Improving Production, Chlorophyll a and Carotenoids Contents of *Gracilaria* sp. with Liquid Organic Fertilizer from Alginate Waste

Ervia Yudiati[1,2]*, Ali Djunaedi[1], Dea Shinta Kharisma Adziana[1],Ayunda Ainun Nisa[1], Rabia Alghazeer[3]

[1]Department of Marine Science, Faculty of Fisheries and Marine Science, Diponegoro University
[2]Laboratory of Biology, Faculty of Fisheries and Marine Science, Diponegoro University
Jl. Prof. Sudarto, SH, Tembalang, Semarang, Jawa Tengah, Indonesia 50275
[3]Department of Chemistry, Faculty of Sciences, University of Tripoli, Libya
Tariq Sayyidi al Misri Road, Tripoli, Libya
Email: erviayudiati@lecturer.undip.ac.id

Abstract

Chlorophyll is a photosynthetic pigment used in the food sector as a natural dye in food. Carotenoids is used in the health sector to prevent several diseases in humans. The production and pigment contents are influenced by nutrient availability. The aim of this study was to increase the production of chlorophyll-a and carotenoids contents in Gracilaria sp. Seaweed obtained from Demak aquaculture ponds, then cultivated in aquarium for 28 days. Alginate from Sargassum sp. waste fertilizer was prepared by adding some compounds, fermented by commercial Saccharomyces cerevisiae. Five different treatments (FB (basal formulation: 75 g alginate waste + 22.5mL molasses + 7.5 g S. cerevisiae + 250 mL aquadest)), FBL (basal formulation+100% Lamtoro leaves), FBLU (basal formulation+50%Lamtoro leaves+50% carapace shrimp waste), FBU (Basal formulation + 100% carapace shrimp waste) and control-without fertilizer) was applied. Analysis of pigments was determined using the spectrophotometric method. The research design was CRD with 4 treatments and a control. Data were analyzed using Kruskal-Wallis statistical analysis. The result showed that fertilization in culture media could increase the production (DW) and pigment contents. The highest levels of dry weight, chlorophyll a and carotenoids ($P<0.05$) were resulted from FBLU ie. 6.58 ± 0.07g dry weight; 5.47 $mg.L^{-1}$ and 0.16 $\mu mol.L^{-1}$.The application of organic fertilizer from alginate extract waste to Gracilaria sp. culture media had a significant effect towards growth, dry weight, chlorophyll a and carotenoids pigments content. This inexpensive fertilizer expected to be the solution of green and zero waste management which provide the enviromentally friendly fertilizer.

Keywords: *Alginate, Carotenoids, Chlorophyll a, Fertilizer, Gracilaria* sp.,

Introduction

The rise in consumer need for high value food products had led to development in the use of innovative technologies and components to produce functional foods (Chan and Matanjun, 2017), cosmetic (chlorophyll and carotenoids), pharmaceutical, and nutraceutical industries Chlorophyll and carotenoids may help protect against reactive oxygen species and free radicals, producing compounds that can act in cosmetics against the harmful effects of UV radiation (Ariede *et al*., 2017). Chlorophyll constitutes unique pigment with green color and can be found in diverse plants, algae, and cyanobacteria (Pareek *et al*., 2018). Chlorophyll is found in almost every part of green plants, which are leaves, stems and it is found highest in chloroplast organelles. Besides, another pigment that exist in plants is carotenoids. Carotenoids is a photosynthetic pigment included in a terpenoid compound and has a yellow-red-ishcolor (Maoka, 2019). Carotenoids, particularly b-carotene isfavoredby the market of natural products, asit consistsof trans and cis isomers that may have anticancer activity (Chan *et al*., 2017) and also useful for preventing cardiovascular disease (Babadi *et al*., 2020).

Several information concerning the nutrient composition of *Sargassum* sp for liquid fertilizer had been reported (Basmal *et al*., 2019) and applied for mustard greens (*Brassica*sp.) (Dewi *et al*., 2019). Some studies have also reported from other seaweed (*Ulvalactuca*) (Suryaningrum and Samsudin, 2020) on mung bean seedlings (Castellanos-Barriga, 2017), as well as *Enteromorpha* sp. (Yousif *et al*., 2004). Fertilizer from seaweed is non-toxic compounds and naturally degraded, so, therefore, environmentally friendly (Hernandez *et al*., 2016). Up to now, the application of alginate waste extracted alginate is very limited, particularly with some relevant application in *Gracilaria* sp. culture.

