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Optimisation of Bioflocculation Using *Anabaena* sp. and *Navicula* sp. for Harvesting of Glagah Microalgae Consortium

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ABSTRACT

One of the problems in microalgae is harvesting. Currently, many chemical methods are used that impact the environment. Not all of them can be used as a filter, so bioflocculation is used because there is no need to change the medium. This method is an environmentally friendly and efficient alternative to chemical flocculants that usually cause contamination of biomass and health. Previous studies have shown that different ratios of auto-flocculated microalgae in cocultures affect the flocculation rate. This research was carried out by the Glagah Consortium bioflocculation using *Anabaena* sp. and *Navicula* sp., which had never been done before. The study aims to study the effect of the mixing ratio on the flocculation rate, carbohydrates, and lipid content of the Glagah Consortium. The consortium uses *Anabaena* sp. and *Navicula* sp. as bioflocculants. Glagah and *Anabaena* sp. consortium was cultured in Bold Basal Medium, while *Navicula* sp. was cultured in F/2 medium. Cell density was measured every 24 hr for 8 days with a hemocytometer. The cultures were harvested in the stationary phase, then mixed between non-flocculated microalgae (Glagah Consortium) and flocculated microalgae (*Anabaena* sp., *Navicula* sp.) in a ratio of 1:1, 1:0.5, and 1:0.25 for 24 hr. Bioflocculation was measured by spectrophotometer at 750 nm 0 and 24 hr after mixing. Carbohydrate levels were measured using the phenol sulfuric acid method, while lipid measurements were performed using the Bligh and Dyer method. The addition of *Anabaena* sp. and *Navicula* sp. as bioflocculant in Glagah Consortium culture results in an increase in flocculation rate with an effective ratio of 1:0.25 for *Anabaena* sp. (81%) and 1:1 for *Navicula* sp. (95%). Mixing of *Anabaena* sp. and Glagah Consortium results in carbon source competition, reducing carbohydrate content at higher mixing ratios (0.172, 0.364, and

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0.500 mg/ml on 1, 1:0.5, and 1:0.25) while increasing lipid content as a result of lipid production in stationary phase (highest on ratio 1:1 = 0.011 mg/ml). *Navicula* sp. and Glagah Consortium mixture caused no significant changes to carbohydrate content but showed an increased amount of lipid at all ratios as a result of osmotic stress on Glagah Consortium from saline F/2 medium (highest on ratio 1:1 = 0.162 mg/ml).

Keywords: *Anabaena* sp., bioflocculation, co-culture, Glagah Consortium, *Navicula* sp.

INTRODUCTION

Biomass harvesting is an extensive process in microalgae biomass production, taking up 20-30% of the total production costs. Methods commonly used to harvest microalgae are centrifugation, filtration, and flocculation. Centrifugation is inefficient due to high upkeep costs, while filtration is ineffective in harvesting strains with smaller cell sizes (Matter et al., 2019; Salim et al., 2011). Flocculation is the most efficient method to harvest microalgae because it is simple, fast, and does not incur significant damage or changes to the harvested biomass (G. Singh & Patidar, 2018). The conventional flocculation method uses chemicals such as aluminium sulphate and iron chloride, which are pH-dependent and cause contamination and health risks. For example, in the flocculation process, using either alum or chitosan (Gupta et al., 2018). An alternative, more efficient method is required for microalgal biomass harvesting, and one such method is bioflocculation (Matter et al., 2019; Rahman, 2020). Bio-flocculation is the formation of flocs by adhesion and interparticle contact by microorganisms, usually done by co-culturing non-flocculating cultures with self-flocculating microorganisms or adding bio-flocculant agents into the culture (A. Singh et al., 2011; Lutfi et al., 2019; Matter et al., 2019; Y. Li et al., 2018). Bio-flocculation is an environmentally friendly and efficient method since it is biodegradable and allows the reuse of cultivation medium after dewatering (Matter et al., 2019; Salim et al., 2011). This process is attributed to the exopolymer substances (EPS) produced by auto-flocculating microalgae. In nature, EPS is used by microalgae to attach to substrates, gather nutrients, reduce diffusion, and protect cells from desiccation. EPS contribute to the formation of flocs in biofilm via the bridging effect, where EPS forms a matrix that holds non-flocculating microalgae in place (Klock et al., 2007; Salim et al., 2011).

