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Mixed Culture with *Monoraphidium* sp. for the Increase of Metabolites Production in Glagah Strain Consortium

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The feasibility of microalgae-derived products became a major issue on an industrial scale. Mixed culture is one of the improvements that can be made. The mixed culture of Glagah Strain Consortium and *Monoraphidium* sp. was investigated in terms of biomass, lipid, protein, and carbohydrates production, as well as productivity. Glagah Strain Consortium is a local consortium strain composed primarily of *Cylindrospermopsis* sp., *Cyclotella* sp., *Corethron* sp., *Golenkinia* sp., *Chlamydomonas* sp., and *Syracosphaera* sp., native to Glagah Lagoon in Yogyakarta, Indonesia. The mixed culture of Glagah Strain Consortium and *Monoraphidium* sp. was compared to the monocultures of Glagah Strain Consortium only and *Monoraphidium* sp. only. It is then found that mixed cultures can increase lipid productivity up to 0.1808±0.010 g/L/day, which is greater than monocultures. Mixing the culture with *Monoraphidium* sp. was shown to increase production and productivity of lipid, biomass, protein, and carbohydrates, though not as much as *Monoraphidium* sp. monoculture. Further research is needed to understand the mechanisms in the mixed culture and to increase the culture's production and productivity.

Keywords: Glagah Strain Consortium; Microalgae Yield; Mixed Culture; *Monoraphidium* sp.

I. INTRODUCTION

With ever-increasing energy demands, available conventional energy sources are incapable of meeting human demands. As a result, biodiesel is being pushed to fill the void. Microalgae is one potential source for biodiesel production. Microalgae is a single cell organism that is able to do photosynthesis and produce several useful metabolites as a way to counter their environmental conditions. Glagah Strain Consortium is not made up of just one species; rather, it was named after several species found in a specific body of water and coexisting in Glagah Lagoon in Kulon Progo, Daerah Istimewa Yogyakarta, Indonesia. Consortium is a grouping of several microorganisms that coexisted in one place. Glagah Strain Consortium, mainly composed of microalgae such as *Chlamydomonas* sp. *Cylindrospermopsis* sp., *Syracosphaera* sp., *Golenkinia* sp., *Corethron* sp., *Cyclotella* sp., and bacteria such as *Pediococcus parvulus*, *Corynebacterium ulcerans*, *Bacillus cereus*, *Corynebacterium bovis*, *Staphylococcus vitulinus*, *Bacillus megaterium*. However, other microalgae species, such as *Desmodesmus* sp. and *Selenastrium* sp., could be found within the consortium (Suyono *et al.*, 2016; Suyono *et al.*, 2018). Glagah Strain Consortium can produce dry weight up to 0.12 g/L, around 50 g/L of Lipid, and 12 g/L of total carbohydrate at day four of cultivation (Muttaqin & Suyono, 2021).

Monoraphidium sp. is a microalgae species that is known for its high lipid production. It can produce up to 6% lipid per gram biomass, while its lipid productivity reaches 240 mg/L/day (Namitha *et al.*, 2021; Zhao *et al.*, 2020). *Monoraphidium* sp. has a maximum biomass productivity of 0.46 g/L/day, with a 32.34% ash content (Hawrot-Paw *et al.*, 2020). *Monoraphidium* griffithii, one of the *Monoraphidium* sp. species, can produce 32.97% protein and 15.36% carbohydrate per biomass produced (Vinitha *et al.*, 2022). However, this monoculture way of cultivation is

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not yet feasible to fulfil the industrial needs for biofuel production.

Mixed culture is a method to increase microalgae metabolite yield by mixing a culture with another microalgae strain. Previously, a mixed culture of *Monoraphidium* sp. and *Streptomyces nojiriensis* demonstrated that the *Streptomyces* was able to increase the biomass, growth, and lipid of *Monoraphidium* sp. by up to 150% when compared to control monoculture treatments (Li *et al*., 2022).

