

## Studies on bioflocculant exopolysaccharides (EPS) produced by *Anabaena* sp. and its application as bioflocculant for low cost harvesting of *Chlorella* sp.

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### Abstract

Microalgae harvesting is critical to remove water from algal growth media with solid-liquid separation. Bioflocculation has the same principle as flocculation. Using solid-liquid separation, microalgae harvesting removes moisture from the algal growth substrate. The same idea underlies flocculation and bioflocculation. Using fungal and bacterial bioflocculants requires a special medium that is different from the microalgae medium, that fungi and bacteria can contaminate microalgae, so it is not recommended to be used as a bioflocculant agent. Microalgae *Anabaena* sp. was chosen in this study as a bioflocculant agent since it can produce exopolysaccharides (EPSs). Dissolved proteins and carbohydrates make up EPSs. This investigation looked into employing *Anabaena* species to extract *Chlorella* species. The harvest day was used to measure the parameters. A spectrophotometer was used to measure the precipitation percentages. Bligh and Dyer's methods were used to measure lipid contents. The phenol-sulfate was used to perform carbohydrates. Bradford method was used to quantify proteins. The ratio of 1:1.25 was determined to have the best proportion of flocculation and carbohydrate content (*Chlorella* sp. : *Anabaena* sp.). The ratio of 1:1 was determined to have the maximum cell lipid and protein content (*Chlorella* sp.: *Anabaena* sp.). The application of this study will be beneficial to design effective methods for harvesting microalgae using biological materials such as other microalgae.

**Keywords:** Bioflocculation, *Anabaena* sp., *Chlorella* sp., Exopolysaccharides

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### Introduction

Global warming is driving the development of

renewable energy. Renewable energy resources are becoming essential in the primary energy supply. Nowadays, The International Energy Agency



reported that renewable resources supply 15% of global primary energy. The renewable resources are in the form of bioenergy (10%), hydropower (3%), and the rest in other renewables (2%) such as photovoltaic (PV) and wind energy (Gernaat et al., 2021). The rapid growth of the human population and technological advancements have led to mounting energy demands, estimated to increase by 50% by the year 2030 (Maness et al., 2009).

Nowadays, the biodiversities in Indonesia that have not been exploited yet, especially microorganisms that have an essential role for humans (Erfianti et al., 2021), including microalgae. Due to their enormous application potential in the renewable energy, biopharmaceutical, and nutraceutical industries, microalgae have recently attracted significant attention around the world (Khan et al., 2018). As functional food, microalgae also contain antioxidants including  $\beta$ -carotene, lycopene, vitamin C, and vitamin E (Zulkarnain et al., 2020). Additionally, microalgae can adapt to extreme environmental conditions, which is able to survive in extreme environments, and efficiently converts solar energy into chemical energy (Erfianti et al., 2023). The most important factor for converting microalgae into valuable products is the cultivation process and the harvesting and extraction technology. Currently, many technologies have been developed for harvesting microalgae. One of the renewable energies comes from microalgae through the extractive transesterification process (Pradana et al., 2018). One of the abundant species of microalgae is *Chlorella* sp. *Chlorella* sp. As a source of renewable energy that contains lots of lipids, it is a source of biodiesel. In this lipid content, there are hydrocarbons and fuel-forming basic compounds (Chisti, 2007). *Chlorella* sp. is a cosmopolitan algae. It is found in brackish water, seawater and freshwater (Kumar and Singh, 1979). *Chlorella* sp. can absorb carbon dioxide and convert it to oxygen (Lavens and Sorgeloos, 1996). Besides that, *Chlorella* is also a promising microalga for CO<sub>2</sub>-neutral biorefineries since it simultaneously produces a variety of biochemicals like antioxidants, sugars, biopolymers, biosorbents, food supplements, feeds and others.

Harvesting microalgae is essential to cultivating microalgae (Kawaroe et al., 2016). Microalgae harvesting removes water from algal growth media by increasing solid content from <1.0% to 20% solids (Santoso, 2017). The harvesting process can be done

with several techniques, such as centrifugation, filtration, sedimentation, flocculation, flotation, ultrasonic vibrations, and screening (Salim et al., 2011). Salim et al. (2011) state that bioflocculation techniques are energy-efficient and cheaper than other techniques. Bioflocculation using microalgae is the same principle as flocculation. The use of microalgae as flocculants will be more efficient than bacteria because microalgae do not require special media. If there are no specific media, it will avoid additional costs.

