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The Agar Production, Pigment and Nutrient Content in *Gracilaria* sp. Grown in Two Habitats with Varying Salinity and Nutrient Levels

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Abstract

Gracilaria sp. is rich in agar, pigment, carbohydrate and mineral contents. This study aims to determine the content of agar, chlorophyll a, carotenoids and nutrient content (protein, carbohydrate, total lipid, ash, water) in Gracilaria sp. grow in different habitats with high and low salinity. Samples were brought from high salinity (44±0.33) ppt from the reservoir habitat and low salinity (26.98±0.15 ppt) from biofilter's shrimp waste pond habitat. Salinity and water quality parameteres were observed in three different subsites. Agar was extracted by alkali methods. Agar spectra were compared to standard agarose and characterized by FT-IR analysis. Results showed that agar content (25.79 ± 0.28%), chlorophyll a (25.79 ± 0.28 mg/g), carotenoid (5.90 ± 0.07 µmol/g) on low salinity was significantly higher ($P<0.05$) than high salinity. In high salinity, the agar (6.6±0.34%), chlorophyl a (3.62±0.15, carotenoids (0.71±0.1 µmol/g) contents was lower. The protein level of low salinity (14.4 ± 0.70%) was also significantly higher compared to high salinity (8.15±0.25%), respectively. The total lipid, carbohydrate, water, and ash content were similar. FT-IR analysis spectra show the presence of 3,6-anhydro-L-galactose. These can be useful data concerning optimum salinity in Gracilaria sp. culture.

Keywords: Salinity, Gracilaria sp., Agar, Pigment, Nutrient content

1. Introduction

Naturally, *Gracilaria* sp. is a euryhaline macroalga (Kumar *et al*., 2010), has grown all over the coast with high economic value due to its nutrient content (Du *et al*., 2016; Hernandez, 2017). *Gracilaria*/*Gracilariopsis* have been mostly cultivated in Asia, especially Indonesia and China. The production is about 98% of global production (FAO, 2016; Kim *et al*., 2017). Asian people consume seaweed daily (Cikos *et al*., 2018). Seaweed empirically improves health and able to reduce the chronic disease incidence such as cancer, cardiovascular and heart diseases (Rioux *et al*., 2017; Xu *et al*., 2017). *Gracilaria* sp. serves as an excellent source of polysaccharide, i.e. agar (Xu *et al*., 2017) also rich in pigmented antioxidants such as chlorophyll and carotenoids (Stengel *et al*., 2015; Asih *et al*., 2019). Polysaccharides from seaweed are frequently related to pharmacological activities (Hamed *et al*., 2015) such as anticoagulant, antioxidant (Yudiati *et al*., 2018a; 2018b), antitumor, and immunomodulatory of shrimp (*Litopenaeus vannamei)* (Yudiati *et al*., 2016, 2019) as well as Zebrafish (Yudiati *et al*., 2020)

Common salinity at sea in marine waters is around 35 ppt. Precipitation or freshwater influxes, as in reservoir and mangrove areas, may lead to salinity variation from 10 to 70 (Graham & Wilcox, 2000; Kumar *et al*., 2010). Some former researchers have reported the response of estuarine macroalgae to the abiotic factors such as salinity light, pH, temperature, nutrient load (Kumar *et al*., 2010; Choi *et al*., 2010) associated to agar yield (Israel *et al*., 1999) and photosynthetic performance (Phooprong *et al*., 2007). Later studies have also discussed the possible effects of environmental stresses on seaweed extracts according to its kinetic parameters (Deyab, 2016).

Salinity has been displayed to the reason of osmotic (Kumar *et al*., 2010) as well as turgor pressure regulation (Pereiera *et al*., 2017). Salinity will improve the upregulation and accumulation of the essential enzymes (Odat, 2018). The seaweed tolerance to high salinity is supported by the internal and external osmotic capacities and on elasticity of the cell wall (Wu *et al*., 2018). When the cell is located in hypertonic solution, water will flow quickly out of the cell, and turgor pressure will be decreased affecting plasmolysis, which is commonly permanent. If the cell is located in hypotonic solution, water will go into the cell, causing an enlarge of cell volume. Salinity in plants also stimulates the ROS generation which produces cellular oxidative damage when overproduced in large amounts (Luis *et al*., 2018). These mechanisms play a role in combating the accumulation of reactive oxygen species (ROS) by a diverse set of enzymes such the superoxide dismutase (SOD), which dismutase the O_2-radicals to H_2O_2(Luis *et*