Glagah Consortium is a local microalgae strain isolated from Glagah Beach, Yogyakarta, Indonesia, which contains 6 different species of microalgae and a symbiotic relationship with bacteria (Suyono et al., 2015, 2018). This strain has the potential to be used as a source of biofuel due to its high lipid content, reaching up to 13.58% and a lower ratio of polyunsaturated fatty acids (Sadaatkhah et al., 2020; Suyono et al., 2016). Using microalgae, bacteria, and fungi as bioflocculants resulted in higher coculture flocculation rates. However, it should be noted that bacteria and fungi may cause microbial contamination in microalgal biomass. Microalgae as

bioflocculant has a lower contamination risk, easier biomass purification, and does not require additional nutrients in the media for bioflocculant growth (Barros et al., 2015; Matter et al., 2019).

The genus *Anabaena* and *Navicula* are microalgae capable of forming aggregations in the form of cyanobacterial mats and biofilms as a defensive response to environmental change (Klock et al., 2007). EPS plays an important part in forming this aggregate by providing adhesion. Both genera can be used as bioflocculants as they can produce large amounts of EPS (Congestri & Albertano, 2011; Gómez-Ramírez et al. 2019). EPS release depends on microalgal cell growth under optimal temperature, pH, luminosity, nutrition, and salinity (Moreira et al., 2022). Previous research on Glagah Consortium bioflocculation using *Anabaena* sp. and *Navicula* sp. has yet to be done. The use of *Navicula* sp. as a microalgae culture bioflocculant still rarely does. This research is important to find a potential bioflocculant as a biofuel material, which can be seen from its lipid content. This research aims to discover the efficiency of Glagah Consortium flocculation using *Anabaena* sp. and *Navicula* sp. The research measures the effect of the mixing ratio on the flocculation rate, carbohydrates, and lipid content.

MATERIALS AND METHODS

Algal Cultures

Glagah isolates were obtained from the Laboratory of Biotechnology, Faculty of Biology, Universitas Gadjah Mada, while *Anabaena* sp. isolates were obtained from Indonesia Culture Collection (InaCC) Lembaga Ilmu Pengetahuan Indonesia (LIPI). *Navicula* sp. isolates were obtained from Pertamina Ltd. Cultivation of Glagah Consortium and *Anabaena* sp. were done in Bold's Basal Medium (BBM, PhytoTech Labs, USA) (Bold, 1949) while *Navicula* sp. cultivation was done in F/2 medium (PT Pertamina, Indonesia) with modification of silicate removal (Guillard, 1975). Cultures were grown for 8 days in 500 ml glass bottles under aeration, with an inoculum: medium ratio of 1:4. The biomass of *Anabaena variabilis* significantly increased when grown on the optimized medium (Refaay et al., 2022).

Cultivation Consortium of Glagah and *Navicula* sp. was done in a bottle culture volume of 500 ml with an inoculum: medium ratio of 1: 4 and a final volume of 500 ml at 28±2°C. The homogenization of nutrients was carried out by aeration and light shaking of *Navicula* sp. as much as 2 times a day. Ratio 1:4 was carried out between the Glagah Consortium and *Anabana* sp. and *Navicula* sp. because both have been proven to have the potential to be used as bioflocculants as they have the capability to produce large amounts of EPS.

Determination of Cell Density

Cell density in cultures was measured by cell count method using a light microscope and Haemocytometer Neubauer 1 mm (Electron Microscopy Sciences, USA) every 24 hr from the start of cultivation.