This research was to study *Anabaena* sp. as a bioflocculant agent because it can produce EPSs. Exopolysaccharides (EPSs) have a physicochemical protective role against several dangerous factors, representing the boundary between the cell and the immediate outer environment and keeping cells from antibacterial agents and protozoa predation. In addition, EPSs can maintain the growth of other species that are nearby (Zumriye, 2005; Tiwari et al., 2015; Rossi and Philippis, 2014). EPSs production will be optimum at the end of the growing phase (Bhaskar and Bhosle, 2005). Therefore, *Anabaena* sp. is promising for harvesting. Microalgae harvesting can be determined based on microalgae turbidity by calculating the density of microalgae on the top surface of the culture. If the density at the surface is small, harvesting will be optimal.

## Material and Methods

### Bioflocculant Determination

Microalgae stock from Indonesian Culture Collection (InaCC) Laboratory was added to a 500 ml culture bottle containing BBM (Bold's Basal Medium). The samples were cultivated for 14 days to determine the growth curve. *Anabaena* sp. and *Chlorella* sp. were cultivated with the same cell density. Bioflocculant *Anabaena* sp. was added to *Chlorella* sp. culture in the end log phase (highest cell number). *Chlorella* sp. was harvested on the 8<sup>th</sup> day, while *Anabaena* sp. was on the 4<sup>th</sup> day (based on a preliminary test). Parameter measurements were performed at the end log phase. There were three treatments (1:1; 1:0.5; 1:0.25) and three repetitions. Each sample was put into a 15 ml conical bottle and allowed to stand overnight (24 hours). The supernatant was taken as much as 1 ml and put into a cuvette. The cuvette was placed into the spectrophotometer and measured with a wavelength of 750 nm.



After that, it was calculated using the following formula:

$$\% \text{ Precipitation} = \frac{\text{OD}_{750}(t_0) - \text{OD}_{750}(t_n)}{\text{OD}_{750}(t_0)} \times 100\% \quad (1)$$

### Calculation of total carbohydrates

The method used to measure carbohydrate content was the phenol-sulfate method (Dubois et al., 1956). Samples were centrifuged at a speed of 3000 rpm for 10 minutes. The cells were separated from the supernatant. Pellets were added with 0.5 ml of 5% phenol. They were homogenized and incubated for 10 minutes. After that, the pellets were added to 1 ml of H<sub>2</sub>SO<sub>4</sub> and homogenized. The samples were incubated for 20 minutes. The samples were transferred into a cuvette, and a spectrophotometer measured their concentration at 490 nm. After that, it was calculated using the formula on the standard carbohydrate curve.

### Lipid content

The method used to measure lipid content was the Bligh and Dyer method (1959) using some modifications. Each sample was taken as much as 15 ml and put in a conical bottle. Samples were centrifuged at 4000 rpm at 4° C for 15 minutes. After that, the supernatants were removed so that the pellets remained. The pellets added as much as 2 ml of methanol reagents and 1 ml of chloroform. Then they were vortexed for 1 minute. Then 1 ml of chloroform and 1 ml of aquadest were added and vortexed for 1 minute. After that, they were centrifuged for 15 minutes to form three layers. The clear solution (top layer) was removed then the yellow liquid (bottom layer) was taken and placed on a petri dish. The Petri dishes were put in the oven for one day. After that, the lipid content is estimated using the following formula:

$$\text{Lipid content} \left( \frac{\text{mg}}{\text{ml}} \right) = \frac{\text{weight of extracted total lipid}}{\text{weight of alga cells-culture volume}} \quad (2)$$

### Protein calculation techniques

Measurement of protein content was carried out using Bradford. A total of 15 ml of samples were put into a conical bottle. It was centrifuged at 1800 rpm at 28°C for 10 minutes. The supernatants were removed so that the pellets were obtained. Pellets were taken and inserted into the microtube. Then, they were centrifuged at 3000 rpm at 25°C for 10 minutes. The supernatants were removed so that the pellets were

completely obtained. A 45µl SDS of 10% was added to the microtube, which already contained pellets. Microtube samples were placed in a water bath at 95°C for 5 minutes. After that, they were cooled in the refrigerator for 5 minutes. 8µl of pellets and 200µl of Bradford solution were added to the microplates. Furthermore, measurements were taken with an ELISA reader at a wavelength of 595 nm. Calculating the total protein content of microalgae was done using the formula on a standard protein curve.