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al., 2018). *Gracilaria* sp. is abundant in chlorophyll a and carotenoids. The pigment important role is to neutralize the free radicals (Sedjati *et al*., 2020). In some periods of time, these mechanisms influence the agar, pigment as well as protein and mineral content caused by salinity stress. The objective of this study is to determine the agar, pigment (chlorophyll a and carotenoids) and nutrient content (water, protein, total lipid, ash, carbohydrate) from low (reservoir) and high (biofilter habitats) salinity of *Gracilaria* sp.

2. Materials and Methods

2.1. Water Quality Parameters

Measurements of water quality parameters such as salinity (Atago Refractometer), pH (pH meter RoHS), dissolved oxygen and water temperature (Water Quality Checker "Amstast") nitrate and phosphate content was administered. Assessment on nitrate content (SNI-06-2480-1991) was applied spectrophotometrically (Shimadzu 1900) while the phosphate content was done by Badan Standarisasi Nasional methods (SNI-06-2412-1991).

2.2. Site Location and Sample Preparation

Sample was collected from low salinity/LS (reservoir) and shrimp (*Litopenaeus vannamei*) pond waste (biofilter), which represents high salinity/HS of Center of Brackishwater and Aquaculture Development Center, Jepara, Central Java, Indonesia (Figure 1). Cordinates of sampling sites in HS was S 06°35'12.74" E 110°39'10.32" while in LS was S 06°35'05.64" E 110°38'49.03". The salinity of the reservoir from this study tends to be mild-low salinity. In contrast, biofilter shallow waters illustrate the different condition. The water supply of biofilter came from shrimp (*L. vannamei*) pond waste from uneaten feed and shrimp's fecal. Due to the static and unchanged water supply, biofilter media was relatively high in salinity.

The water and *Gracilaria* sp. sample was taken at three different subsites from two different habitats at 800 cm in depth. Seaweed was taken randomly. Soon as arrived at the laboratory, samples were rinsed with tap water to clean up from debris and salt. This was followed by dried up samples indoor with room temperature. Dried *Gracilaria* sp. was cut off to 0.5 cm and then stored in a clean and dry pack and sealed with aluminium foil

Figure 1. Site location of *Gracilaria* sp. samples (reservoir and shrimp pond biofilter).

2.3. Pigment Extraction

Pigment extraction was administered by single extraction. Ethyl acetate was used (1:10) to extract the dried seaweed for 24 hr maceration at room temperature (Hidayati *et al*., 2019). Whatman no. 41 was used to sieve the extract. The extract was then concentrated with a rotary evaporator (Bucchi) and stored in a refrigerator and ready to use. Percentage of yield was calculated using the formula:

Yield (%) = weight of extract (g)/weight of seaweed x 100%

2.4. Determination on Chlorophyll a and Caroteniods Contents

Chlorophyll a and carotenoids (Dere, 1993; Harborne, 1984) content were done spectrophotometrically (Shimadzu 1800). Five g of extract was diluted with acetone p.a. (1:1) The absorbance of samples was recorded at 645 nm, 663 nm dan 470 nm wavelength. The chlorophyll a and carotenoids content was determined based on this formulation

Chlorophyll a µg/g sample (Ca) = 12.21 × A663 – 2.81× A646

Carotenoids µmol/g sample (Cx+c) =

$$\frac{A470 + 0.114xA663 - 0.638xA646\,)xVx1000}{112.5x0.1x10}$$

2.5. Agar extraction

Agar extraction methods were basically done by Jayasinghe *et al*., (2016) with some modifications. Fifty grams of dried seaweed was extracted for 1.5 hr in hot water (85°C) added with 750 mL NaOH 5%. The extract was then rinsed with continuous tap water for discoloration. This was followed by aquadest 750 mL and homogenized. Acetic acid (CH_3COOH) was then added, stirred and boiled at ± 90-95°C for two hrs. The agar was then sieved, dropped with KCl 6 g, homogenized and poured into the container. Finally, 18 hrs later, the gel was performed and yield (%) was counted.

2.6. Nutrient Content

The proximate analysis was referred to as the Association of Official Analytical Chemists (AOAC, 2005) and conducted to 5 g samples.