Samples were homogenized by shaking; 0.8 ml of samples were taken and mixed with 0.2 ml of 70% alcohol (Sigma-Aldrich, USA) for fixation (Sudibyo et al., 2017). The following formula calculated cell density:

$$\text{Cell density (cell/ml)} = n \times 10{,}000 \quad (1)$$

where, n = Average cell counted (Chalid et al., 2010).

Mixing of Cultures on Different Ratios

Samples of Glagah Consortium, *Anabaena* sp., and *Navicula* sp. were taken on day 3, 4, and 5 of cultivation, respectively. The sample was then put into a 15 ml tube with a mixing ratio between non-flocculating microalgae (Glagah Consortium) and flocculated microalgae (*Anabaena* sp., *Navicula* sp.) of 1:1, 1:0.5, and 1:0.25 (Salim et al., 2012). Samples were left undisturbed for 24 hr to determine bioflocculation rate, carbohydrate, and lipids content.

Determination of Bioflocculation Rate

The bioflocculation rate of the mixed cultures was measured using a UV-Vis spectrophotometer (MRC Laboratory-Instruments, United Kingdom) at 750 nm wavelength at T0 and T24 of mixing. The resulting absorbance was used to determine the sedimentation rate of the samples.

$$\text{Sd}(\%) = \frac{\text{OD}_{750}(\text{T0}) - \text{OD}_{750}(\text{Tn})}{\text{OD}_{750}(\text{T0})} \times 100\% \quad (2)$$

where, Sd (%) = Sedimentation rate; OD_{750} T0 = Optical density at 750 nm in 0 hr; Tn = Optical density at 750 nm in 24 hr (Salim et al., 2011).

Determination of Carbohydrate Content

Carbohydrate content was measured using the phenol sulphuric acid method (DuBois et al., 1956). Fifteen (15) ml of samples were centrifuged at 2,058 × *g* for 10 min, supernatant was removed, and 0.5 ml 5% phenol (Spectrum Chemicals, USA) was added into the pellet. Samples were then homogenized using a vortex and left for 10 min. One ml of concentrated sulfuric acid (Merck, USA) was added to the sample, followed by homogenization by the vortex. The sample was left for 20 min. Absorbance was measured using a UV-Vis spectrophotometer (MRC Laboratory-Instruments, United Kingdom) at 490 nm. Carbohydrate concentration was calculated from absorbance reading using the glucose standard curve as follows:

$$y = 0.0884x + 0.0095$$

Determination of Lipid Content

Lipid content was measured using Bligh and Dyer (1959) method. Fifteen (15) ml of samples were centrifuged at 2,683 × *g* for 15 min at 4°C. The supernatant was removed, and 2 ml methanol (Sigma-Aldrich, USA) and 1 ml chloroform (Sigma-Aldrich, USA) were added to the pellet. Samples were homogenized with vortex for 1 min, 1 ml equates (Sigma-Aldrich,

USA), and 1 ml chloroform was added, followed by homogenization for 1 min and centrifugation at 544 × *g* 4°C for 15 min. The sample will form 3 layers with lipids at the bottom. The layer containing lipids was moved into a Petri dish, then incubated at 33°C for 12 hr. Lipid content was calculated using the following:

$$\text{Lipid content } \left(\frac{\text{mg}}{\text{ml}}\right) = \frac{\text{Wn} - \text{W0}}{\text{W0} \times \text{V}} \quad (3)$$

where, Wn = Weight of filled Petri dish (g); W0 = Weight of empty Petri dish (g); V = Sample volume (ml) (Novaryatiin et al., 2011)

RESULTS

Cell Density

Figure 1 shows the cell density of Glagah Consortium, *Anabaena* sp., and *Navicula* sp. culture over the period of 8 days. The highest cell density of Glagah Consortium was found on day 3 (2.07 × 10^6 cell/ml), *Anabaena* sp. on day 4 (2.58 × 10^6 cell/ml), and *Navicula* sp. on day 6 (3.02 × 10^6 cell/ml). *Navicula* sp. culture showed a higher cell count from inoculation until the end of the culture period compared to Glagah Consortium and *Anabaena* sp. culture.

