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Bioflocculation of Glagah strain consortium using *Skeletonema costatum* (Greville) cleve

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ABSTRACT

Along with the increasing energy demand, microalgae-based renewable energy is currently being developed. One of the local Indonesian microalgae strains with high potential for biodiesel production is the Glagah strain consortium. Although biodiesel from microalgae is sustainable, the production costs are still relatively high, especially for harvesting (requires 25%–30% of total production cost). Bioflocculation is an alternative method of harvesting that employs inexpensive and environmentally friendly microorganisms as flocculant agents. Bioflocculant agents for floc formation may consist of diatoms with a high EPS output, such as *Skeletonema costatum*. Harvesting the local strain of *S. costatum* as a bioflocculant has not been the subject of extensive research on bioflocculation. This study determined the flocculation percentage, carbohydrate, lipid, and pigments content after harvesting Glagah strain consortium culture using *S. costatum*. The cultivation of Glagah strain consortium was carried out in Bold Basal Medium, then *S. costatum* in f/2 medium for seven days. Bioflocculation was performed by mixing *S. costatum* and Glagah strain consortium at 1:1, 1:2, and 1:4 ratios. The results showed that the addition of *S. costatum* to Glagah strain consortium culture increased the production of flocculation percentage, lipid, carbohydrate, and pigment content. The combining ratio of SG 1:2, on the other hand, resulted in the highest percentage of flocculation and a significant induction of biological materials, including carbohydrates, chlorophyll, and carotenoids. Then, the highest lipid content was produced in the mixing ratio of SG 1:1 cultivation.

Keywords: Bioflocculation, Glagah strain consortium, *Skeletonema costatum*.

1. INTRODUCTION

With the expansion of the human population comes an increase in the amount of energy required for industry and transportation. As alternative fuels, many biological-based biofuels have been produced. One of them is using microalgae, the third generation of biodiesel sources (Sabilil and Suyono, 2021). Microalgae are photosynthetic microorganisms with simple cell structures and apparatus to have high photosynthetic efficiency. Microalgae biomass may be harvested quickly and contains metabolites such as lipids, proteins, carbohydrates, and other chemical components created by absorbing CO_2 during the photosynthetic

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process (Nigam et al., 2011). Lipids are the main components needed in the production of biofuels from microalgae. Rodolfi et al. (2009) state that microalgae possess the capability to rapidly generate lipids, with lipid production exceeding palm oil production by a factor of ten.

Mixed cultures are currently being developed in addition to optimizing local strains and improving culture stability with faster growth (Friday, 2010). According to Kazamia et al. (2012), a mixed culture has many advantages. Each microalgae species that interact in a mixed culture plays a part in maintaining its stability. The high biomass production is a result of this stability preventing contamination and promoting growth. One of the benefits associated with the utilization of symbiotic bacteria in coexisting phytoplankton systems is the prevention of bacterial contamination. Other bacteria are deterred from invading the niche due to the fact that it is already occupied by the bacteria in the culture. This phenomenon can be elucidated by the competitive exclusion principle of community ecology (Kazamia et al., 2012).

Consequently, species with complementary niches will develop in a stable synthetic community without the presence of contaminating species (Kazamia et al., 2012). Investigating natural mixed cultures, Suyono et al. (2015) examine the Glagah strain consortium, which originates from Glagah beach in the special region of Yogyakarta, Kulonprogo Regency. The Glagah strain consortium consists of six genera of local microalgae, namely *Cyclotella*, *Clyndospermopsis*, *Golenkinia*, *Corethron*, *Chlamydomonas*, *Syracosphaera* (Suyono et al., 2016). In the mixed culture, bacteria and microalgae occupy their respective niches to increase lipid production (Suyono et al., 2015).

Mutualistic symbiosis is one of the microorganism interactions that occur in mixed cultures. Each organism benefits from mutualism symbiosis, one of which is related to the availability of CO_2 and O_2 that can support their growth (Santos and Reis, 2014). Glagah strain consortium is a result of a natural interaction between bacteria and microalgae (Suyono et al., 2015). The bacteria provide the vitamin B12 that microalgae need. Presence of the highest lipid-producing algae in the largest proportion in this mixed community, the overall lipid yield can be maximized. The situation shows how important microorganism interactions are when sterile environments are unavailable and is a great deal for algae production on a large scale intended for the production of biodiesel. Furthermore, microbial symbioses can be tools to improve growth and productivity (Santos and Reis, 2014).

In order to be a part of the G-3 biofuel feedstock, microalgae produce biomaterials that may be utilized to make biofuels (bioethanol, biodiesel, and bio-crude oil) (Sudibyo et al., 2017). Microalgae are the most promising biodiesel feedstock since they can produce a high biomass per hectare and can be cultivated in any conditions (Chisti, 2008; Demirbas, 2011). Considering microalgae are active in CO_2 fixation, producing biofuel from them can also help to reduce carbon emissions. Future biofuels made from microalgae can take the place of the fuels currently utilized around the world, such as fossil fuels, which are in high demand but have not been followed by the discovery of new reservoirs with comparable capacity (Daniyanto et al., 2016). Despite the potential of microalgae-derived biofuels to supplant existing fuels, their cost remains higher in comparison to fossil fuels (Mata et al., 2010).