### Modelling growth kinetic of *Anabaena* sp. and *Chlorella* sp.

For predicting the performance and optimisation of photobioreactor operating conditions, kinetic modelling can be used. Suitable kinetic modelling is helpful for learning the dynamics of biomass growth of microalgae (Galvao et al., 2013). For rapid population growth of the organism, the two non-linear models, the Logistic and Gompertz models, are commonly used (Lam et al., 2017). The Logistic and Gompertz model was the simple model in microbial growth because it is not limited by substrate type and consumption. Therefore, Logistic and Gompertz were chosen for *Anabaena* sp. and *Chlorella* sp. growth kinetic modelling. 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<sub>0</sub> is the initial cell density, X<sub>max</sub> is the maximum cell density, and µ<sub>max</sub> is the maximum specific growth rate (Phukoetphim et al., 2017; Hanief et al., 2020).

#### Logistic Model

$$\frac{dX}{dt} = \mu_{max} \left( 1 - \frac{X}{X_{max}} \right) X \quad (3)$$

$$X = \frac{X_0 \exp(\mu_{max} t)}{1 - \left[ \frac{X}{X_{max}} (1 - \exp(\mu_{max} t)) \right]} \quad (4)$$

The Gompertz model is also used to determine the cell population of the exponential phase. However, the parameters used in this model are more complex, including maximum cell production (r<sub>m</sub>) and lag time (t<sub>L</sub>).

#### Gompertz Model

$$X = X_0 + \left[ X_{max} \cdot \exp \left[ -\exp \left( \left( \frac{r_m \cdot \exp(1)}{X_{max}} \right) (t_L - t) + 1 \right) \right] \right] \quad (5)$$



The determination of the model was carried out using the following formula where SSR is sum square residual and SST is sum square total (Phukoetphim et al., 2017; Hanief et al., 2020).

$$R^2 = \left(1 - \frac{SSR}{SST}\right) \quad (6)$$

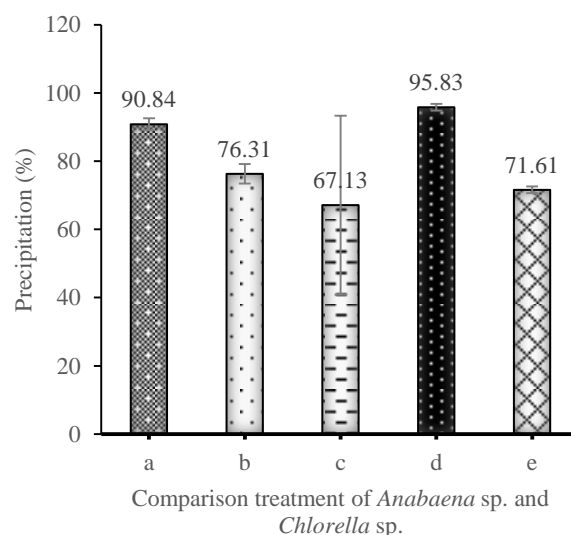
## Results

Microalgae bioflocculation was performed to determine the optimal ratio between flocculant and non-flocculant microalgae. The results showed that the most optimal percentage of flocculation was found at the ratio of *Chlorella* sp. and *Anabaena* sp. at 1: 0.25, which was 90.84%. While the lowest percentage of precipitation was *Chlorella* sp. and *Anabaena* sp. at the ratio of 1:1. It accounted for 67.13%. The results showed that the addition of several flocculants could decrease the precipitation percentage. It could be shown *Anabaena* sp. (control) (d) had the highest precipitation percentage compared to *Chlorella* sp. control (e), and treatments were equal to 95%. *Chlorella* sp. has a diameter size range of 2-10 μm (Barsanti and Gualtieri, 2006) and *Anabaena* sp. has a larger size range than *Chlorella* sp.