Pigment synthesis is influenced by environmental factors, particularly nutrients such as nitrates and phosphates. Nutrients in the photosynthesis process play a role in the formation of chlorophyll so that the increase of nutrient content in

*) Corresponding author

https://ejournal.undip.ac.id/index.php/ijms
DOI: 10.14710/ik.ijms.26.1.1-6
Received : 30-11-2020
Accepted : 27-02-2021

the water will increase the production of chlorophyll a (Yudiati *et al*., 2020). Therefore, in controlled culture media, many nutrient enrichment technologies have been carried out to increase the growth and production of macroalgae pigments. Media enrichment can be formed as fertilizer.

Based on the facts approaching organic fertilizer, in related to the utilization of waste materials and the contribution on *Gracilaria* sp. production for human needs and health, this research needs to be fulfilled. The aim of this research was to increase the biomass production, chlorophyll a and carotenoids content in *Gracilaria* sp. with alginate waste liquid fertilizer. The inexpensive organic fertilizer from alginate waste is expected to be the solution of green and zero waste management, inexpensive fertilizer as well as environmentally friendly materials.

Materials and Methods

The composition of alginate waste fertilizer refers to Dewi *et al*. (2019) with modifications. Basically, 75 g of dry alginate waste from *Sargassum* sp. was added with 250 mL sterile water, heat up at 90^oC for 15 mins. Leave it for normal temperature and added with 22.5 mL of molasse and 7.5 g commercial *Saccharomyces cerevisiae.* After 14 days, the fermentation was stopped and the liquid fertilizers were then harvested.

The fertilizer was prepared in four treatments (FB, FBL, FBLU, and FBU). FB was basic formulation (alginate waste + molasse + *S. cerevisiae* + aquadest). FBL was basic formulation + 100% lamtoro leaves. FBLUwas basic formulation + 50% lamtoro leaves + 50% shrimp carapace waste. FBU was basic formulation +100% shrimp carapace waste. All alginate waste, lamtoro (*Leucaena leucocephala)* leaves, commercial *S. cerevisiae*, and shrimp carapace waste were in dry weight base.

The nutrient content was analyzed by AOAC (2005). Nitrogen total was determined by Kjedahl method by three main steps namely destruction, distillation and titration. Phosphate content was analyzed by destruction methods, using 0.5 g in 5 mL HNO_3 65% and 0.5 mL $HClO_4$ in Kjeldahl apparatus. After boiling up for 1 hr at 100^oC, the temperature was raised to 150^oC and 200^oC, at last. The sample was cooled down and 50 mL aquadest was added. The extract (1 mL) was pipetted into a 10 mL volume flask added 1 drop of PP indicator and 40% NaOH to arise pink color. This then followed by adding 2 mL of reagent vanadate into the flask and diluted. Leave it for 20 mins and measured by spectrophotometer (400 nm). The assessment of Potassium content was measured using the Flame Photometry method. The microminerals analysis used AAS-flame method. This has a 0.01 ppm limit of detection.

In this study, the application of fertiliser was Gracilaria sp, which obtained from the cultivation from Karangtengah pond, Demak. *Gracilaria* sp. acclimatized for 7 days. Fifty grams of seaweed were reared in the 15 aquariums (25 cm x 25 cm x 25 cm). Each aquarium was equipped with aeration to allow air diffusion and nutrient mixing. The media used was seawater with 25 ppt salinity. Each aquarium was filled with 10L of filtered seawater. The maintenance media was added with liquid organic fertilizer at 1 ppm. Four treatments of media fertilizer (FB, FBL, FBLU, FBU) and one control (seawater only without any fertilizer) was applied and replicated in three times.

The *Gracilaria* sp. dry weight were measured every week. The harvested *Gracilaria* sp. were dried for five days at room temperature to avoid direct sunlight. The pigment content of chlorophyll a and carotenoids were analyzed spectrophotometrically at the end of the experiment (28 days of rearing). The 0.5 g of dry *Gracilaria* sp. was weighed, crushed and extracted with 5 mL acetone and macerated for 24 hours. The extract was filtered with filter paper. The filtrate were then placed in a cuvette. The contents of chlorophyll a and carotenoids were measured using spectrophotometer at 480 nm, 645 nm and 663 nm wavelengths. Chlorophyll-a and carotenoids were calculated using the following formula (Gross, 1991):

$$\text{Chlorophyll a} = 12.7x\ A_{645} - 2.69\ x\ A_{663}$$

$$\text{Carotenoids } (\mu mol/L) = \frac{(A480+(0.114\ x\ A663))-(0.638\ x\ A645\ x\ V x1000)}{112.5\ x\ W}$$

Data in tables and figures are expressed as mean±SD. Analysis of variance was performed non-parametrically (Kruskal Wallis test). Mann Whitney analysis was used to determine differences between sampletreatments. $P<0.05$ were considered as significantly different.