Bioflocculation Rate

The flocculation rate of the cultures under different mixing ratios is shown in Figure 2. The highest flocculation rate was achieved on the mixing ratio of 1:0.25 for Glagah and *Anabaena* sp. mix (Figure 2a) with a flocculation rate of 81%, mixing ratio of 1:1 is observed on Glagah and *Navicula* sp. mix with a flocculation rate of 95% (Figure 2b). The lower ratio of *Anabaena* sp. in the mix showed an increase in bioflocculation rate, while the larger mixing ratio of *Navicula* sp. showed an increase in bioflocculation rate.

Carbohydrate Content

Figure 3 shows the relation of the mixing ratio of microalgae to the carbohydrate content.

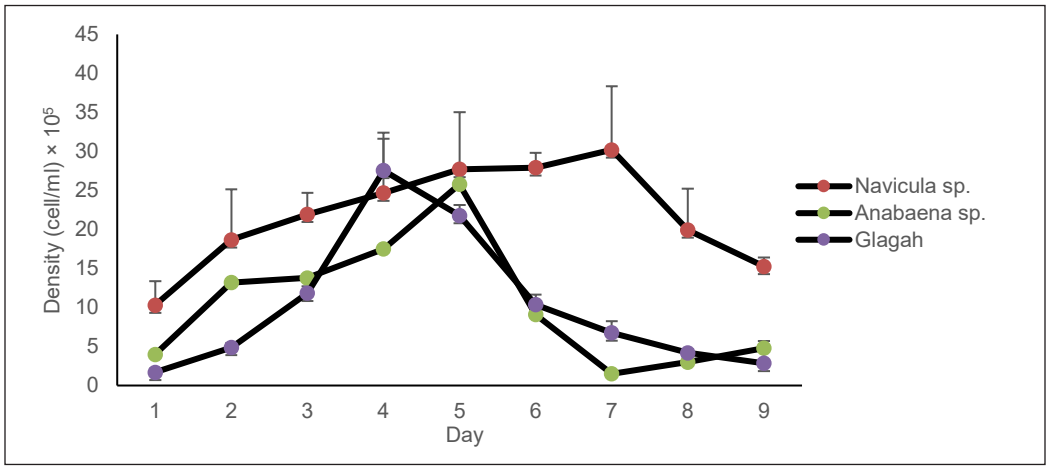
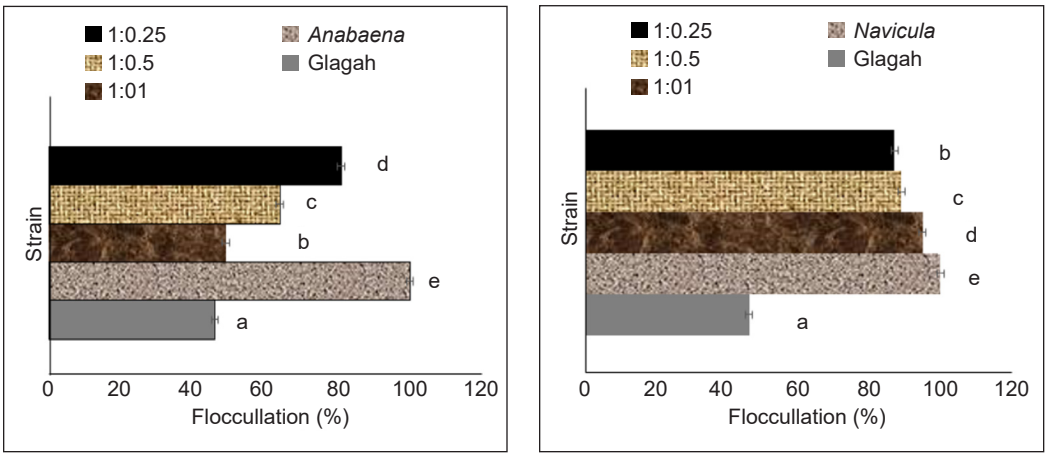
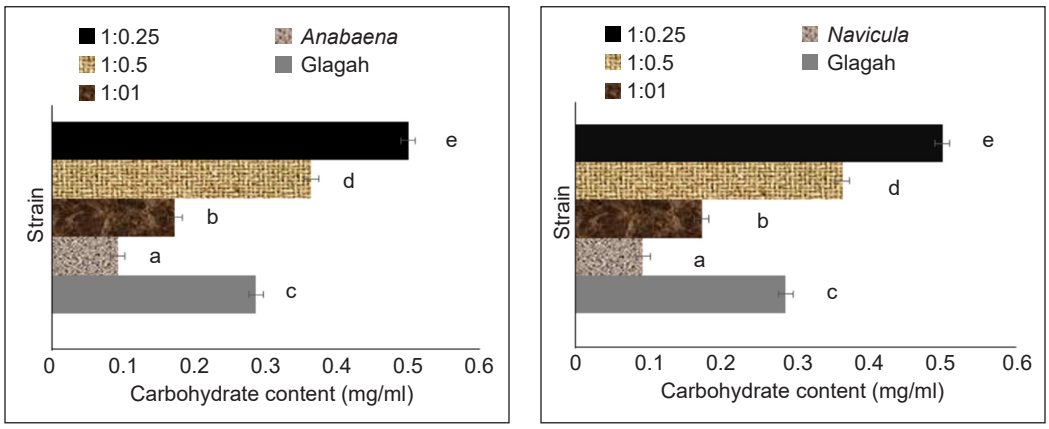