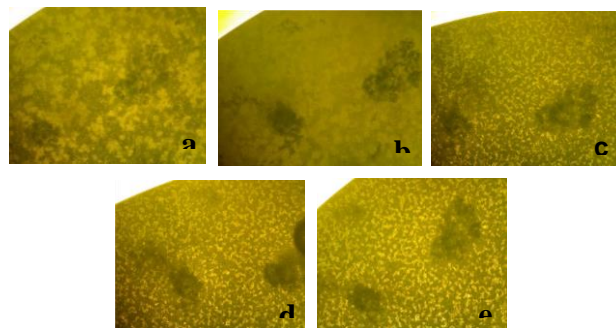
Bioflocculant observations for *Anabaena* sp. and *Chlorella* sp. under a microscope with a magnification of 10×4 could be seen in Fig 2. At the beginning of the mix of flocculant and non-flocculant microalgae, the flocculant and non-flocculant microalgae cells were still separated and not yet completely bound (0-hour). Gradually the binding process began to take place because the cells began to experience stress due to reduced nutrition and triggered microalgae to secrete extracellular polymers. The result of extracellular polymer would create bonds between microalgae cells. Bonding between flocculant and non-flocculant microalgae cells at the 4<sup>th</sup> hour was seen with the formation of bonds between species of microalgae. Bonding started to appear more at the 8<sup>th</sup> hour and so on, microscopically visible non-flocculant species were bound and formed a collection of biomass with flocculant species, causing cells to settle.

The carbohydrate content of microalgae varies depending on the species and its living conditions (Basmal, 2008). The histogram showed that the lower the microalgae flocculants are given, the higher the carbohydrate content produced. According to Rinanti

and Purwadi (2018), strong acids have broken down the cell walls of microalgae that contain cellulose, so it increases the percentage of carbohydrates. The amount of microalgae cells that were too abundant required more time to carry out the hydrolysis process. The acid added to the hydrolysis process acted as a catalyst to accelerate the polysaccharide chain termination reaction to glucose. The basic principle of hydrolysis is to cut the α-1,4-glucoside bond and the α-1,6-glucoside bond from ecto pectin to produce smaller starch (glucose) (Shanavas et al., 2011).

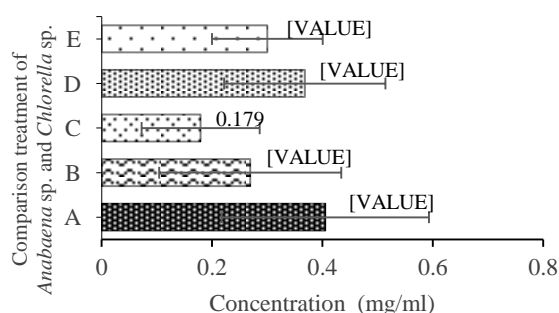


**Figure-1. Percentage of microalgae treatment and control flocculation on harvest day (a) treatment of 1:0.25 (b) treatment of 1:0.5 (c) treatment of 1:1 (d) *Anabaena* sp. control (e) *Chlorella* sp. control (Irawan, 2020).**

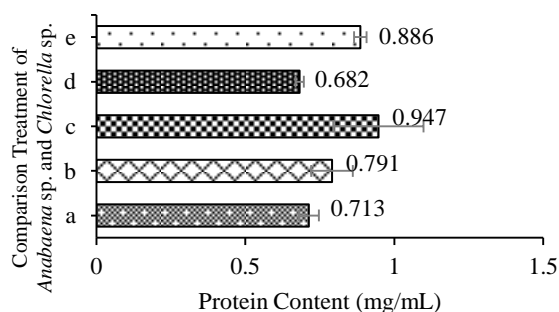


**Figure-2. Microalgae before flocculating and after flocculating (a) 0 hour (b) 4<sup>th</sup> hour (c) 8<sup>th</sup> hour (d) 12<sup>th</sup> hour (e) 16<sup>th</sup> hour (Irawan, 2020).**

The results of each treatment with three ratios showed significant differences. The more bioflocculant agents (*Anabaena* sp.) added to *Chlorella* sp., the higher the concentration of protein produced. The highest protein content was performed by a ratio of 1:1 was 0,947 mg/ml. While the lowest protein content in the treatment was performed by treatment 1:0,25 which was 0,713 mg/ml. Extracellular products from *Anabaena* sp. produced amino acids (serine and threonine) in complex pigmented and neon compounds (Ambarwati et al., 2014). So along with the addition of bioflocculant *Anabaena* sp. on *Chlorella* sp. could increase the protein content produced.



