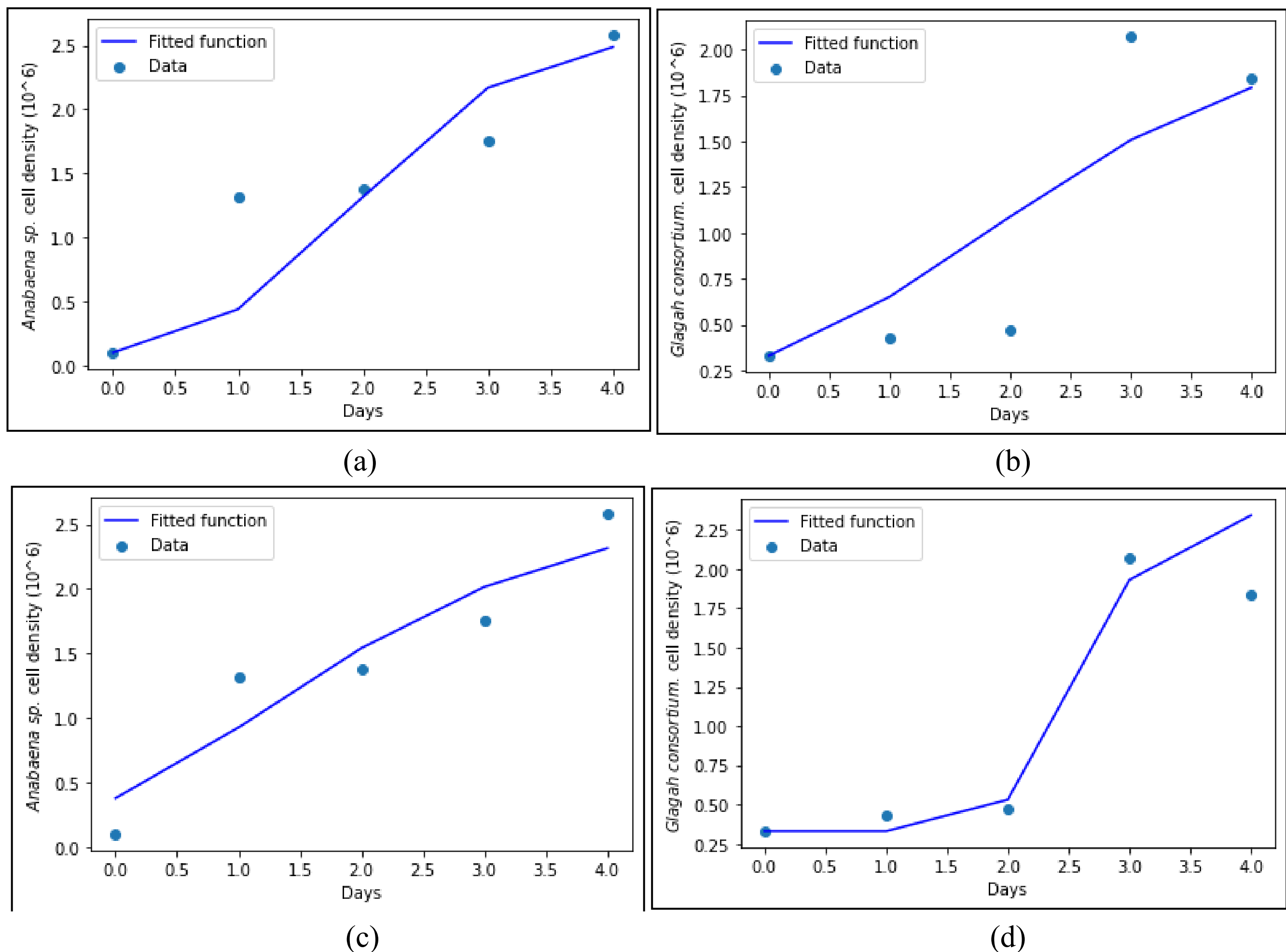


**Figure 2.** Modeling Growth Kinetic of *Anabaena* sp. and Glagah consortium (a)Logistic *Anabaena* sp. (b) Logistic Glagah consortium (c) Gompertz *Anabaena* sp. (d) Gompertz Glagah consortium.