Producing biofuel derived from microalgae presents a number of obstacles, despite the fact that it has the potential to replace existing fuels. Harvesting expenses account for 20%–30% of total microalgae biomass production (Grima et al., 2003; Mata et al., 2010; Uduman et al., 2010; Branyikova et al., 2018). By employing highly effective harvesting techniques, this cost could be reduced. This amount can be reduced if more effective and efficient harvesting methods are applied. The standard techniques for harvesting microalgae are filtration, centrifugation, flotation, flocculation, and sedimentation. However, because they require a lot of tools and apparatus, the filtration and centrifugation methods are relatively expensive (Mata et al., 2010).

Bioflocculation is an alternative method that is cost-effective, environmentally beneficial, and conserves energy. Bioflocculation is accomplished through the co-cultivation of self-flocculating microorganisms or biopolymers with the target microalgae, resulting in the formation of aggregates known as flocs (Matter et al., 2019). Biopolymers, including polysaccharides and proteins, are biomolecules comprising microalgae and consist of long carbon chains and a high molecular weight (Xiao and Zheng, 2016). Biopolymers as flocculant agents can be extracted from microorganisms that produce extracellular polymeric substances (EPSs), such as algae, bacteria, yeast, and fungi (Matter et al., 2019). Exopolysaccharide (EPS) is the primary component of EPSs generated mostly by bacteria. However, the number of bacteria that may be employed as flocculants is limited (Wang et al., 2012). Furthermore, the necessity for extra media for co-culture with bacteria or fungi as bioflocculants can induce contamination and incur additional costs (Salim et al., 2011).

Co-culturing and incorporating auto-flocculant microalgae into non-flocculant cultures is regarded as a safer alternative because it helps prevent contamination and reduces the need for additional media costs (Gultom and Hu, 2013; Matter et al., 2019). Auto-flocculant microalgae can produce EPS, which is essential in the harvesting process of non-flocculant microalgae. Cultures with high EPS content will have high efficiency in the bioflocculation process (Matter et al., 2019). EPS, naturally produced by microalgae, is a response to environmental changes. EPS production in microalgae is influenced by several factors, such as salinity, pH, and light intensity (Kumar et al., 2018). Several groups of microalgae that secrete EPS are Chlorophyceae (green algae) and Cyanobacteria (blue-green algae) (Kumar et al., 2018). The diatom group (Bacillariophyta) is also a microalgae producing high EPS (Salim et al., 2011).

Diatoms are prevalent microalgae, specifically within marine ecosystems. They perform an essential function as a substantial contributor to worldwide oxygen generation, accounting for around 20% of worldwide carbon fixation (Gao et al., 2018). The protein content of diatoms reaches 60%, carbohydrate production reaches 50%, and lipids reach 20% of the dry weight (Granum et al., 2002). Diatoms produce EPS which contains mostly heteroglycans, especially polysaccharides, and sometimes forms a thick secretion or a mucilage layer surrounding the cell (Granum et al., 2002). One of the diatoms that produce high EPS is *Skeletonema* which is widely used in aquaculture, especially for fish and shrimp larvae feed (Cuzon et al., 2004).

EPS is mostly composed of polysaccharides, although it also contains proteins and uronic acid. Polysaccharides, characterized by their predominantly positive zeta potential, exhibit the capacity to function as bioflocculant agents. Uronic acid, on the other hand, is commonly employed as a heavy metal remediation agent due to its anionic nature (Gupta and Diwan, 2017). Therefore, the EPS component produced by *S. costatum* can help the formation of aggregates. In addition, *S. costatum* is one of the microalgae that can perform an auto-flocculation mechanism. However, it has a high potential to become a bioflocculant agent (Schenk et al., 2008).

A suitable harvesting method is necessary for intense cultivation in order to produce large amounts of microalgal biomass, particularly for large-scale production. The technique for properly obtaining microalgal metabolites is extremely crucial (Shyam and Saramma, 2018). However, bioflocculation is a promising technique to obtain microalgal biomass that is simple to use, affordable, safe, and environmentally friendly (Ummalyma et al., 2017). To ensure that it can be used for mass cultivation in the microalgae industry, bioflocculation research is required.

Consequently, research on bioflocculation is needed so that it can be adopted for mass culture in the microalgae industry. This study focused exclusively on flocculation utilizing diatoms, with particular attention given to *S. costatum*. Similarly, the Glagah strain consortium, an undeveloped natural local mixed culture, has been the subject of investigating by only a limited number of individuals. To optimize flocculation percentage, lipid, carbohydrate, and pigment content, this study determined the optimal ratio for adding *S. costatum* bioflocculant to the Glagah strain consortium cultivation prior to harvest.