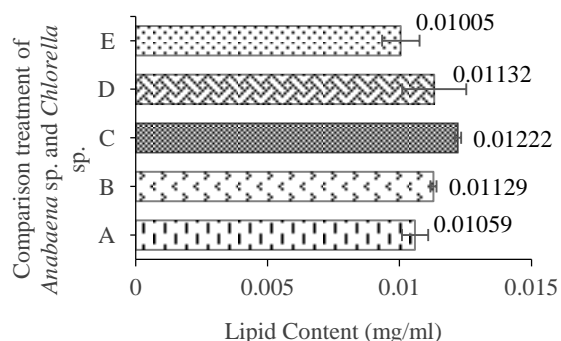
**Figure-3. Total carbohydrates in the treatments and control of microalgae on harvest day (a) treatment of 1:0.25 (b) treatment of 1:0.5 (c) treatment of 1:1 (d) *Anabaena* sp. control (e) *Chlorella* sp. control (Irawan, 2020)**



**Figure-4. Protein content in the treatments and control of microalgae on harvest day day (a) treatment of 1:0.25 (b) treatment of 1:0.5 (c) treatment of 1:1 (d) *Anabaena* sp. control (e) *Chlorella* sp. control (Irawan, 2020)**

The lowest cell lipid content was indicated at a ratio of 1:0.25. While the highest cell lipid content was obtained at a ratio of 1:1. *Anabaena* control sp. and *Chlorella* sp. control showed lower lipid levels compared to 1:1 treatment. Lipids are a series of

secondary metabolite processes in microalgae. Microalgae can produce various lipid levels (Manullang et al., 2012). Mixed cultures are potentially used to enhance lipids' content and productivity (Suyono et al., 2016). According to Suyono et al. (2016), commercial single-cell cultivations of microalgae cannot overcome the problem of low biomass because it also still produces low biomass and lipid content due to various problems such as culture conditions and susceptibility to contaminants. The mixed culture shows that microalgae cells can grow faster and have a high biomass yield because the cells are capable of utilising organic carbon sources optimally. Nur et al. (2023) also reported that the lipid and other metabolites production of microalgae increased significantly through mixing consortia of microalgae and other microorganisms.



**Figure-5. Lipid content in the treatments and control of microalgae on harvest day (a) treatment of 1:0.25 (b) treatment of 1:0.5 (c) treatment of 1:1 (d) *Anabaena* sp. control (e) *Chlorella* sp. control (Irawan, 2020).**

In this study, the highest cell lipid content was obtained at a ratio of 1:1 with the cell lipid content of 0.01222 mg/ml.

**Table-1. Growth rate parameter of Logistic Model**

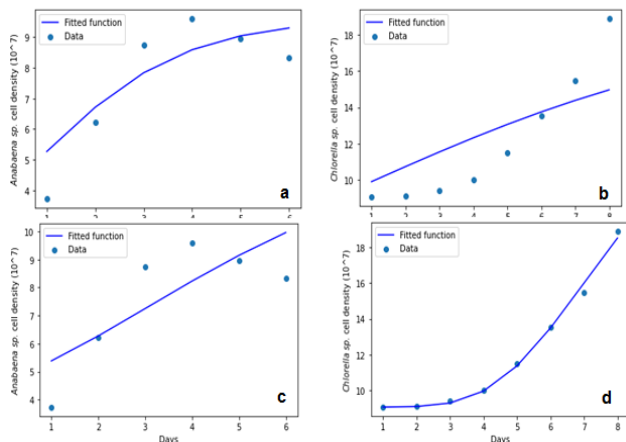
Parameter	<i>Anabaena</i> sp.	<i>Chlorella</i> sp.
$\mu_{max}$	0.6492	0.1776
$R^2$	0.78	0.64

Based on logistic modeling, the maximum specific growth rate ( $\mu_{max}$ ) of *Anabaena* sp. and *Chlorella* sp. were 0.6492/day and 0.1776/day respectively. The  $R^2$  error were 0.78 and 0.64 for *Anabaena* sp. and *Chlorella* sp. (Table 1, Fig. 6).



**Table-2. Growth rate parameter of Gompertz Model**

Parameter	Monoculture Glagah	Mixed Culture Glagah and <i>Euglena</i> sp.
rm	$0.99 \times 10^7$	$2.58 \times 10^7$
tl	-0.6	4.3
R <sup>2</sup>	0.61	1.00



**Figure-6. (a) Logistic model for *Anabaena* sp. cell density, (b) Logistic model for *Chlorella* sp. cell density (c) Gompertz model for *Anabaena* sp. cell density, (d) Gompertz model for *Chlorella* sp. cell density (Irawan, 2020).**

For the Gompertz modeling, the maximum cell production rate (rm) of *Anabaena* sp. was  $0.99 \times 10^7$  cells/mL. The maximum cell production rate (rm) of *Chlorella* sp. was  $2.58 \times 10^6$  cells/mL. The lag time (tL) for *Anabaena* sp. and *Chlorella* sp. was -0.6/day and 4.3/day respectively. Each of the R square error values of *Anabaena* sp. and *Chlorella* sp. were 0.61 and 1.00.