Result and Discussion

Nutrients available in each alginate waste fertilizer is presented inTable 1.The FBU reached the highest Nitrogen total but lowest Phosphate and Potassium, and microminerals (Mn, Zn and Fe) content. On the other hand, the FBLU have the smaller Nitrogen total compare to FBU, but was leading in Phosphate and Potassium, and microminerals (Mn, Zn and Fe) content. The growth of *Gracilaria* sp. and final dry weight biomass in each treatment is shown in Figure 1 and Table 1. *Gracilaria* sp. maintained a positive growth during the period of rearing for entirely thalli grown at the five different treatments. The highest growth and biomass was obtained from FBLU treatment.

The dry weight results confirmed the growth and dry weight biomass. This strengthened that FBLU was the best treatment. This due to the macronutrients and micronutrients compounds obtained from alginate waste fertilizer. Nutrient content obtained from fertilizer is one of the factors that influence *Gracilaria* sp. growth. The fertilizer used in this study was a combination of alginate, lamtoro leaves and shrimp waste. The more diverse and balanced macro and micronutrient obtained from alginate fertilizer was able to improve the production of *Grcilaria* sp., though the Nitrogen of FBLU was less than FBU treatment. This finding was in agreement with research by Dewi *et al*. (2019) in Chinese cabbage. The combination of raw materials reached the leaves count but not in root. The root growth of may be influenced by N content (Ji *et al*., 2017; Dewi *et al*., 2019). The combination of seaweed *Ulvarigida* and *Fucusspiralis* was found to have greatestinfluencer on growth parameters of Bean plant (Latique *at al*., 2013). The total nitrogen combination of lamtoro leaves and shrimp carapace waste in our present research (0.54%) was higher than extract shrimp fertilizer from Ji *et al*. (2017). Similar to our present research, high percentages of macroalgae extract in medium culture (75% and 100% of *Ulvarigida*; 100% of *Fucusspiralis*) were found to demonstrate negative effect. Mamun *et al*. (2020) is strengthened our findings. From the empirical model analysis, they indicated that Total Phosphate is the key regulating factor for algal growth, rather than Total Nitrogen. The phosphate role is transferring high energy such as ATP (adenosine triphosphate) in the respiration and photosynthesis process. Chlorophyll synthesis needs ATP and NADPH (Nicotinamide adenine dinucleotide) and this need adequate phosphate (Lobban and Harrison, 1997). Hence, it suggested that providing the right and various fertilizer concentrations can support growth and dry weight of *Gracilaria* sp. while excessive amount of nutrients will inhibit the growth of *Gracilaria* sp.

The addition of fertilizer in culture media also significantly affected the chlorophyll and carotenoid content of *Gracilaria* sp. after cultivation. Chlorophyll *a* (5.51 $\mu g.g^{-1}$) and carotenoids (0.16 $\mu mole.L^{-1}$) in the FBLU treatment was the highest compared to other treatments, as depicted from Figure 2 a and b.

Fertilization in culture media has managed to increase the chlorophyll content of *Gracilaria* sp. compared to culture without fertilization. Chlorophyll-a is an important part of the photosynthesis process in algae. The main components of chlorophyll are N and Mg. Chlorophyll is the compound in conjunction with free electrons, structured from N atoms (Sedjati *et al*., 2020). Nitrogen is needed as the basic material for building and forming chlorophyll in the photosynthesis process. FBLU treatments have the highest Mg mineral (54.67 $mg.100\ g^{-1}$) and other microminerals. Mg plays a role in capturing light in photosynthesis. Furthermore, the formation of chlorophyll-a is also influenced by nutrients. Ismail and Osman (2016); Yudiati *et al*. (2020) stated that nutrients influence the growth and development of seaweed in nitrate and phosphate. This is confirmed by Ahmad *et al*. (2011) that if the N content in water increases, the chlorophyll contained by aquatic plants also increases. Nitrate and phosphate are needed in the protein preparation and chlorophyll formation during

Table1. The Macro and Microminerals Nutrient Analysis ondifferent Treatment of Alginate Waste Liquid Organic Fertilizer