Figure 1. The cell density of Glagah Consortium, *Anabaena* sp., and *Navicula* sp. culture (Lawijaya, 2022)



(a) (b)

Figure 2. Flocculation rates of: (a) Glagah strain and *Anabaena* sp.; (b) Glagah strain and *Navicula* sp. under different mixing ratios (Lawijaya, 2022)
Note. Different superscript letters above bars indicate significant differences (p<0.05)



(a) (b)

Figure 3. The carbohydrate content of: (a) Glagah strain and *Anabaena* sp.; (b) Glagah strain and *Navicula* sp. under different mixing ratios (Lawijaya, 2022)
Note. Different superscript letters above bars indicate significant differences (p<0.05)

Glagah Consortium and *Anabaena* sp. mix (Figure 3a) showed higher carbohydrate content on lower mixing ratios, with the highest carbohydrate content at 1:0.25 ratio (0.500 mg/ml), *Anabaena* sp. control showed lower carbohydrate content than mixed samples at 0.092 mg/ml. A different trend was observed in Glagah Consortium and *Navicula* sp. mix (Figure 3b), which showed increased carbohydrate content at a higher mixing ratio, with the highest carbohydrate content found on a 1:1 mixing ratio (0.290 mg/ml). All mixed samples showed a lower amount of carbohydrates compared to the *Navicula* sp. control sample.