Saputra (2013) recorded that the flocculation percentage of the Glagah consortium is relatively low. *Anabaena* sp. was used as a bioflocculant agent because it produced EPS. According to Pillai (1997), particle flocculation speed is influenced by particle size. The smaller particle size is more challenging to flocculate. Therefore, it was extended for the Glagah consortium to form floc and precipitate compared to *Anabaena* sp. Sathe (2010) declares that bioflocculation is a spontaneous flocculation of microalgae cells due to the secretion of the EPS when the microalgae are under stress. This EPS causes the formation of clumps of cells, which become biomass so that it precipitates. Limited nutrients are stress conditions that trigger EPS secretion (Lee et al. 2009).

The result indicated that the highest percentage of flocculation was obtained at a ratio of 1: 0.25, equal to 80.5%, while the lowest rate of flocculation was obtained at a ratio of 1: 1 equivalent to 49% (Figure 3). Salim et al. (2011) stated that adding flocculant species with high concentrations in harvesting could increase the percentage of precipitation. If referring to this journal, the highest rate of rainfall obtained should be at a ratio of 1: 1, but in this study the flocculation percentage was optimum at 1: 0.25. This is due to EPS, which is produced by *Anabaena* sp., some of the constituents are proteins (Tiwari et al. 2015). It is assumed that one of the proteins produced is anatoxin (Gangl et al. 2015).

According to Suyono et al. (2016b), The Glagah consortium is mixed culture. Mixed culture has rapid growth and high lipid level. According to Chisti (2007), Facilitation between species in diverse culture increase productivity. Thus, it causes the complex mechanism to produce

lipid (Behl et al. 2011). Suyono et al. (2016b) reported that the lipid percentage of the Glagah consortium was 1.25% and could be increased to 13.58% by stressing the treatment of environmental factors such as salinity. Based on Figure 4, it was found that the lipid concentration of *Anabaena* sp. was 0.007 mg/ml while the Glagah consortium was 0.009 mg/ml. The lowest lipid in treatment percentage was obtained at a ratio of 1: 0.25. This was assumed that microalgae were left alive without significant stress.

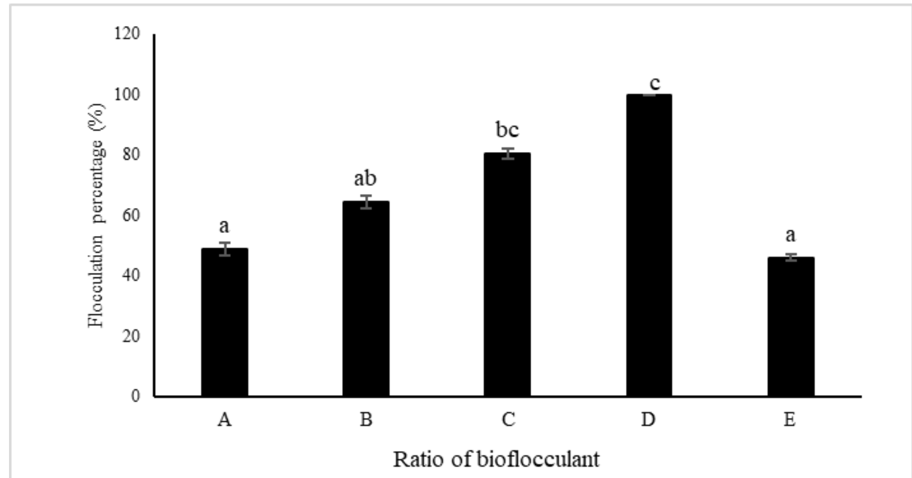


**Figure 3.** Bioflocculation percentages on harvest day using a medium of BBM with the ratio between non-flocculant and flocculant microalgae (A) 1:1, (B) 1:0.5, (C) 1: 0.25, (D) *Anabaena* sp., and (E) Glagah consortium (Rahmawati 2020). Data were means ±SD (n=5). Different bioflocculation percentages on harvest day using a medium of BBM with the ratio between non-flocculant and flocculant microalgae indicated significant differences between treatments and were calculated by one-way ANOVA followed by Duncan Multiple Range Test (DMRT) ($p$ <0.05).