Parameter	Treatment			
	FB	FBL	FBLU	FBU
N-Total (%)	0.13±0.002	0.13±0.005	0.33±0.002	0.54±0.003
P_2O_5(%)	0.08±0.002	0.05±0.002	0.11±0.007	0.05±0.002
K_2O (%)	0.29±0.009	0.32±0.008	0.35±0.008	0.29±0.007
Mn ($mg.kg^{-1}$)	5.37 ± 0.04	6.63 ± 0.26	6.81 ± 0.35	5.14 ± 0.10
Mg($mg.100g^{-1}$)	29.57 ± 0.52	36.24 ± 1.11	54.67 ± 1,4	54.94 ± 2.39
Zn ($mg.100g^{-1}$)	0.76 ± 0.01	0.81 ± 1.12	0.85 ± 0,03	0.59 ± 0.02
Fe ($mg.100g^{-1}$)	4.58 ± 0.07	2.77 ± 0.13	3.16 ± 0,15	2.26 ± 0.04

Table 2. The Result of Average Dry Weight Measurement of *Gracilaria*sp. in each Treatment. Different superscript indicates significantly difference (P<0.05)

Parameter	Treatments				
	C	FB	FBL	FBLU	FBU
Dry Weight (g)	6.10 ±0.25[a]	6.21 ± 0.14[a]	6.49 ± 0.08[ab]	6.58 ± 0.07[b]	5.88 ±0.40[a]

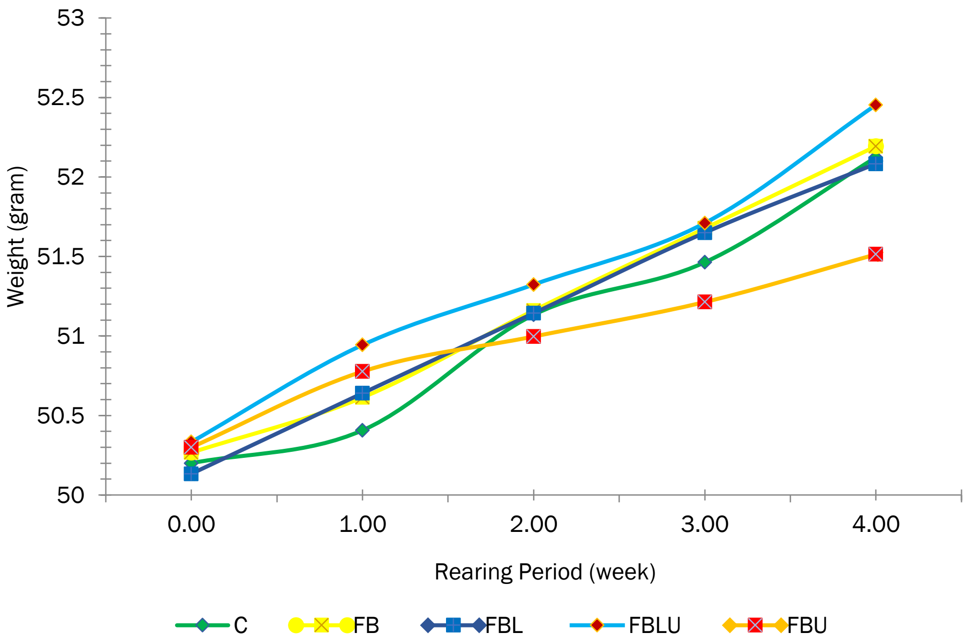


Figure 1. Growth of *Gracilaria* sp. with different media fertilizer reared in every week

photosynthesis. Results form this study shows that the highest N content was reached from FBU treatment. Though the N content of FBLU was lower than FBU treatment, the highest phosphate content was reached from FBLU treatment. According to our results, research from Ji *et al*. (2017)stated that the N content was in a dose dependant manner. FBL treatment revealed that a sufficient and balanced amount of nitrate and phosphate are fulfilled.

Chlorophyll, the predominant constituent present in green plants and algae (Sakthivel and Devi, 2014). As happened in the FBU treatment, the FBU fertilizer had the highest N content (0.54%), but the FBU treatment had a lower growth rate and chlorophyll content. Algae as filter plants can reduce nutrients which present in their growth media (Briggs and Funge-Smith, 1993). Briggs and Funge-Smith(1993) also reported that all the nutrients in the water are absorbed, so there is an excess of nutrients which causes the inhibition of growth and formation of chlorophyll. The content of chlorophyll a from the North Java coast, Indonesian our study was lower (5.51 μg.g^{-1}) compared to the research from Thondi Coast, India (8.96 μg.g^{-1}) (Rosemary *et al*., 2019). This may due to the small volume of culture, short periods of culture and the fertilizer dose (1 ppm). Moreover, seaweed has a nutrient composition that differs significantly depending on environmental conditions, species, geography, season and harvest age and (Denis *et al*., 2010). Even though, our chlorophyll-a was higher than *G. edulis* 3.06 μg μg.g^{-1} from Palk Bay (Sakthivel and Devi., 2014).