Lipid Content

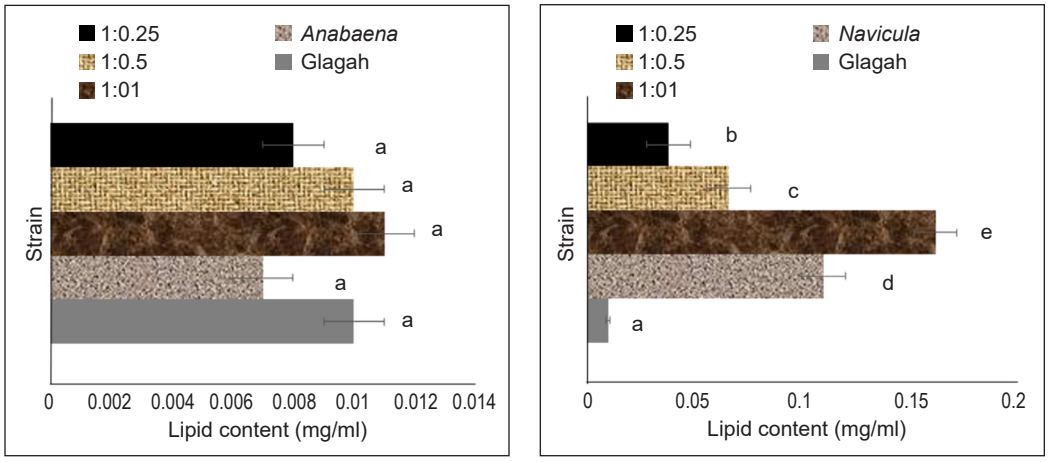
The lipid content of cultures is shown in Figure 4. Glagah Consortium and *Anabaena* sp. mix (Figure 4A) showed a decrease in lipid content on a lower mixing ratio. The same pattern is observed on Glagah Consortium and *Navicula* sp. mix. The mixing ratio 1:1 showed the highest lipid content in Glagah and *Anabaena* sp. mix (0.011 mg/ml) and Glagah and *Navicula* sp. mix (0.162 mg/ml). *Navicula* sp. produced significantly higher lipid content than Glagah Consortium and *Anabaena* sp.

DISCUSSION

Cell Density

An increment of cell density in the culture indicates the usage of nutrients by microalgae for cell metabolism. In fast-growing microalgae, photosynthetic products are used for asexual reproduction and are kept as carbohydrates in microalgae with slow metabolisms (Jiménez et al., 2003). Cell density is used to indicate the growth phases of the culture. The lag phase of the Glagah Consortium occurs from day 0 to day 1 with a peak population of 4.3 × 10^5 cell/ml. This phase is characterized by a small increase in cell density due to cells adjusting to new living conditions (Fachrullah, 2011). The exponential growth phase occurs from days 1 to 3, with a peak cell density of 2.07 × 10^6 cell/ml on day 3. The stationary phase occurs from days 3 to 4, which shows no significant change in cell density, while the death phase occurs from day 4 to day 8, where a significant drop in cell density from 1.84 × 10^6 to 7.7 × 10^5 cell/ml. The mutualistic interaction between Glagah causes a large increase in cell density during the exponential growth phase.

Consortium and bacteria. Glagah Consortium supplies oxygen and organic substances via photosynthesis, while the



(a) (b)

Figure 4. Lipid content of: (a) Glagah strain and *Anabaena* sp.; (b) Glagah strain and *Navicula* sp. under different mixing ratios (Lawijaya, 2022)
Note. Different superscript letters above bars indicate significant differences (p<0.05)

bacteria provide vitamin B and indole-3-acetic-acid (IAA), which in turn increases the growth speed of Glagah Consortium (Fuentes et al., 2016; Rahmawati et al., 2020; Suyono et al., 2018).