Thus, this study aims to understand the effect of mixing the culture with *Monoraphidium* sp. towards the improvements of biomass, lipid, protein, and carbohydrate total production and productivity of Glagah Strain Consortium

II. MATERIALS AND METHOD

The experiment was carried out at the Laboratory of Biotechnology, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia. The study lasted from August 2020 to June 2021. Glagah Strain Consortium was isolated from Glagah Lagoon (GPS: S7°54'45.8" E1,1.0°3'59.5") and cultured in laboratory scale on BBM (Bold's Basal Medium) medium after two months of optimisation. BBM (Bold's Basal Medium) was composed of NaNO3, MgSO4·7H2O, CaCl2·2H2O, KH2PO4, K2HPO4, Alkaline EDTA Solution (EDTA and KOH), NaCl, acidified iron solution (FeSO4·7H2O and H2SO4), Boron Solution (H3BO3), and Trace Metals Solution (ZnSO4·7H2O, MnCl2·4H2O, MoO3, CuSO4·5H2O, and Co(NO3)2·6H2O). The isolated Glagah Strain Consortium consisted of microalgae *Cyclotella polymorpha*, *Golenkinia radiata*, *Corethron criophilum*, *Scenedesmus bijuga*, *Scenedesmus* sp., *Scenedesmus subspicatus*, *Monoraphidium contortus*, *Scenedesmus bernardii*, *Merismopodia glauca*, *Cyclotella meneghiniana*, *Navicula lanceolata*, *Nitzcshia vemicularis*, and also bacteria such as *Corynebacterium ulcerans*, *Corynebacterium bovis*, *Bacillus cereus*, *Bacillus megaterium*, *Pediococcus parvulus*, and *Staphylococcus vitulinus* that was identified by matching the microalgae found with nomenclature book. *Monoraphidium* sp. was obtained from the Indonesian Culture Collection (InaCC) as InaCC M8, which had previously been isolated from Pekanbaru, Riau. On BBM Medium, the collected culture was optimised for two months. The BBM was prepared according to Bold's methodology (Bold, 1949).

Treatments used were monoculture of *Monoraphidium* sp. only, Glagah Strain Consortium only, and a Mixed culture of *Monoraphidium* sp. and Glagah Strain Consortium. Mixed culture treatments began with approximately equal cell density of 1:1 combination of *Monoraphidium* sp. and Glagah Strain Consortium culture. All treatments were cultured under laboratory conditions, at pH 7 at 25°C in a temperature-controlled room under continuous illumination for 24 hours (25.000 lux). Monoculture of *Monoraphidium* sp. and Glagah strain Consortium were also analysed as controls. Five replications were used for each treatment. During the seven-day experiments, the yield of biomass, lipid, carbohydrates, and protein of each culture were analysed.

Total lipid production and productivity were calculated using the Bligh and Dyer method (Bligh & Dyer, 1959). 5 mL of sample was collected and centrifuged at 3300 rpm for 10 minutes to separate the wet weight from the media. Furthermore, 2 mL methanol and 1 mL chloroform, as well as 1 mL chloroform and 1 mL distilled water, were added. The second centrifugation phase used a speed of 4000 rpm at a temperature of 10°C. The bottom part of the formed layers will then be extracted and dried in a petri dish. The lipid content was calculated using following equation:

$$Total\ lipid\ production\ (g/L) = (Final\ weight\ of\ petri\ dish\ (g) - initial\ weight\ of\ petry\ dish) \times 200 \quad (1)$$

%Lipid was calculated by dividing obtained crude total lipid production results to gram biomass.

Carbohydrate analysis was done using Phenol-Sulphuric Acid method (Nielsen, 2017). Standard curve was made using standard glucose solution of 2.25 g/L; 2 g/L; 1.6 g/L; 1 g/L; 0.75 g/L; 0.50 g/L; 0.45 g/L; 0.35 g/L; 0.15 g/L; concentration. From this, 10 mL of sample from each treatment was collected and centrifugated at 3300 rpm for 15 minutes. The remaining pellet was added with 1 mL H_2SO_4 and 0.5 mL 5% phenol. This was followed by the incubation of this mixture at room temperature for 30 minutes. The absorbance of the mixture was measured at

595 nm. The final equation used to convert absorbance result into concentration format (g/L) is y=0.0045x+0.0727.