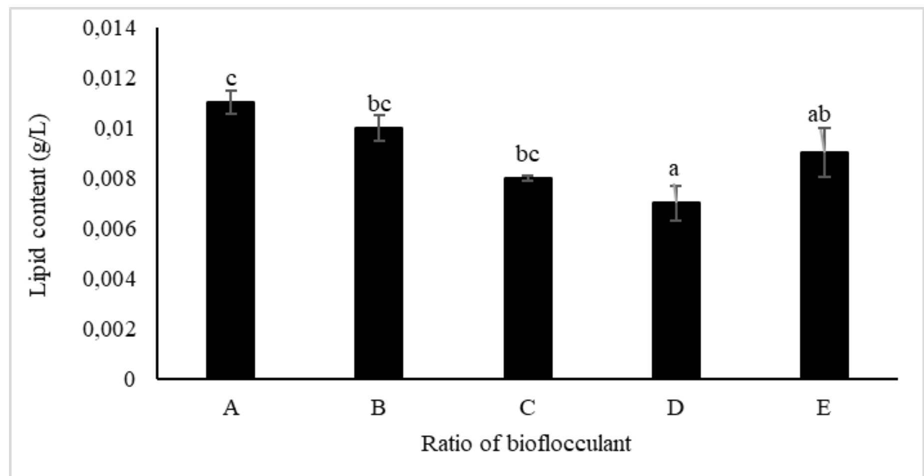


**Figure 4.** Lipid content harvest day using medium of BBM with the ratio between non-flocculant and flocculant microalgae (A) 1:1, (B) 1:0.5, (C) 1: 0.25, (D) *Anabaena* sp., and (E) Glagah consortium (Rahmawati 2020). Data were means ±SD (n=5). Different lipid content harvest days using a medium of BBM with the ratio between non-flocculant and flocculant microalgae indicated significant differences between treatments and were calculated by one-way ANOVA followed by Duncan Multiple Range Test (DMRT) ($p$ <0.05).

Glagah consortium had a higher lipid percentage than *Anabaena*. It is caused by the symbiosis between species, especially with bacteria which helped absorb and supplied nutrients (Croft et al. 2005). The highest lipid concentration was at a ratio of 1: 1. This was caused by stress in the

treatment. Stress was possible because of limited nutrients and efficiency in using photosynthetic active radiation. This is a microalgae response to environmental changes that occur (Guschina & Harwood 2006; Ho et al. 2011).

Carbohydrate is a product of photosynthesis and component of cell walls, mainly in cellulose form. The carbohydrate content of the microalgae is used as an alternative fuel, for example for biofuels (Domozych et al. 2012; Yen et al. 2013). The carbohydrate content in the Glagah consortium is higher than *Anabaena* sp. (Figure 5). Mixing of Glagah and *Anabaena* sp. increased carbohydrate content. Because of the carbohydrate synergy that was contained in each species. The highest carbohydrate content was found at a ratio of 1: 0.25. The lowest carbohydrate concentration was found at a ratio of 1: 1. Addition of bioflocculant concentration decreases protein concentration. According to (Ritanti & Purwadi 2018), increasing the number of cells extends the time for hydrolysis of cell walls.

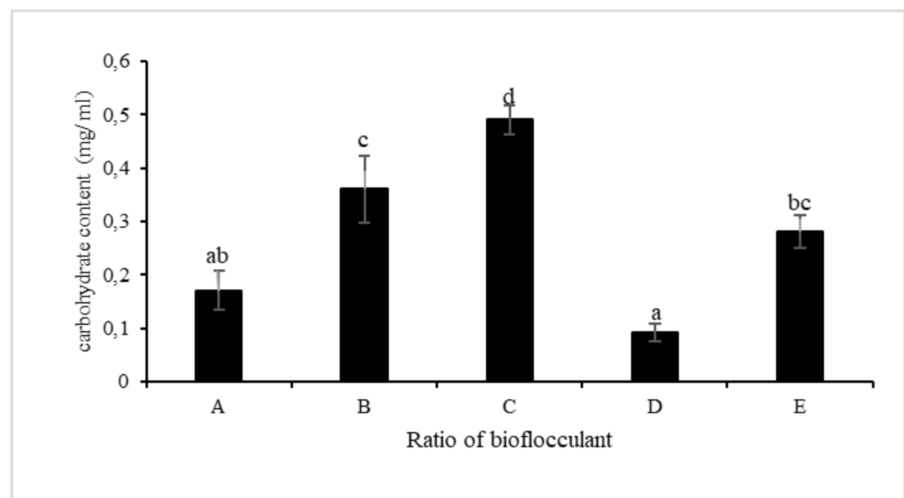


**Figure 5.** Carbohydrate content on harvest day using medium of BBM with the ratio between non-flocculant and flocculant microalgae (A) 1:1, (B) 1:0.5, (C) 1: 0.25, (D) *Anabaena* sp., and (E) Glagah consortium (Rahmawati 2020). Data were means ±SD (n=5). Different carbohydrate content on harvest day using a medium of BBM with the ratio between non-flocculant and flocculant microalgae indicated significant differences between treatments and were calculated by one-way ANOVA followed by Duncan Multiple Range Test (DMRT) ($p$ <0.05).