Anabaena sp. culture showed a lag phase on day 0 to 1 of cultivation, followed by exponential growth until day 4, with a peak cell density of 2.58×10^6 cell/ml. The stationary phase occurs on days 4–5, and the death phase on days 5–8, indicated by the decline of cell density. *Navicula* sp. culture experienced a lag phase from day 0 to day 1, a log phase until day 5, and a stationary phase until day 6 of cultivation. The highest cell density was obtained on day 6 of cultivation at 3.02×10^6 cell/ml. The death phase occurs from day 6 to day 8. Silicate is an important component in the growth and division of diatom cells (Pandit et al., 2017). The silicate removal from the F/2 medium does not cause a slower growth rate in the culture due to the slow consumption cycle of silicate. Silicate deficiency usually affects older cultures (Sadaatkhah et al., 2020) as well as the growth inhibition of *A. platensis* was significantly ($p<0.01$) increased as a result of salt stress (Z. Li et al., 2022). The death phase of microalgae is caused by the scarcity of nutrients or accumulation of organic matter (NO_2^- and NH_4^+), which is toxic to microalgae, disturbing oxygen and nutrient intake (Nugroho, 2006; Suantika et al., 2009). Harvesting of the microalgae is done at the early stationary phase, where peak cell density is achieved. At this phase, nutrient stress occurs due to the limited nutrient in the media. It causes a drop in cell density and changes in cell metabolism (Guschina & Harwood, 2006).

Bioflocculation Rate

Harvesting of microalgae was done at the end of exponential growth, where secondary metabolite production is abundant (Cruz et al., 2020; Lutfi et al., 2019; Suyono et al., 2015). Mixing the Glagah Consortium with *Anabaena* sp. and *Navicula* sp. was done to induce the formation of flocs between microalgae, increasing the flocculation rate of the Glagah Consortium.

Anabaena sp. bioflocculation showed a higher rate of flocculation on a lower mixing ratio of flocculants. It is caused by the composition of EPS produced by *Anabaena* sp., mostly protein. It could be assumed that one of the proteins produced and excreted by the cyanobacteria is classified as an anatoxin (Gangl et al., 2015; Tiwari et al., 2015). Anatoxin could potentially inhibit the metabolism of Glagah Consortium and causes cell death in the culture mix, lowering the flocculation rate at a higher mixing ratio.

Navicula sp. is a benthic diatom with a tendency to form biofilms. As *Navicula* cells come into contact with Glagah Consortium, the cells form flocs between each other and fall onto the bottom of the media. Contact between *Navicula* sp. and Glagah Consortium could be attributed due to polysaccharides in the cell wall and colloidal exopolysaccharides excreted by *Navicula* sp., which helps in the formation of biofilm (Salim et al., 2011). This

interaction between microalgae causes the increase of flocculation rate on the addition of *Navicula* sp. to Glagah Consortium. The increase of bioflocculant rate to a higher flocculant ratio showed similar trends to Salim et al. (2012).

Carbohydrate Content

Carbohydrate is a product of photosynthesis and the main component in cell wall construction (Yen et al., 2013). It is commonly stored in the cell wall or plastids for energy storage (Domozych et al., 2012; Ho et al., 2012). Carbohydrate and lipid content are usually inversely proportional to each other. Lower amounts of carbohydrates in *Anabaena* sp. are caused by culture growth entering the stationary phase. In this phase, microalgae prioritize the hydrolysis of carbohydrates and accumulation of lipids in the cell (Sayanova et al., 2017). This results in the higher carbohydrate content on a lower mixing ratio because of lower amounts of *Anabaena* sp. competing for a carbon source.

Navicula sp. and Glagah Consortium produced a different result; a higher mixing ratio produced more carbohydrate content caused by a difference in medium salinities. *Navicula* sp. is cultivated in an F/2 medium with a salinity of 30%. When the cultures are mixed, osmotic stress will occur in Glagah Consortium cells, increasing carbon storage in cell walls, such as cellulose and hemicellulose (Suyono et al., 2015). No significant differences were observed between Glagah Consortium control and the mixed samples. Exopolysaccharides excreted by diatoms can act as substrates for heterotrophic bacteria growth, which also suggests the possibility of interaction between Glagah Consortium symbiotic bacteria and *Navicula* sp., causing a slight decrease in carbohydrate content (Amin et al., 2012).

Lipid Content

Glagah Consortium is a strain composed of multiple microalgae and bacteria species capable of fast, exponential growth. Interaction between species increases the productivity of lipids because the cells can use carbon sources optimally (Behl et al., 2011; Rahmawati et al., 2020; Suyono et al., 2015). Glagah Consortium contains 1.25% lipid, which could be increased up to 13.58% in response to saline stress (Suyono et al., 2016).