Total production and productivity of biomass was determined using high-rate laboratory filtration with Büchner funnel method (Shapiro, 1961). In this, 10 mL of one of the treatments of microalgae sample was passed through a Büchner funnel part of the Vacuum Pump Kit that is already equipped with a dry glass microfiber filter (GF/C) paper. Furthermore, 10 mL of distilled water was used to wash the Büchner funnel three times. This was followed by overnight drying of glass microfiber filter paper in 50°C oven. The pellets that remain on the glass microfiber filter paper can be classified as dry biomass weight (DW). The equation used for obtaining DW was

$$Total\ Biomass\ Production\ (g/L) = (Final\ Weight\ of\ GF/C\ paper\ (g) - initial\ weight\ of\ GF/C\ paper) \times 100 \quad (2)$$

Protein assay was done according to Bradford method procedure (Bradford, 1976). The standard curve was made by the absorbance gathered from 0.5 g/L; 1.0 g/L; 1.5 g/L, 2.0 g/L and 2.5 g/L of Bovine Serum Albumin (BSA). Pellet collected from 2 mL sample was added with 1 mL 10% SDS. This mixture was incubated at 95°C in water bath. After five minutes of incubation, the mixture swiftly incubated in -5°C freezer for five minutes. 8µL of the mixture was then placed into microplate and added with 200µL Bradford reagent. Absorbance of the sample was calculated using ELISA Reader Bio Tek in 490 nm wavelength. The final equation used to convert absorbance result into concentration format (g/L) is y=0.4482x+0.0416.

Statistical analysis was done by making average from five replications of lipid, biomass, carbohydrate, and protein analysis as well as measuring the standard deviation and standard error. Results were then assessed by Analysis of Variance (ANOVA) and then continued with Duncan's Multiple Range Test (DMRT) at P<0.05 in SPSS software.

III. RESULT AND DISCUSSION

The effectiveness of mixed cultures can be determined by analysing the total production and productivity of each metabolite for seven days of cultivation. For the aims of biofuel production, high lipid, carbohydrate, and protein profile was favoured.

Analysis of cells morphological characteristics from the consortium found in Glagah Beach shows that there are several microalgae found. The details of the species found described in this picture below:

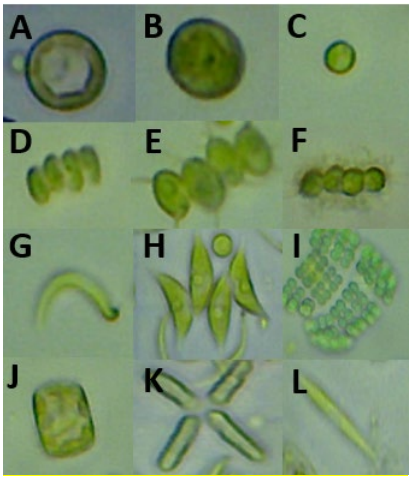


Figure 1. Some microalgae found in the Glagah Strain Consortium upon identification through morphological characteristics comparison: (A) *Cyclotella polymorpha*, (B) *Golenkinia radiata*, (C) *Corethron criophilum*, (D) *Scenedesmus bijuga*, (E) *Scenedesmus* sp., (F) *Scenedesmus subspicatus*, (G) *Monoraphidium contortus*, (H) *Scenedesmus bernardii*, (I) *Merismopodia glauca*, (J) *Cyclotella meneghiniana*, (K) *Navicula lanceolata*, dan (L) *Nitzcshia vemicularis*