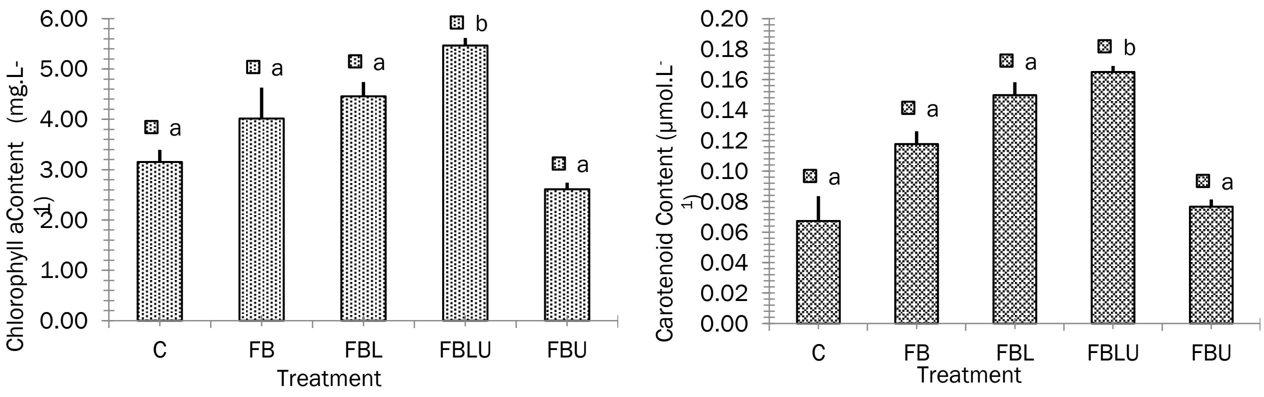
Carotenoids constitute tetraterpene pigments that display orange, red, yellow and purple colours. These are crucial pigments in photosynthetic organs alongside chlorophylls (Maoka, 2019). Carotenoids helps chlorophyll for absorbing light energy. The conjugated double bonds in carotenoids make this pigment high chemical reactivity easily isomerized and oxidized (Mezzomo and Ferreira, 2016). Carotenoids is influenced by several factors, including the availability ofN and K nutrient elements. Fertilization with elements of N and K is able to produce carotenoids that are higher than other fertilizer variations. According to the Mamun *et al*. (2020), the C: N: P: Mg ratio plays a role in the formation of carotenoids. Carotenoids levels in each treatment had a linear relationship with nutrient content in fertilizers. The best combination of alginate extraction waste fertilizer towards carotenoids content was FBLU fertilizer

Carotenoids levels in all treatments had a lower value (0.07–0.16 μmol.L^{-1}) compare to our previous findings (Yudiati *et al*., 2020) from *Gracillaria* sp.'s natural habitat (1.12–7.39 μmol.L^{-1}). This is presumably because *Gracilaria* sp. from natural habitat produced higher carotenoids content due to the supported environment, nutrients availability, photoperiodicity etc. (Denis *et al*., 2010).

The water quality in media during the rearing period (Table 3.) showed that all parameters (salinity, temperature, pH and DO) was in accordance to SNI

Table 3. Water Quality Measurements during 28 days of *Gracilaria* sp. Culture

Parameter	Measurement	SNI 7904:2013	Description
pH	8.3	6.5-8.5	Suitable
Temperature (°C)	25.9	25-30	Suitable
Salinity (ppt)	25.8	15-30	Suitable
Dissolved Oxygen (mg.L^{-1})	6.3	>5	Suitable



(a) (b)

Figure 2. **a)** Chlorophyll a content of *Gracilaria* sp. in different media, reared for 28 days. **b)** Carotenoids Content of *Gracilaria* sp. In different media, reared for 28 days. Different superscript indicates the significantly difference (*P*<0.05).

7904:2013. Measurements of water quality parameters such as salinity (Atago Refractometer), pH (ATC-2011, USA), dissolve oxygen and water temperature (Water Quality Checker "Amstast") have conducted weekly. The range of water quality parameters was in the optimal range to support the growth of *Gracilaria* sp.

Conclusion

The application of organic liquid fertilizer from alginate extract waste to *Gracilaria* sp. culture media had a significant effect on growth, dry weight and also affect its pigment composition of chlorophyll a and carotenoids. The best combination of alginate waste fertilizer to influence the growth of *Gracilaria* sp. is FBLU treatment, which is the basic alginate waste fertilizer with the addition of 50% lamtoro leaves and 50% shrimp waste

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