2. MATERIALS AND METHODS

2.1 Cultivation

This study employed *S. costatum* and Glagah strain consortium. The stock culture utilized in this study is Glagah strain consortium, which is maintained at the Faculty of Biology, Biotechnology Laboratory, Universitas Gadjah Mada. This stock culture was obtained from the Glagah beach lagoon, Kulon Progo, Special region of Yogyakarta. *S. costatum* was obtained from Balai Perikanan Budidaya Air Payau/Government Research Center for Marine Aquaculture (BPBAP) Situbondo, East Java, Indonesia. Bold Basal Medium and f/2 (Guillard) modified without $NaSiO_3$ were used for cultivation. Glagah strain consortium and *S. costatum* were cultured under aseptic laboratory conditions in 500 mL (100 mL culture was mixed with 400 mL distilled water) of capacity for each culture with 3 repetitions.

2.2 Microalgae Cell Density and Biomass

The rate of cell density of Glagah strain consortium and *S. costatum* were counted using Hemocytometer Improved Neubauer's Chamber and Opti lab every day for 7 days of observation.

2.3 Sample Preparation

S. costatum was harvested on the 6th day, while the Glagah strain consortium was collected on the 4th day (based on preliminary tests). Samples were placed in 15 mL conical tubes with a 1:1, 1:2, and 1:4 ratio of flocculant microalgae (*S. costatum*) to non-flocculant (Glagah strain consortium). Treatment measurements were carried out 3 times. The samples for lipid, carbohydrate, and pigment analysis were let to stand for 24 h in order to allow *S. costatum* and the Glagah strain consortium to produce flocculant.

2.4 Parameter Measurements

2.4.1 Bioflocculation

Upon reaching the final exponential stage on the sixth day of cultivation, *S. costatum* was harvested and subsequently combined with Glagah strain consortium, which had been harvested on the fourth day, in three repetitions at the following ratios: 1:1, 1:2, and 1:4. Each sample was placed in a 15 mL conical tube. Following the mixing, 1 mL of supernatant was collected and deposited in a cuvette, which was then placed in a spectrophotometer and measured at 750 nm to obtain the absorbances at t_0 ($OD_{750}(t_0)$). Glagah strain consortium culture also have been measured as a control. Each sample in the 15 mL conical tube was allowed to stand for 24 h before being examined using a spectrophotometer at 750 nm to obtain the absorbances at t_{24} ($OD_{750}(t_{24})$). The absorbances were then calculated using Equation (1) (Salim et al., 2011):

$$\% \text{ Flocculation} = (OD_{750}(t_0) - OD_{750}(t_{24})) / (OD_{750}(t_0)) \times 100\% \quad (1)$$

where t_0: Absorbance at 750 nm before incubation, t_{24}: Absorbance at 750 nm after 24 hours incubation.

2.4.2 Lipid content

The lipid content was measured using Bligh and Dyer's method (Bligh and Dyer, 1959). The 15 mL sample was centrifuged at 4000 rpm for 15 min. Then, the supernatant

was removed, and 2 mL of methanol and 1 mL of chloroform were added to the pellets. Next, the sample was vortexed until homogenous. The samples were treated with 1 mL distilled water and 1 mL chloroform. The material was vortexed again for 1 min before being centrifuged at 1800 rpm for 15 min. The sample was divided into 3 layers with lipid at the bottom with a yellow to green color. Lipids were taken and placed on a petri dish. After that, the petri dish was put in the oven. The chloroform evaporation process is carried out with an open petri in an oven at 30°C until the weight was constant. The empty weight of a petri dish and the weight after the oven were then estimated using Equation (2):

Lipid content (g/L) = (Weight after oven – the empty petri dish) (g) / sample volume (L) (2)

2.4.3 Carbohydrate content

The analysis of carbohydrate content was carried out using the phenol sulfuric acid method (Dubois, 1956). 15 mL sample was centrifugated at 3300 rpm for 10 min at room temperature. After removing the supernatant, 0.5 mL of 5% phenol was added to the pellet. The sample was vortexed until homogenous and allowed to stand for 10 min. 1 mL of sulfuric acid was added, and the sample was vortexed again and then allowed to stand for 20 min. After that, absorbance readings were performed using a spectrophotometer at a wavelength of 490 nm. Standard curve formula is needed to determine the glucose standard curve for calculating the concentration of carbohydrates. Glucose anhydrate standard solutions with concentrations of 0.1, 0.2, 0.3, 0.4, 0.6, 0.9, and 1.0 g/L were prepared and extracted using the Dubois method. The absorbance was measured, and a standard curve was obtained using Equation (3):

$$Y = 0.0884x + 0.009 \text{ (with } r^2 = 0.9826) \quad (3)$$

where Y: Absorbance at 490 nm, X: Concentration of carbohydrate.