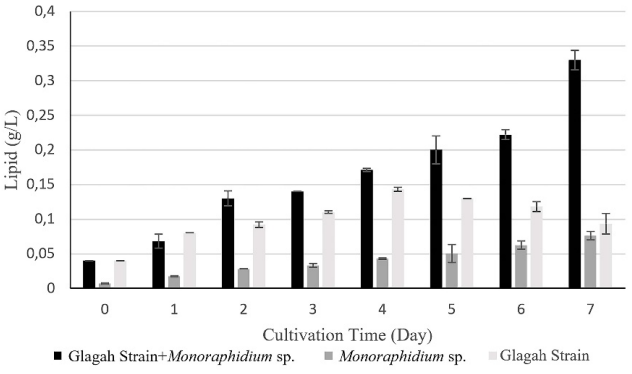


Figure 2. Total Lipid Production of mixed culture, monoculture of *Monoraphidium* sp., and Glagah Strain Consortium

Table 1. Total Lipid Production

Day	Lipid (g/g)		
	Glagah + *Monoraphidium* sp.	*Monoraphidium* sp.	Glagah Strain
0	(0.5503±0.013)[a]	(0.0152±0.0001)[b]	(0.0649±0.003)[c]
1	(0.6833±0.023)[a]	(0.0163±0.0001)[b]	(0.1247±0.004)[c]
2	(0.9761±0.033)[a]	(0.0253±0.001)[b]	(0.2465±0.028)[c]
3	(1.0318±0.049)[a]	(0.0480±0.002)[b]	(0.7727±0.064)[c]
4	(1.3500±0.132)[a]	(0.0896±0.012)[b]	(1.0633±0.089)[c]
5	(2.0000±0.000)[a]	(0.1267±0.009)[b]	(0.7386±0.016)[c]
6	(2.7021±0.082)[a]	(0.1361±0.002)[b]	(0.3494±0.013)[c]
7	(3.3809±0.067)[a]	(0.2392±0.003)[b]	(0.2757±0.073)[c]

Table 2. %Lipid of mixed culture and monoculture of *Monoraphidium* sp. and Glagah Strain Consortium

Culture	**%Lipid (crude lipid/gram biomass)**
Monoraphidium sp.	18.9
Monoraphidium sp. + Glagah Strain Consortium	17.4
Glagah Strain Consortium	16.6

Results presented in figure one revealed that the mixed culture of Glagah Strain Consortium and *Monoraphidium* sp. produced highest total lipid compared to the other two monocultures. The highest amount of lipid produced was at day seven, reaching 0.3300±0.014 g/L. The lowest total lipid produced shown by monoculture of *Monoraphidium* sp. (0.076±0.006 g/L). Meanwhile, for the crude lipid analysis, the mixed culture of *Monoraphidium* sp. and Glagah Strain Consortium was higher (17.4%) compared to the monoculture of Glagah Strain Consortium (16.6%).

Mixed cultures of *Monoraphidium* sp. and Glagah Strain Consortium were expected to produce more lipid than monocultures of *Monoraphidium* sp. only or Glagah Strain Consortium only. The high lipid production could be attributed to the ability of mixed culture, which has been shown to increase lipid production by up to 40-50%, carbohydrates by three times, and biomass production by 20-30% when compared to monoculture (Qin *et al.*, 2019; Grover *et al.*, 2020).

At day 7, the mixed culture showed three times higher lipid as compared to a monoculture of Glagah Strain Consortium (0.0933±0.015 g/L). It is above the expected amount of increase compared to available literature. Not only that, graph one also shows that monoculture experienced the decrease of lipid production at day five of treatments. Based on this data, it is possible to conclude that mixed culture treatments were able to sustain lipid production for more than seven days of cultivation. It is possible that this is because mixed cultures have been shown to increase microorganism tolerance to external stress through a synergetic relationship between species. Furthermore, mixed culture was simpler and less expensive to implement on an industrial scale than other types of modifications (Tang *et al.*, 2021).