## Discussion

*Anabaena* sp. is blue-green microalgae that can produce exopolysaccharides (EPS) in response to adverse conditions. EPS are present mainly around *Anabaena* cells or filaments as an enveloped layer and released outside the cell (Kumar et al., 2018). Due to its ability to produce EPS, *Anabaena* sp. was used in this study as a biofloculant candidate to harvest *Chlorella* sp. Our study indicated that the percentage of precipitation decreased with the addition of flocculants. This statement can be caused by *Anabaena* sp. producing EPS, but the highest EPS content was protein. One example of protein was anatoxin. Anatoxin was an amine alkaloid compound

that could kill cells because it damages cellular function of the cell (Devlin et al., 1976). Therefore, with an increase in the ratio, the resulting anatoxin was bigger. It caused damage and even death in *Chlorella* sp. cells. The smaller the number of cells that bind with polysaccharides in *Anabaena* sp., the percentage of precipitation produced will be lower.

Sathe (2010) states that bio-flocculation is spontaneous flocculation of microalgae cells that occurs due to the secretion of extracellular polymeric substances (EPS) when microalgae are under stress conditions. Lack of nutrients is a major factor causing microalgae cells to secrete extracellular polymeric substances (Lee et al., 2009). Therefore, extracellular polymers produced by microalgae trigger the formation of clumps of cells (Sathe, 2010). According to Pillai (1997), particle deposition speed is influenced by the size of the particle. The larger the size of a particle, the easier it will settle. This research showed that all treatments were not able to flocculate 100% of microalgae. This was due to the experimental time that was still too short in period.

Control of *Anabaena* sp. had lower protein concentration than the control of *Chlorella* sp. Christwardana et al. (2013) stated that the content of microalgae protein is influenced by environmental conditions such as light intensity, nutrient limits (especially nitrogen), salinity, temperature, pH, and culture age. Complete nutrient composition and proper nutrient concentration determine the production of biomass and the nutrient content of microalgae. Stockenreiter et al. (2013) explained the effect of biodiversity on lipid content in microalgae. Culture with the addition of other microalgae (functional groups) could produce more lipids. There were two mechanisms proposed to explain the increase in lipid content: (1) diversity increased the efficiency of the use of photosynthetically active radiation (PAR) thereby increasing the primary productivity of culture, primary productivity increased, and lipid production from biomass also increased; (2) mixed culture has increased the efficiency of nutrition use due to the complementary effects of niche division so that it creates a nutritional stress condition. The limited amount of nutrients would stimulate microalgae to accumulate lipids (Hu et al., 2008).

Data modeling to predict growth rate parameters results that the logistic model is not suitable for predicting *Anabaena* sp. and *Chlorella* sp. growth rates due to the low coefficient of determination R<sup>2</sup>.



The Gompertz model fits the *Chlorella* sp. growth curves indicated by the high value of the coefficient of determination  $R^2$ . On the contrary, the Gompertz model is not suitable for predicting *Anabaena* sp because of the low value of the coefficient of determination  $R^2$ .

## Conclusions

In conclusion, the addition of bioflocculant *Anabaena* sp. was proven to increase the carbohydrate content at an optimum ratio of 1:0.25, while the highest lipid and protein content was shown by 1:1 ratio. *Anabaena* bioflocculant was also proven to increase the percentage of precipitation with an optimum ratio of 1:0.25 which was 90.84%. The results of this study have proven that *Anabaena* sp. is an energy-efficient bioflocculant that has a promising future as a feasible bioflocculant for *Chlorella* sp. compared to previously known techniques. As a recommendation, other microalgae or diatoms can be used in future observations to increase the effectiveness of harvesting *Chlorella* sp.

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**Conflict of Interest:** None

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### Contribution of Authors

Irawan AP: Participated in research framing, conducted the research stages, analysis and interpretation of data, drafting the manuscript

Rahmawati A & Fahmi UA: Participated in preparing tools and materials, analysis and interpretation of data

Budiman A, Putri RAE, Nurafifah I & Sadewo BR: Acquisition of data, analysis and interpretation data

Maghfiroh KQ, Erfianti T, Andeska DP: Acquisition of data, analysis and interpretation data, drafting the manuscript

Suyono EA: Conceptual design, analysis and interpretation data, drafting the manuscript, final approval.