2.4.4 Pigment content

The pigments including chlorophyll-a, chlorophyll-b, total chlorophyll, and total carotenoids were analyzed in this study. The suspension was vortexed in a 15 mL container before being centrifuged at 3300 rpm for 15 min. The supernatant was then removed, and 3 mL of acetone was added before homogenization. Following that, 2 mL of the resultant mixture was extracted and placed in a cuvette. Then, each sample was measured using a UV-Vis spectrophotometer with multiwavelength at 470, 645, and 662 nm (Dere, 1998). With the following equation, total chlorophyll can be obtained as a mixture of chlorophylls a and b components and the carotenoid contents based on the absorbance determined by the spectrophotometer. Equation (4):

- Chlorophyll a (g/L) = ((16.72 × A665.2) – (9.16 × A652.4)) / 1000
- Chlorophyll b (g/L) = ((34.09 × A652.4) – (15.28 × A665)) / 1000
- Carotenoids (g/L) = (1000 A470 – 1.63 chlorophyll a – 104.96 chlorophyll b) / 221
- Total Chlorophyll (g/L) = (20.2 × A645) + (8.02 × A663) / 1000 (4)

where A_{662}: Absorbance at 662 nm, A_{645}: Absorbance at 645 nm, A_{470}: Absorbance at 470 nm.

2.5 Statistical Analysis

The analysis of variance was performed on the data using a one-way method and the statistical software SPSS (Version 26, IBM Corporation, USA). Differences were determined to be significant at $a < 0.05$. When the treatment effect was significant, the means were separated using Duncan's Multiple Range Tests (DMRT).

3. RESULTS AND DISCUSSION

3.1 Cell Count and Biomass

As can be seen in Fig. 1, this study used microalgae that had been grown for 7 days. The evidence of nutrient absorption and utilization by microalgae was demonstrated by an increase in cell count. The lag-phase period of *S. costatum* was observed approximately 0–3 days after cultivation, as shown in Fig. 1. On day six of the exponential phase, the greatest number of cells of *S. costatum* matured. The highest cell number of *S. costatum* was 9.87 × 10^5 cells/mL.

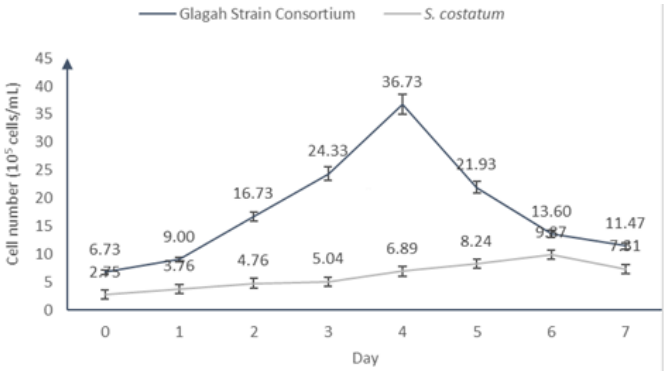

Fig. 1. Cell number of *S. costatum* sp. and Glagah strain consortium (n = 3)

The rapid increase in cell count is a characteristic of the exponential phase that is succeeded by the lag phase. The adaptability of microalgae is supported by the nutrient source in the culture and the age of the inoculum culture. The growth of microalgae cells was influenced by both the cell density and phase of the inoculum. During the exponential phase, appropriate nutrition and microalgal culture conditions were maintained, resulting in non-linear

development. The cell division phase often corresponded with the apex of microalgae growth, resulting in a significant rise in biomass. This state is often found during the exponential stage and is characterized by a considerable increase in cell numbers. The exponential phase indicates that the cells are efficiently absorbing nutrients (Yaakob et al., 2021).

The Glagah strain consortium had the largest cell number during the exponential phase on the 4th day, which equaled 36.73 × 10^5 cells/mL. This demonstrated that the energy produced in cells with relatively fast metabolic rates is used for cell multiplication through asexual division (Jiménez et al., 2003). The greater quantity of cells observed in Glagah strain consortium as opposed to *S. costatum* may be attributed to the association of Glagah Strain Consortium with bacteria capable of generating organic and inorganic nutrients, as well as vitamin B12, which may facilitate the development of microalgae (Novoveská et al., 2016).

The Glagah strain consortium is comprised of numerous microalgae that support nitrification reactions in the medium and engage in mutualistic symbiosis with bacteria as vitamin suppliers (Suyono et al., 2016). The cell density of *S. costatum* was low because of the limitation of Si in the growth media (Setyaningsi et al., 2018). In contrast to *S. costatum*, the Glagah strain consortium exhibited a greater cell count during the late exponential phase on the day of harvest. The Glagah strain consortium was collected for analysis on the 4th day of cultivation, when it had significantly increased in cell count from its initial state. Following this, on the sixth day of cultivation, *S. costatum* was harvested at its maximum cell density of 9.87 × 10^5 cells/mL.

The highest cell density occurred in the late exponential phase. High cell density will be causing difficulties for microalga cells to absorb light radiation for photosynthesis. This condition arose due to the fact that cells are in competition to obtain the light radiation necessary to sustain their photosynthetic processes. After this stage, microalgae cells start to decline because most of them cannot absorb light radiation. However, it cannot conduct photosynthesis and finally die. The nutrient depletion, which leads to a decline in metabolic activity, also contributes to the death of microalgae cells. In order to produce a significant amount of high-quality microalgal biomass, it is crucial to provide adequate nutrients for microalgae growth.