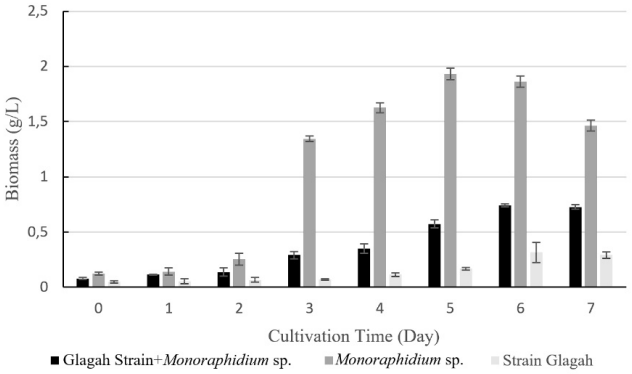


Figure 3. Total Biomass Production of mixed culture, monoculture of *Monoraphidium* sp. and Glagah Strain Consortium

Table 3. Total Biomass Production

Day	**Biomass (g/L)**		
	Glagah + *Monoraphidium* sp.	***Monoraphidium* sp.**	**Glagah Strain**
0	(0.078±0.009)[a]	(0.122±0.015)[b]	(0.050±0.010)[c]
1	(0.113±0.005)[a]	(0.140±0.033)[a]	(0.053±0.023)[b]
2	(0.137±0.037)[a]	(0.253±0.055)[b]	(0.067±0.022)[c]
3	(0.290±0.034)[a]	(1.343±0.025)[b]	(0.070±0.017)[c]
4	(0.350±0.042)[a]	(1.626±0.045)[b]	(0.113±0.017)[c]
5	(0.573±0.037)[a]	(1.930±0.052)[b]	(0.167±0.011)[c]
6	(0.747±0.015)[a]	(1.863±0.051)[b]	(0.316±0.091)[c]
7	(0.726±0.027)[a]	(1.463±0.055)[b]	(0.290±0.034)[c]

In contrast to total lipid production, total biomass, protein, and carbohydrate production in a mixed culture of *Monoraphidium* sp. and Glagah Strain Consortium was

lower than in a monoculture of *Monoraphidium* sp. but higher than in a monoculture of Glagah Strain Consortium.

In terms of biomass production, the highest biomass production was obtained by a monoculture of *Monoraphidium* sp. on day five (1.930±0.052 g/L), while the lowest biomass production was obtained by a monoculture of Glagah Strain Consortium on day 5 (0.167±0.011 g/L) (Figure 2).

Higher cell density is usually associated with higher biomass. In theory, mixed culture should have higher cell density than monoculture. The higher cell density can sometimes obstruct mass transfer within the culture (Overyielding). Because of the retained water within the biomass, the obstruction may cause slower culture growth and lower biomass production (Luo *et al.*, 2020). It did not happen, however, in a mixed culture of *Monoraphidium* sp. and Glagah Strain Consortium. If overyielding occurs, the logarithmic phase of the culture may be shorter in time. The graph shows that the growth phases of Glagah Strain Consortium's mixed culture and monoculture were nearly identical.

The increase in biomass is most likely related to the limited amount of nutrients available in culture. The Glagah Strain Consortium is made up of many different species. As a result, it is possible that only some of the species in the consortium were able to form a symbiotic relationship with *Monoraphidium* sp. This allows competition for nutrient collection among microalgae species, resulting in the accumulation of lipids and carbohydrates as the primary energy storage in microalgae. Lipid and carbohydrates account for almost 80% of microalgae biomass. Thus, the increase in the lipid, carbohydrates, and protein content will increase the amount of biomass produced. However, another species capable of symbiotic relationship with *Monoraphidium* sp. may be able to maintain the culture condition and make it more stable, as can be seen from the graph that the biomass production of Glagah Strain Consortium monoculture began to decline at day seven, while the mixed culture remained in stationary phase.

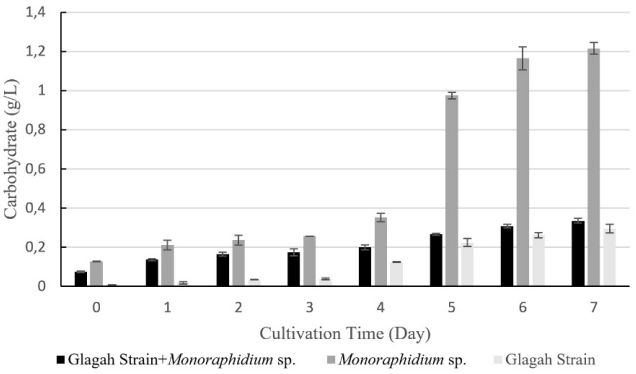


Figure 4. Total Carbohydrate Production of mixed culture, monoculture of *Monoraphidium* sp. and Glagah Strain Consortium