2.6.1. Water content

Sample was put into the oven (105°C) for three hrs. Dried samples were then placed into dessicator until the weight was constant.

Water content (%) = $\frac{a-b}{c}$ x 100%

a = cup + dried sample
b = cup without sample
c = initial samole weight

2.6.2. Protein Analysis

Protein analysis was done by Kjeldahl methods, by located samples into Kjeldahl vial and destructed using 20 mL of hot sulfuric acid. The process was continued until samples were colorless and clear. This was then diluted and distilled with 10 mL of NaOH 10%. The distillate was then put into 25 mL of 3% H_3BO_3 and titered with HCl standard using methyl red as an indicator. The volume of titrants was used to calculate the percentage of total

nitrogen. The protein level was counted by multiplied the total nitrogen and correction factor.

Total nitrogen % = mL HCl x NHCl/sample weight x 14008 x f

Total protein % = total nitrogen x 6.25

denoted : f = correction factor (6.25).

2.6.3. Total Lipid Content

Total lipid analysis was done by soxhlet methods. Dried samples were wrapped with cotton wool. Sample was then placed into soxhlet extractor, using diethyl ether as solvent. Reflux was conducted by the samples until done. Vial was then put into the oven (105°C) and weight. The percentage of total lipid was determined by this formula:

Total lipid (%)= Lipid weight/Sample weight x 100%

2.6.4. Ash Content

Sample was weighed and burned at the top of bunsen until smoke produced and put in the muffle furnace at 500 - 600°C until turned to ash formation. Cup was cooled down and weighed. The ash content was counted using formula:

Ash content (%) = ash weight/sample weight x 100%

2.6.5. Total Carbohydrate

Total carbohydrate was counted simply by this equation:

Total carbohydrat (%) = 100% - (% ash + % water + % protein + % total lipid).

2.6.6. Characterization of Agar

Agar characterization was determined by Fourier Transform Infrared (FT-IR) spectroscopy (Thermo Nicolet 380 FTIR, Germany). Samples were mixed with KBr pellets (10% w/w). Similar to alginate, the pellet was recorded at 4000-500 cm^{-1} (Yudiati and Isnansetyo, 2017).

3. Statistical Data Analysis

All data surveys were analyzed non parametrically (Mann Whitney test) using SPSS version 20.0 computer software. The laboratory data were analyzed using One Way ANOVA (Analysis of variance) using the same software, with a 95% level of significance.

4. Results

4.1. Water Quality Parameters

Salinity, nitrate and phosphate contents and others water quality parameter LS and HS with three subsites are shown in Table 1. There was a differences in salinity, nitrate and phosphate contents, while other parameters were similar.

Tab1e 1. Water quality parameters of *Gracilaria* sp. grown in low salinity (reservoir) and high salinity (pond waste biofilter)

Parameter	LS	HS
Temperature (°C)	29.74 ± 0.15[a]	29.70 ± 0.03[a]
Salinity (ppt)	26.89 ± 3.15[a]	44.00 ± 0.33[b]
pH	8.0 ± 0[a]	8.3 ± 0[a]
Dissolve Oxygen (ppm)	3.55 ± 0,72[a]	3.45 ± 0,05[a]
Nitrate (mg/L)	0.43 ± 0.56[b]	1.77 ± 1.44[a]
Phosphate (mg/L)	0.78 ± 0.26[b]	0.29 ± 0.19[a]

4.2. Yield of pigment extraction, chlorophyll a and carotenoids content

Yield, chlorophyll a and carotenoids content in reservoir and shrimp pond bioflilter is shown in Figure 1. Yield were similar, chlorophyll a and carotenoids of *Gracillaria* sp. sample in reservoir was higher.

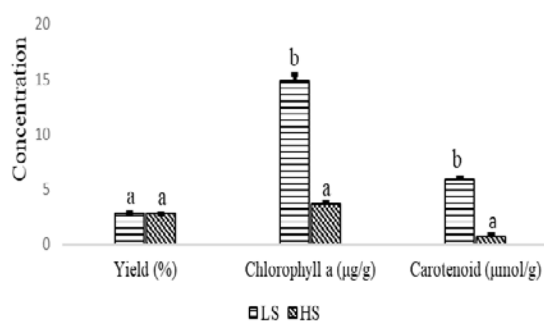

Figure 2. Yield, Chlorophyll and Carotenoids Content of *Gracilaria* sp. grown in high and low salinity. Different superscript indicates a significantly difference (p<0.05).