Nutrient content, especially nitrogen and phosphorus, is important to consider while growing microalgae. Other important considerations include culture method, temperature, light intensity, light : dark ratio, salinity, pH, mixing, turbulence, and carbon dioxide concentration. Nitrogen and phosphorus, which are essential macronutrients for facilitating algal growth, also modulate metabolic processes when supplied in suitable forms. Under conditions of nutrient depletion, cellular division, and development ceased, directing the stored carbon towards metabolic processes instead of growth. An additional factor contributing to the decline in cell count and biomass generation is the accumulation of organic compounds (specifically NO_2^- and NH_4^+), which may have detrimental effects on microalgae by impeding their nutrient and dissolved oxygen absorption capabilities (Suantika et al., 2009). The Glagah strain consortium entered its mortality phase between the conclusion of the 5th day of cultivation and the day's end. The mortality phase is characterized by a substantial reduction in the quantity of cells.

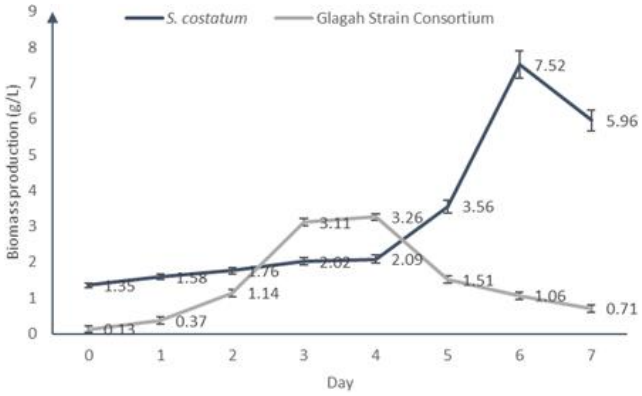

Fig. 2. Biomass production of *S. costatum* and Glagah strain consortium (n = 3)

Despite having fewer cells than Glagah strain consortium, *S. costatum* generates a greater amount of biomass. This is because of the different morphology, like cell size and cell walls. *S. costatum* typically generates a chain consisting of multiple cells, with each chain containing a maximum of 20 cells (Popovich et al., 2012). Each cell in the chain is between 4 and 15 µm in diameter. Following that, the Glagah strain consortium comprises numerous species characterized by their diminutive cell sizes. Variations in the medium utilization may also contribute to this phenomenon. Variations in the medium utilization may also contribute to this phenomenon. The study employed two distinct media, namely Guillard medium (f/2) and Bold Basal Medium (BBM). The medium used for cultivating Glagah strain consortium was BBM, while the medium for cultivating *S. costatum* was f/2 medium. The nutritional contents of these two media are different. In contrast to f/2 medium, BBM medium has a high N-P (nitrogen-phosphorus) ratio. The high N-P ratio composition assists in the formation of chlorophyll, accelerating photosynthesis (Suyono et al., 2016). Additionally, this will influence the quantity of carbohydrates generated. BBM medium consists of K_2HPO_4 and KH_2PO_4 as their phosphorus nutrient sources. According to Yuarrina et al. (2018), BBM medium is the optimal cultivating medium for bioethanol-producing microalgae and for facilitating the synthesis of carbohydrates in microalgae. Additionally, BBM is a medium that is commonly employed to cultivate green algae.

Despite the comprehensive nutritional value of BBM medium, the biomass production in cultures utilizing f/2 medium can be enhanced by the vitamin content of f/2 medium, which facilitates photosynthesis. This medium is

also widely used for marine microalgae cultivation because of its nutritional content and the use of seawater, especially for diatoms. f/2 medium is composed solely of NaH_2PO_4 as a source of phosphorous nutrients. In contrast, f/2 medium contains a greater variety of trace elements than BBM medium. It has been discovered that the presence of micronutrients, such as vitamins, in the culture media, is another important component that promotes microalga growth. Microalgal axenic culture requires a more exact nutritional composition in their growing media. For growing microalga monoculture at the laboratory scale, specific formulations of standard media such as Guillard or f/2 media are typically utilized. The vitamins biotin, cyanocobalamin (B12), and thiamin (B1) are all classified as micronutrients. Consequently, f/2 medium is one of the most extensively utilized enrichment media and is suitable for the development of the vast majority of algae.

The cellular walls of *S. costatum* consist of silica, characterized by an overlapping structure known as a frustule (Endar et al., 2012). In this study, SiO_2 was not added to growth medium f/2 as a source of silica to construct the cell wall of *S. costatum*. This process was intended to reduce the amount of silica in the harvested biomass and to make it easier to extract metabolites and easier to break down the cell wall. Without requiring additional effort to remove the silica component from the biomass, silica-free biomass can be used right away.