Anabaena sp. and Glagah Consortium mix produced significantly lower amounts of lipids compared to *Navicula* sp. and Glagah Consortium mix lipid content. It results from cultivation under normal conditions without stress (Rawat et al., 2013). A higher ratio of *Anabaena* sp. caused an increase in lipid production due to nutrient stress. At the stationary phase, microalgae compete in a limited nutrient condition, thus causing a change in metabolism (Guschina & Harwood, 2006). Microalgae will convert carbohydrates into lipids during this period.

Navicula sp. and Glagah Consortium mixture produced the highest lipid content at a 1:1 ratio, which is caused by exposure of Glagah Consortium to osmotic shock after mixing with *Navicula* sp., causing an

increase in lipid production as an adaptation to higher salinity. The impact of salt media increases the lipid content of the Glagah Consortium from 5.86% in brackish water to 13.58% in saltwater media (Suyono et al., 2015). Removal of silicate affects the growth and division of the cell as it is a component of the diatom frustule. Silicate deficiency and saline cause an increase in lipid production and the ratio of saturated and monounsaturated fatty acids while decreasing polyunsaturated fatty acids (Sadaatkhah et al., 2020; Seckbach & Kociolek, 2011).

The individual *Navicula* sp. or *Anabaena* sp. exposure gave a higher flocculation rate, but the contents of carbohydrate and lipid of the *Navicula* sp. or *Anabaena* sp. exposure were much lower than the mixed exposure because the lipid and carbohydrate content not only from the two microalgae but also from the Glagah Consortium.

Mixing Glagah Consortium and *Navicula* sp. cause the mixing of fuel growth medium with medium F/2, which has high salinity. In the ratio of mixing to the volume of *Navicula* sp., which is higher, the volume of medium F/2 mixed with medium BBM will be increased, thus causing a hyperosmotic condition in the culture. The Glagah Consortium and *Navicula* sp. would be hypoosmotic conditions because the medium F/2 dissolves in a non-saline BBM medium. Hyperosmotic conditions in the Glagah Consortium increased carbohydrate production, as seen in the research results. The increase is due to the influence of salinity in medium F/2 when mixing, which causes salinity stress at the Glagah Consortium. Salinity stress and water composition sea in medium F/2 results in carbon storage in cell walls as cellulose and hemicellulose (Suyono et al., 2015). One factor in increasing carbohydrate production is the Glagah Consortium, which is still in the progress log phase, so the production of carbohydrates as a cell wall material still takes precedence. Salinity stress on the Glagah Consortium also resulted in oxidative stress, increasing lipid content. On the Consortium mix Glagah with volume *Navicula* sp. lower ratios like 1:0.5 and 1:0.25, Glagah Consortium does not experience stress due to salinity, which can be seen from the lipid data that it shows lower content, and it does not significantly different from the Glagah Consortium control.

CONCLUSION

Adding *Anabaena* sp. and *Navicula* sp. as bioflocculant in the Glagah Consortium culture increases the flocculation rate with an effective ratio of 1:0.25 for *Anabaena* sp. and 1:1 for *Navicula* sp. Mixing of *Anabaena* sp. and Glagah Consortium results in carbon source competition, reducing carbohydrate content at a higher mixing ratio while increasing lipid content because of lipid production in the stationary phase. *Navicula* sp. and Glagah Consortium mixture caused no significant changes to carbohydrate content but showed an increased amount of lipid at all ratios because of osmotic stress on Glagah Consortium from saline F/2 medium. This study proves

that bioflocculation is an effective way to harvest microalgae, as evidenced by using *Anabaena* sp. and *Navicula* sp. to gather the Glagah Consortium from the coast of Yogyakarta, Indonesia.

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