The phenol-sulfate method was used for carbohydrate estimation. The reagent of the phenol-sulfate method is Phenol 5% and concentrated sulfuric acid ($H_2SO_4$). Phenol is used to detect simple sugars, while $H_2SO_4$ produces orange color (Agustini & Febrian 2019). Carbohydrate content in Glagah control was 0.28 mg/ml higher than *Anabaena* sp. 0.09 mg/ml. The highest carbohydrate content was obtained at a ratio of 1: 0.25, which is equal to 0.49 mg/ml, while the lowest carbohydrate concentration was obtained at a ratio of 1: 1, which is equal to 0.17 mg/ml. Their reverse trend of lipid and carbohydrate content casued by the metabolic pathways of high energy. This is due to harvesting microalgae carried out in the stationary phase, where microalgae change their metabolism by breaking down carbohydrates into energy reserves such as lipids, because the precursor of TAG is glyceraldehyde-3-phosphate (G3P). G3P is the result of carbohydrate catabolism (Sayanova 2017). Microalgae that produce high amounts of carbohydrates tend to have small amounts of lipids. Therefore, microalgae is identified as a reliable source of protein. The Bradford method was used for protein estimation. The Bradford is staining based on the measurement of absorbance (Yasmine 2011).

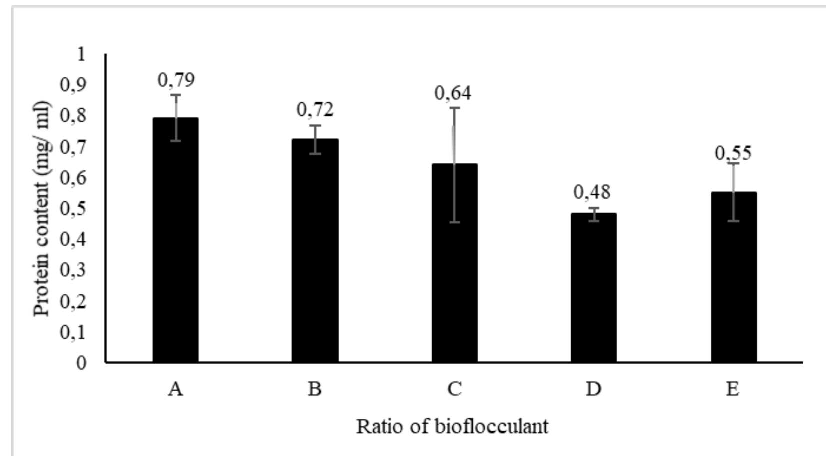


**Figure 6.** Protein content on harvest day using medium of BBM with the ratio between non-flocculant and flocculant microalgae (A) 1:1, (B) 1:0.5, (C) 1: 0.25, (D) *Anabaena* sp., and (E) Glagah consortium (Rahmawati 2020). Data were means ±SD (n=5). Different protein content on harvest day using a medium of BBM with the ratio between non-flocculant and flocculant microalgae indicated insignificant differences between treatments and were calculated by one-way ANOVA ($p$ >0.05)

Based on Figure 6, the protein content in the Glagah consortium control was 0,55 mg/ml, while in *Anabaena* sp. was 0.48 mg/ml. The highest protein content was found at a ratio 1: 1. The lowest protein content was found at a ratio 1: 0.25. Glagah consortium protein content was higher when compared to *Anabaena* sp. This is due to the optimum utilization of nutrients in the Glagah consortium due to the effect of niche division in culture.

*Anabaena* sp. can produce EPS consisting of soluble protein and polysaccharides (Tiwari et al. 2015). The increased protein content along with the increased concentration of bioflocculants added. Increased bioflocculant concentrations cause an increased in EPS. EPS accumulation increased protein content, this is because protein is a constituent component of EPS.

## CONCLUSION
Compared to *Anabaena* sp., the Glagah consortium took longer to precipitate and produce bioflocculation. The result indicated that the addition of bioflocculant *Anabaena* sp. increased the percentage of deposition of the Glagah consortium, with an optimum ratio of 0.25:1. The increased protein content, along with the increased concentration of bioflocculants added.

## AUTHOR CONTRIBUTION
A.R. contributed to doing research, data interpretation, and editing. I.R. contributed to the interpretation of the data, review, and editing. I.N. contributed to the review and editing. B.R.S. contributed to data modeling, and E.A.S. contributed to designing the research, reviewing, editing, and supervising the process.

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## CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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