Bioflocculation determination

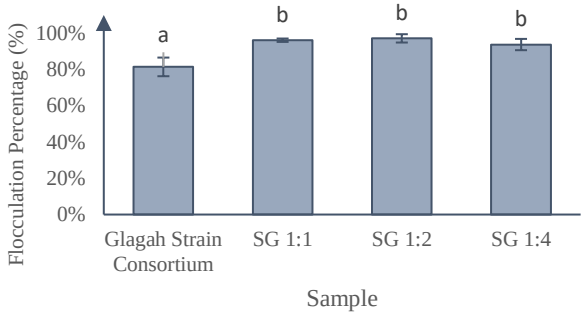

Fig. 3. Flocculation percentage in Glagah Strain Consortium and SG mixed ratios of 1:1, 1:2, and 1:4. Data are means ± SD (n = 3).

Saputra (2013) demonstrates that the Glagah strain consortium exhibits a comparatively low flocculation percentage. *S. costatum* is a diatom with bioflocculant potential and a high EPS output. Particle flocculation speed is influenced by particle size. The smaller particle size is more challenging to flocculate. Compared to *S. costatum*, the Glagah strain consortium took longer to precipitate and produce floc. Sathe (2010) defines bioflocculation as the spontaneous flocculation of microalgae cells under stress, which is caused by the secretion of EPS. This EPS causes the formation of clumps of cells, which become biomass so that it precipitated. Limited nutrients are stress conditions that trigger EPS secretion (Lee et al., 2009). However, *S. costatum* known as auto-flocculation microalgae produces high EPS (Schenk et al., 2008). Glagah strain consortium can flocculate because it consists of some diatom (*Cyclotella* and *Conethron*) that produce high EPS and several bacteria like *Bacillus* (Suyono et al., 2015*). Bacillus*, as identified by Salim et al. (2011), is among a variety of bacteria that generate substantial EPS and have the potential to function as flocculant agents.

The result shown in Fig.3 that at a ratio of 1:2, or 97%, the maximum percentage of flocculation is achieved. As a comparison, the proportion of flocculation that is achieved at a ratio of 1:4 is the smallest, at 94%. Salim et al. (2011) state that the percentage of precipitation can be increased by incorporating flocculant species with high concentrations during harvesting. As the mixture ratio increases, a greater quantity of *S. costatum* is incorporated into the Glagah strain consortium culture, resulting in a greater percentage of flocculation. Autoflocculating *S. costatum* microalgae induce sedimentation more rapidly than non-flocculating microalgae, thereby increasing harvesting efficiency. Combining therapies differ significantly from the control (Glagah strain consortium), as demonstrated by the results.

Lipid content analysis

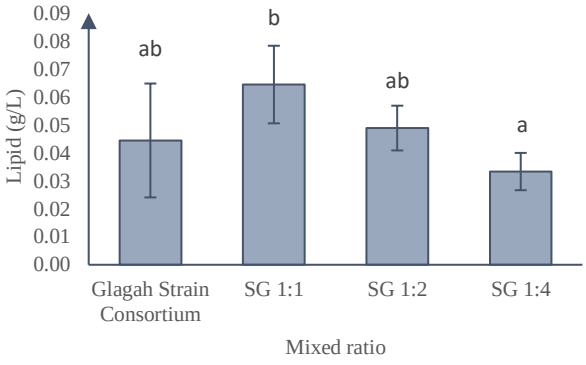

Fig. 4. Lipid content in Glagah Strain Consortium and SG mixed ratios of 1:1, 1:2 and 1:4. Data are means ± SD (n = 3).

Suyono et al. (2016) describe Glagah strain consortium as a mixed culture. Mixed culture grows quickly and has a high lipid content. Mixed culture can boost productivity, according to Chisti (2007). It activates a complicated mechanism that results in the production of lipids (Behl et al., 2011). The lipid content of the Glagah strain consortium was recorded as 1.25% by Suyono et al. (2016). They suggest that by emphasizing the management of environmental factors like salinity, this value could be raised to 13.58%. The Glagah strain consortium lipid concentration is determined to be 0.044 g/L, as shown in Fig. 4. The lowest percentage of lipid is obtained in the treatment

at a ratio of 1:4, indicating that the microalgae are sustained in the absence of significant stress. It turns out that the microalgae mixture does not create a lot of lipids at this ratio. This might happen because, in the 1:1 and 1:2 ratios, the conversion of carbohydrates to lipids occurs more effectively. It is possible that the mixed culture balance at this ratio will not shift the culture balance quickly enough to produce unfavorable circumstances that stress the culture as much as the 1:1 and 1:2 ratios do. The mixed culture at 1:4 has lower lipid content than the Glagah Strain Consortium (control).

Non-structural lipid biosynthesis and structural lipid biosynthesis are the two pathways involved in the biosynthesis of lipids. The process of lipid biosynthesis in microalgae requires considerable energy from the process of forming carbohydrates (Ahmad et al., 2011). Carbohydrates are formed by algae cells through the process of photosynthesis. Carbon dioxide is synthesized into glyceride-3-phosphate. Then, glyceride-3-phosphate will be converted into pyruvate in the process of glycolysis. The resulting pyruvate will then be converted to acetyl-CoA, which is the precursor to fatty acid synthesis with the help of the enzyme pyruvate dehydrogenase complex. Fatty acid synthesis occurs in the plastids and then is carried to the endoplasmic reticulum to be converted into structural lipids and non-structural lipids (Bellou and Aggelis, 2012). Under optimum conditions, more lipids are formed, whether structural lipids in cell components, while non-structural lipids will be produced as energy reserves under stress conditions.