Table 4. Total Carbohydrate Production

Day	Carbohydrate (g/g)		
	Glagah + *Monoraphidium* sp.	*Monoraphidium* sp.	Glagah Strain
0	(0.4741±0.040)^{a}	(0.5601±0.036)^{b}	(0.0224±0.003)^{c}
1	(0.6889±0.006)^{a}	(0.7494±0.028)^{b}	(0.0238±0.00002)^{c}
2	(0.7990±0.033)^{a}	(0.8613±0.026)^{a}	(0.0656±0.014)^{b}
3	(0.9089±0.026)^{a}	(1.0511±0.004)^{b}	(0.1397±0.001)^{c}
4	(1.0493±0.002)^{a}	(1.7746±0.028)^{b}	(0.4141±0.011)^{c}
5	(1.3680±0.004)^{a}	(2.1719±0.032)^{b}	(0.6978±0.015)^{c}
6	(1.5116±0.040)^{a}	(2.3759±0.103)^{b}	(1.0620±0.028)^{c}
7	(1.8573±0.033)^{a}	(2.5161±0.087)^{b}	(1.1262±0.002)^{c}

Furthermore, carbohydrate production was estimated, and the results shown in figure three reveal that total carbohydrate production peaked on day seven for each treatment. The monoculture of *Monoraphidium* sp. produced the most carbohydrates, reaching 1.2152±0.031 g/L, followed by a mixed culture of *Monoraphidium* sp. and Glagah Strain Consortium (0.3349±0.012 g/L), and finally a monoculture of Glagah Strain Consortium (0.2952±0.022 g/L).

Carbohydrates are found in microalgae cells as storage polysaccharides as a result of the photosynthesis pathway. The storage polysaccharide can be further classified as intracellular, cellular, and extracellular (Gouda *et al.*, 2022). The culture could then be predicted to have primarily stored the carbohydrates produced and not yet used them for metabolism. This is due to the high carbohydrate content of the mixed culture. In general, mixed culture has a higher cell count than monoculture. As the number of cells increases, so will the number of storage carbohydrates.

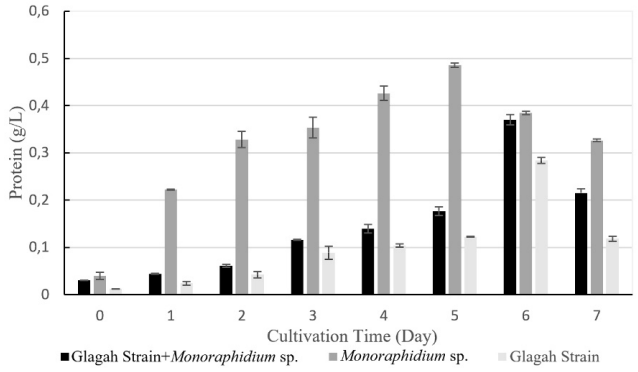


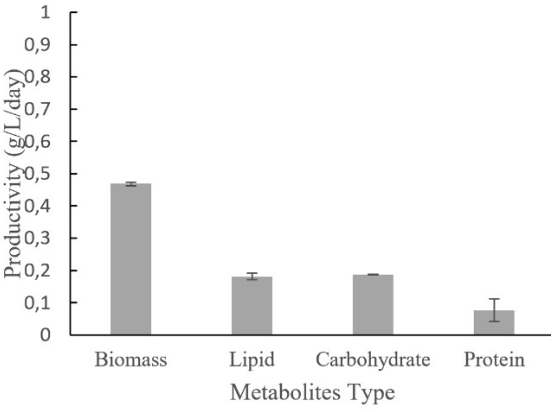
Figure 5. Total Protein Production of mixed culture, monoculture of *Monoraphidium* sp. and Glagah Strain Consortium