4.3. Agar Percentage and Characterization

Percentantion of agar in *Gracillaria* sp. samples from LS and HS is shown at Table 2. Agar characterization of sample and standard agarose (Merck, USA) is depicted in Figure 3a, b and Table 3. Concentration of agar sample from LS habitat was significantly different compared to HS (p<0.05). On the other hand, pairwise comparison spectra of agar either from low or high salinity were fit to the standard.

Table 2. Percentage agar yield of *Gracilaria* sp. samples from low salinity (reservoir) and high salinity (biofilter)

Habitat	Agar (% w/w)
LS	25.79 ± 0.28
HS	6.60 ± 0.34

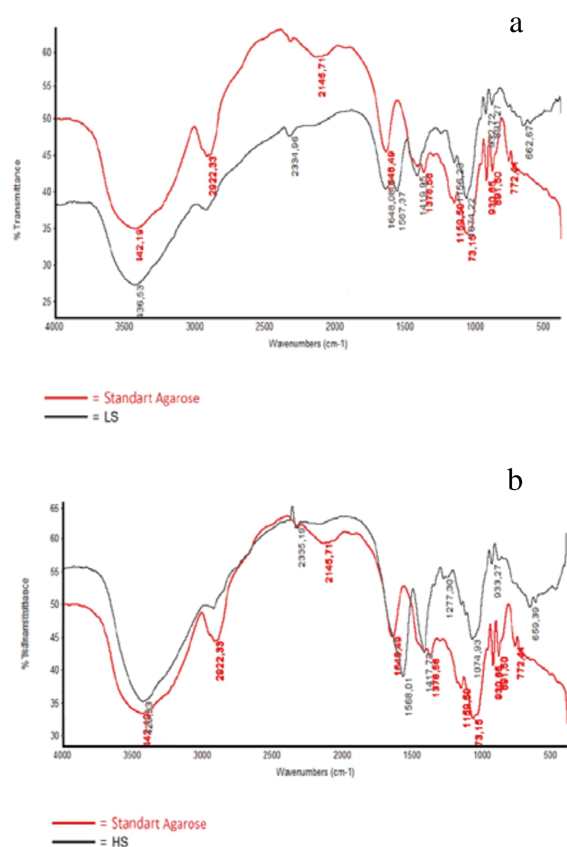

Figure 3. FT-IR spectra of agar from *Gracilaria* sp. from low salinity, LS (a) and high salinity, HS (b)

Table 3. The vibration signal of Standard, Low Salinity (LS) and High Salinity (HS) of Agar (1500- 400 cm^{-1})

Location	Type of Bonding	Wave Number (cm^{-1})
Standar Agarose	O-H	930.65 bending
	C-H	1378.56 deformation 891.50 bending 772.41 bending
	C-O	1159.50 stretching 1073.15 stretching
LS	O-H	932.72 bending
	C-H	1419.95 bending 891.27 bending 662.67 bending
	C-O	1156.23 stretching 1074.22 stretching
HS	O-H	933.27 bending
	C-H	1417.73 bending 1277.30 stretching 659.39 bending
	C-O	1074.93 stretching

4.4. Nutrient Content

Percentage of protein, carbohydrate, total lipid, water and ash of *Gracilaria* sp. samples from LS and HS is presented in Figure 4. Protein content of samples from low salinity is significantly different from the samples from high salinity ($p<0.05$). On the other hand, other concentrations were similar.

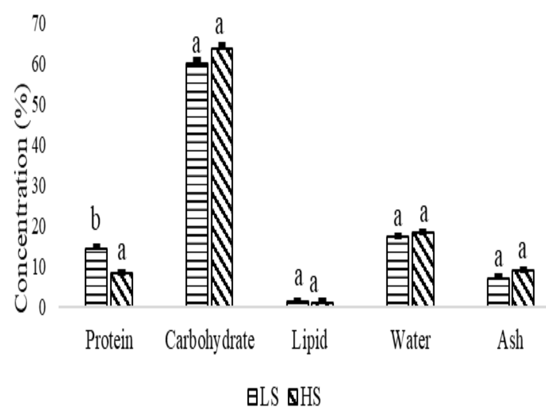

Figure 4. Protein, carbohydrate, lipid, water and ash contents of *Gracilaria* sp. grown in LS and HS