Carbohydrate analysis

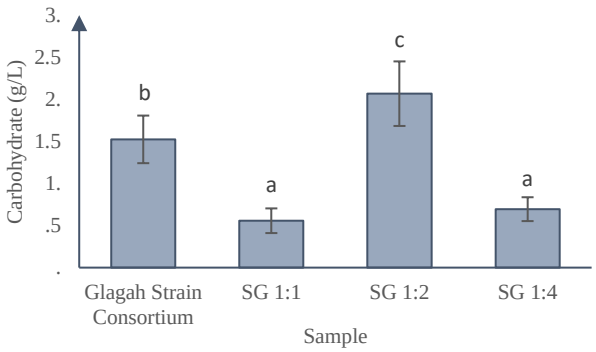

Fig. 5. Carbohydrate content in Glagah Strain Consortium and SG mixed ratios of SG 1:1, 1:2, and SG 1:4. Data are means ± SD (n = 3).

Carbohydrate is a product of photosynthesis and a component of cell walls. The carbohydrate content of the microalgae is used as an alternative fuel to produce bioethanol (Domozych et al., 2012; Yen et al., 2013). Glagah strain consortium has a high carbohydrate content equal to 1.52 g/L. The carbohydrate content is increased when Glagah Strain consortium is combined with *S. costatum* as a bioflocculant. At SG 1:2, the carbohydrate content is at its maximum. The carbohydrate concentration is discovered to be the lowest at an SG 1:1 ratio. The metabolic pathways associated with elevated energy produce an inverse trend in the lipid and carbohydrate content. This is due to harvesting microalgae in the stationary phase, where microalgae change their metabolism by breaking down carbohydrates into energy reserves such as lipids because the precursor of TAG (Triacylglycerol) is glyceraldehyde-3-phosphate (G3P). G3P is the result of carbohydrate catabolism (Sayanova et al., 2017). However, microalgae that produce high amounts of carbohydrates tend to produce small amounts of lipids.

Fig. 5 shows that only the mixture of *S. costatum* and Glagah strain consortium at SG 1:2 has a higher carbohydrate content than Glagah strain consortium. However, SG 1:4 and SG 1:1 show lower results than the Glagah strain consortium culture. This could be due to insufficient dehydration and hydrolysis of carbohydrates in the SG 1:1 and SG 1:4 samples. A mixed culture must have a higher metabolite content than a single culture. The more bioflocculants added, the more metabolites it will contain. A higher bioflocculant ratio leads to a greater percentage of flocculation when it is added. The contribution of extracellular polymeric substances, mostly comprised of exopolysaccharides, which are carbohydrate components, in both bioflocculation and carbohydrate content, is attributed to the relationship between the two.

Moreover, the incubation period after mixing *S. costatum* with the Glagah strain consortium for 24 h may be the reason for the rapid increase in carbohydrate content in SG 1:2. Due to this mixture, adverse conditions may develop for both of them. The observation that the blending treatment contained fewer carbohydrates than the control group (Glagah strain consortium) suggests that the conversion of carbohydrates to lipids may occur under these unfavorable conditions. This can also be altered by the mixing that occurs when each culture is in the late exponential phase. As the exponential phase of growth comes to an end and the amount of nutrients in the medium decreases, microalgae start to produce lipids, which change their metabolism. Differences in carbohydrate content in the mixing treatments may also be caused by a lack of culture equilibrium in the mixture. The Glagah strain consortium itself is a mixture of various kinds of microalgae, including diatoms, green algae, and bacteria. When *S. costatum* and Glagah strain consortium are mixed, the culture equilibrium is altered, enabling the bacteria to utilize the carbohydrate content in the mixed culture. The mixing ratio of SG 1:2 exhibits the maximum carbohydrate content, according to the data. Based on the obtained results, it can be inferred that the carbohydrate content of the mixture is increased by a culture equilibrium at this particular mixing ratio, as opposed to the SG 1:1 and 1:4 ratios.