Table 5. Total Protein Production

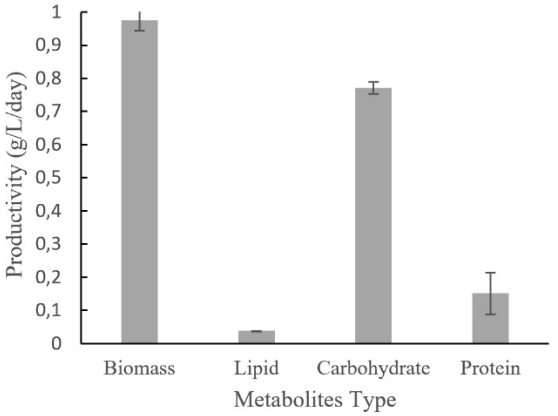
Day	Protein (g/g)		
	Glagah + *Monoraphidium* sp.	*Monoraphidium* sp.	Glagah Strain
0	(0.1200±0.052)^{a}	(0.6012±0.006)^{b}	(0.0071±0.001)^{a}
1	(0.1737±0.027)^{a}	(1.2036±0.027)^{b}	(0.0171±0.005)^{c}
2	(0.2731±0.068)^{a}	(1.5474±0.033)^{b}	(0.0656±0.009)^{c}
3	(0.3428±0.023^{a}	(2.6691±0.024)^{b}	(0.0950±0.071)^{c}
4	(0.3902±0.060^{a}	(2.9702±0.166)^{b}	(0.1213±0.015)^{a}
5	(0.4916±0.0006)^{a}	(3.7870±0.033)^{b}	(0.1642±0.064)^{c}
6	(1.6404±0.078)^{a}	(1.7860±0.019^{a}	(1.2557±0.011)^{b}
7	(0.4155±0.016)^{a}	(0.9270±0.060)^{b}	(0.1098±0.002)^{c}

Analysis of protein from culture provided in figure four shows that total protein produced by *Monoraphidium* sp. monoculture at day five is the highest compared to other treatments, reaching 0.4857±0.005 g/L. *Monoraphidium* sp. and the Glagah Strain Consortium produced the most protein, reaching 0.3701±0.011 g/L on day six. Glagah Strain Consortium monoculture also peaked on day six by 0.2842±0.006 g/L.

The higher protein content in mixed culture could be due to a higher nitrogen supply in mixed culture versus monoculture. The Glagah Strain Consortium is known to contain bacterial strains as well as microalgae. This could result in improved nitrogen fixation in the culture, both from the medium and from atmospheric gas. Nitrogen is a key component in the formation of proteins, nucleic acids, chlorophyl, and phycocyanin (Latsos *et al.*, 2020). The bacteria appear to be more active in mixed culture because there are more microalgae species that use up the nitrogen than in monoculture. The amount of nitrogen may not be higher than in monoculture, but because there are more microalgae in monoculture, the amount of nitrogen that is usually left out in monoculture can be used in mixed culture. In comparison to monoculture, this results in higher protein production. However, more research is needed to fully understand the relationship of *Monoraphidium* sp. with each species in the Glagah Strain Consortium.



(A)



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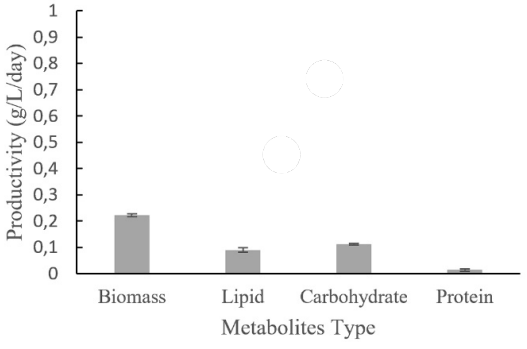

(C)

Figure 6. Productivity of mixed culture (A) and monoculture of *Monoraphidium* sp. (B) and Glagah Strain Consortium (C)