5. Discussion

5.1. Pigment Yield, Chlorophyll a and Carotenoids content of Gracilaria sp. from LS and HS

In terms of pigment extraction yield, the extraction of *Gracilaria* sp. from LS and HS resulted in a similar yield (± 2,8%) (Fig. 2). This value was possibly derived from other source of pigments based on water extraction from phycobiliprotein groups. Kumar *et al*. (2010) reported that decline concentration of chlorophyll *a* and carotenoids was in accordance with higher allophycocyanin (APC) and phycoerythrin (PE) content in hyper-salinity (45-50 ppt). Additionally, the similar researchers stated that the increment approximately from 52% and up to 70% from initial contents. This finding was also in agreement with Pereira *et al*. (2017) that high salinity reducing the chlorophyll contents and depigmentation of apices.

As shown in similar figure, photosynthetic pigments i.e. chlorophyll a and carotenoids content of *Gracilaria* sp. samples from the LS (26.98 ppt) was significantly higher than HS (44 ppt) ($p<0.05$). Chlorophyll is a compound with free electrons structure from nitrogen atoms (Sedjati *et al*., 2020). Meanwhile, Martinez *et al*., 2010 stated that carotenoids neutralize free radicals in three ways which is electron transfer, addition of radical species and the hydrogen abstraction. This indicated that low salinity was more tolerable. So, the physiology was relatively normal. Moreover, the phenomenon of oxidative stress, osmotic and turgor pressure regulation might not appear. In addition, chlorophyll a content from the Indonesian North Java coast in this study (14.81 µg/g) is distinctly higher compared to the study from Thondi Coast, India (8.96 µg/g) (Rosemary *et al*., 2019). In addition, visually, the colour green intensity of pigment from low salinity was much brighter and darker.

In general, visually, *Gracilaria* sp. thallus in this present study from the LS were thicker and bigger that might be indicated the better growth. Pereira *et al*. (2017), Wong and Chang (2000) reported similar results. Moreover, *Gracilaria tenuistipitata* from Songkhla Lagoon in Thailand showed the best growth rates at the salinity of 25 psu (Bunsom and Prathep., 2012). The growth of macroalgae in the marine ecosystem is often decreased in hypersaline water. This is due to the cumulative enzyme effects such as reduction of

turgor pressure, this then leads to restrict division of cells and at last, affected the growth (Lee and Liu, 1999).

Nitrate content at HS in this study was higher than LS (Table 1). This is probably caused by instant efflux nutrient from the shrimp pond. Pond's feed waste and shrimp faecal contribute the nitrate content. Nutrient factor highly fluctuates on photosynthesis especially nitrate and phosphate (Ismail and Osman, 2016). Research by Wu *et al*., (2018) reported that the nitrate and phosphorus uptake were higher at lower salinities (less than 20 psu) than higher salinity conditions (up to 20 psu). Study from Choi *et al*. (2010) exhibits that nitrate and phosphate uptake of macroalgae were greater in certain levels of salinity (20 and 25 ppt). These reports demonstrate similar suggestion to this study. Moreover, the water supply in LS from this study was more diverse, either from upside regimes from the sea as well as freshwater influxes from the river. Table 1 noted that phosphate content in LS was higher. The role of phosphate pathway is by transferring the high adenosine triphosphate (ATP) energy and other high energy compounds. This occurs in respiration and photosynthetic process (Ismail and Osman, 2016). These researchers' results were similar to our previous data, that photosynthetic pigments (chlorophyll a and carotenoids) of *Gracilaria* sp. in LS was higher when compared to the HS habitat (data is not shown).