Pigment analysis

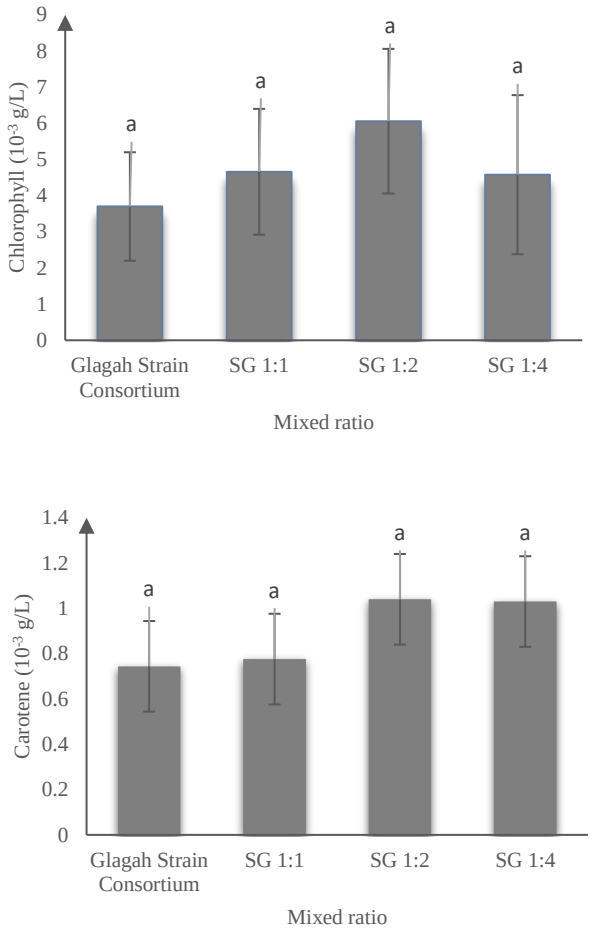

Fig. 6. Pigment content (top) total chlorophyll (down) total carotenoid in Glagah strain consortium and SG mixed ratios of 1:1, 1:2, and 1:4. Data are means ± SD (n = 3).

The results presented in Fig. 6 indicate that the mixture SG 1:2 contains the highest concentration of total chlorophyll, measuring 5.8 × 10^{-3} g/L. In contrast, the solution containing SG 1:4 exhibits the lowest cumulative chlorophyll content at 3.4 × 10^{-3} g/L. With the highest total carotene concentration found in an SG 1:4 mixture and the lowest in an SG 1:1 mixture, the amount difference for carotene is not quite significant. Total carotene content is lower than total chlorophyll content in all regimens, according to the data. Conversely, there is no substantial variation in the overall chlorophyll and carotenoid content among the regimens. Chlorophyll makes up the majority of the pigment in diatoms, which justifies the higher chlorophyll content than carotenoids. The modified composition medium f/2 without Si also affects the production of chlorophyll. Limitations of Si increase chlorophyll-a production (Kuczynska et al., 2015).

According to Arifah et al. (2019), when harvesting occurs during the exponential phase, the supply of nutrients for microalgae is still fulfilled, and the number of cells continues to increase. According to Benavente-Valdes et al. (2016), the photosynthetic process will be greatly affected if the amount of nitrogen in the medium begins to become limited. The main pigment of *S. costatum* is chlorophyll, which absorbs light from the environment. It also has a carotenoid for photoprotection (Kuczynska et al., 2015). The primary photosynthetic pigment, chlorophyll-a, is a nitrogen-rich compound. When nitrogen is depleted, chlorophyll-a will function as an intracellular nitrogen source to facilitate cell growth and biomass production (Benavente-Valdes et al., 2016). The chlorophyll-c and carotene play as the accessory pigment that helps light absorption. Carotenoid does not increase in number despite the high nitrogen content because it only helps in light absorption. Since phosphorus aids in the process of light absorption, it is not anticipated that the accessories will contain a significant amount of pigment as a result (Kuczynska et al., 2015). Consequently, the concentration of carotene is comparatively lower in magnitude when contrasted with chlorophyll chlorophyll-a. An observed yellowish-brown pigment indicates that the presence of carotenoid pigments is significantly elevated in the Glagah strain consortium culture supplemented with *S. costatum*.

The treatment in which a 1:1 SG ratio was utilized contained the most lipids. The highest total pigment (chlorophyll and carotenoids) and carbohydrate content are the results of treating SG with a 1:2 ratio. In addition, the SG ratio of 1:2 produces the greatest flocculation rate. This indicates that the Glagah strain consortium's addition of the bioflocculant *S. costatum* results in an excellent ratio of SG 1:2 for metabolite production and bioflocculation.

4. CONCLUSION

Autoflocculating microalgae-based bioflocculation is a potentially effective method of harvesting. The present study ascertains that the flocculation percentage, as well as the concentrations of lipids, carbohydrates, chlorophyll, and carotene, are all enhanced by the Glagah Strain Consortium and *S. costatum* in their mixing ratio. The use of *S. costatum* as a bioflocculant increases the frequency of flocculation percentage. An increased mixing ratio adds to improved flocculation effectiveness, with the addition of additional bioflocculant resulting in a higher lipid content. The maximum lipid content is observed at the mixing ratio SG 1:1. The mixing ratio of SG 1:2 yields the greatest quantities of chlorophyll and carotene, followed by the SG 1:2 mixing ratio for carbohydrates.

Further research requires an examination of the generated EPS content composition. It is additionally imperative to monitor it during the combining period so as to ascertain the manner in which bacterial activity in the mixed culture influences the bioflocculant process and the generation of metabolite products. To permit large-scale production, further research is required to determine the efficacy of

bioflocculation employing a mixing ratio containing auto-flocculant microalgae.

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