Productivity is closely related to total production in each culture. The higher the productivity of each culture, the better the culture for industrial applications. Figure five shows that the productivity of lipid, protein, carbohydrates, and biomass for each treatment was higher in mixed culture of Glagah Strain Consortium and *Monoraphidium* sp. than in monoculture of Glagah Strain Consortium. Mixed culture of *Monoraphidium* sp. and Glagah Strain Consortium has the highest lipid productivity (0.1808±0.010 g/L/day), while monoculture of *Monoraphidium* sp. has the highest biomass, carbohydrates, and protein productivity (0.9750±0.031 g/L/day; 0.7710±0.018 g/L/day; 0.1514±0.062 g/L/day respectively). When compared to monoculture of Glagah Strain Consortium, mixed culture has a 70% increase in carbohydrates productivity. Meanwhile, when compared to Glagah Strain Consortium monoculture, biomass productivity in mixed culture increases up to two-fold. Thus, it is clear that mixed culture has the potential to be used on a larger scale, though further improvements are required. Mixed culture with *Monoraphidium* sp. is especially suitable for products requiring high protein content, as it can be increased nearly seven-fold in the mixed culture of *Monoraphidium* sp. and Glagah strain Consortium when compared to monoculture of Glagah Strain Consortium.

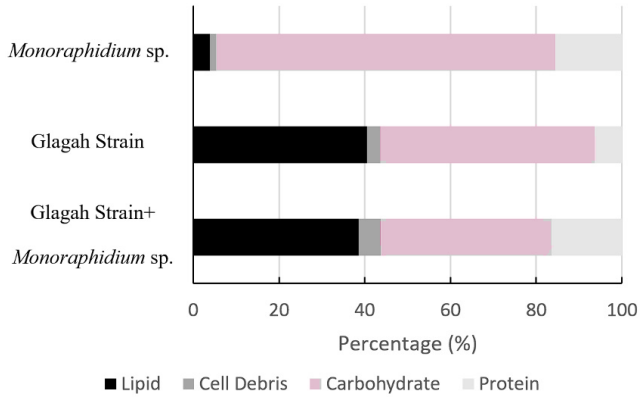

Figure 7. Biomass composition of mixed culture and monoculture of *Monoraphidium* sp. and Glagah Strain Consortium

Further analysis of biomass content was performed to determine the amount of metabolites that are dominant in microalgae cells, as well as the culture's condition. Figure 6 depicts the biomass content of all treatments, which is primarily made up of carbohydrates. The biomass of a mixed culture of *Monoraphidium* sp. and Glagah Strain Consortium was composed of 40% carbohydrates, 39% lipid, 16% protein, and 5% cell debris. When compared to other treatments, *Monoraphidium* sp. has the highest carbohydrate content (79%). It has only 16% protein and 4% lipid. Monoculture of Glagah Strain Consortium has the highest lipid percentage (41%) compared to other treatments. Carbohydrates account for 50% of biomass composition in the monoculture of Glagah Strain Consortium, while the rest are for protein (6%) and cell debris (9%). When compared to Glagah Strain Consortium monoculture, the protein percentage in mixed culture (16%) is higher, while the amount of lipid and carbohydrate per biomass is higher in Glagah Strain Consortium monoculture. The three treatments show an abundance of carbohydrates in comparison to other metabolites. One reason could be the use of wet biomass in this study; it was possible that the EPS produced by microalgae was included in the carbohydrate assay. EPS (Extracellular Polymeric Substances) is a macromolecule with a high molecular polymer that is excreted outside the cells of microorganisms and is primarily composed of polysaccharides (Babiak & Krzemińska, 2021). One of the unique properties of EPS is its ability to protect microalgae cells from a variety of toxic substances (Li *et al.*, 2021)

IV. CONCLUSION

This study concluded that combining Glagah Strain Consortium with *Monoraphidium* sp. increased productivity and total protein, lipid, carbohydrate, and biomass production when compared to monoculture of Glagah Strain Consortium. However, the results were still inferior to *Monoraphidium* sp. monoculture. Therefore, several modifications, such as changes in pH, light intensity, light quality, medium nutrient content, and temperature, may be required to improve the results.

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VI. REFERENCES

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