5.2. Agar Percentage and Characterization

Data from Table 2 pointed that agar percentage of *Gracilaria* sp. in LS was significantly higher than HS. This is probably due to a relationship between salinity and the agar content. Research by Sasikumar *et al*. (1999) stated that a high salinity of *G. verrucosa* in summer (43.8‰) was found to be negatively correlated with agar yield. The best agar production of *Gracilaria tenuistipitata* (24.8 $\pm$ 3.0 %DW) in laboratory was found at 25 psu (Bunsom and Prathep., 2012). Rocha *et al*. (2018) stated that, often, yield of agar of *G. tikvahiae* has been clearly connected with salinity and adversely with nitrogen content, similarly reported to *G. gracil* species (Martin *et al*., 2013). Nitrogen concentration in LS, in fact, is lower than HS (Table 1). Less concentration of nitrogen, synthesis of protein declines in favor of polysaccharide synthesis. However, other factors such as nutrient availability, environmental and geographic factors, seasonal variations can influence the yield, chemistry and biosynthesis of agar (Lahaye and Rochas, 1991). Moreover, Lee *et al*. (2017) also stated that it is not easy to investigate the effects of a single factor on the yield of agar extracted from seaweeds grown in the natural habitat.

Based on spectra of FT-IR analyses, (Figure 2) shows that two *Gracilaria* sp. samples are fit to the standard agarose (Merck) and positively fingerprinted at a specific agar (1500-400 cm^{-1}) with galactose bond (around 1070 cm^{-1}). The vibration signal is slightly different, but overall those are agar characterizations. Agarose has a basic repeating unit of 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-a-L-galactopyranose. Based on figure 3 above, there is a 3,6 anhydrogalactose unit at a wave number of 928-933 cm^{-1} (Hii *et al*., 2015). Observed bands at 930 cm^{-1} indicates O-H bending. The hydroxyl (O-H) unit appears at 3400 cm^{-1} wavenumber. Alkene (CH_3 or CH_2) associated to metoxil appeared at 2900 cm^{-1}. Aldehyde group (-CHO) markedly appears at 1600 cm^{-1} (Pereira *et al*., 2009).

Based on Figure 4, it can be seen that protein content in *Gracilaria* sp. samples from LS was significantly higher compared to HS ($p<0.05$). Protein percentage is related to the nitrate content. In high salinity, nitrate compound has not taken up, easily (Choi *et al*., 2010). However, in special cases, nutrient uptake can be occasionally imbalanced during unfavorable periods of situations (Trimmer *et al*., 2000).

Results from Table 1 show that phosphate compound from the LS habitat was higher, and this may force the production of protein in the algal cell. Energy from photosynthesis will be used for amino acid biosynthesis that comes from the surrounding water. ATP is energy synthesized by photosynthesis, and these surely need phosphate. The reduction of protein synthesis triggered the decrease of the protein content and this was then affected the other cell components such as chlorophyll and other pigments (Ismail and Osman, 2016). This phenomenon was performed in *Gracillaria* sp. from HS habitat. Compared to other research, protein content in this study (14.40%) was higher than *G. changii* from Sarawak, Malaysia (12.57%) (Chan and Matanjun, 2016). Even though, this protein content was lower compared to the study on *G. corticata* by Rosemary *et al.* (2019).

Carbohydrate percentages form *Gracilaria* sp. samples grown in LS and HS were similar ($p>0.05$) (Figure 4). Carbohydrate content in this study was higher than *G. changii* (Chan and Matanjun, 2016) and *G. edulis* (Rosemary *et al*., 2019). Total lipid, water and ash content of *Gracilaria* sp. in both samples was not significantly different ($p>0.05$). Generally, total lipid from all macroalgae, including *Gracillaria* sp. in this research, was relatively low at the range of 0.9-40% (Khairy and El Shafay, 2013). Seaweed is rich in minerals. The high ash content indicates high mineral content. In accordance to our finding, the results from Wu *et al*. (2018) reported that nutrient uptake, tissue nutrient contents were affected by salinity and the ideal salinity was around 20 psu. This information could be beneficial to define optimal salinity in *Gracilari*a sp. culture systems to get the maximal agar yield and pigment quality, protein and mineral content. This initiates better economic incomes for agarophyte farming.

6. Conclusion

Agar, chlorophyll a, carotenoids, protein and mineral contents of *Gracilaria* sp. grown from low salinity habitat (reservoir) were higher compared to high salinity habitat (biofilter shrimp ponds). Other compounds such as the total lipid, carbohydrate, water, and ash content from high and low salinity were similar. In this study, it can be concluded that salinity affected *Gracilaria* sp. nutrient content. Similar to the standard agar, FT-IR spectra from different salinity show the existence of 3,6-anhydro-L-galactose. Our findings can be useful for considering the application of *Gracilaria* sp. culture in the future.

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