Improving the Antioxidant Profile of Yoghurt Using Red Seaweed *Gracilaria* sp.

Septiana Sulistiawati[1,*], Sardila[1], I*rman* Irawan[1], *Andi Noor* Asikin[1], and *Bagus fajar* Pamungkas[1]

[1]Department of Fisheries Product Technology, Faculty of Fisheries and Marine Sciences, Mulawarman University, Samarinda, East Kalimantan, Indonesia, 75123

Abstract. This study investigated the potential of *Gracilaria* sp. as a functional ingredient for yoghurt, focusing on its impact on consumer acceptance and its antioxidant capacity. The research employed a Completely Randomized Design (CRD) with three concentrations of added *Gracilaria* sp. (10%, 20%, and 30%). Key parameters analysed included pH, viscosity, total lactic acid, and proximate composition. Crucially, the antioxidant activity was evaluated using the DPPH free radical scavenging assay, and consumer acceptance was assessed through sensory evaluation. The results showed that increasing the concentration of *Gracilaria* sp. had a significant effect on all observed parameters. As the seaweed concentration increased, there was a notable increase in pH (acidity) and antioxidant activity. Conversely, the viscosity and total lactic acid content tended to decrease. Yoghurt fortified with 20% *Gracilaria* sp. was identified as the most desirable product, achieving the highest consumer preference score. This specific formulation exhibited an optimal balance of quality attributes, including a pH of 3.76, a viscosity of 270 cP, and a robust antioxidant activity of 42.74%. This research confirms that *Gracilaria* sp. can be effectively incorporated into yoghurt to create a functional food product with enhanced antioxidant properties. The 20% concentration proved the most effective and demonstrated high consumer acceptance.

Keywords: Antioxidant, functional, prebiotic, probiotic, seaweed

1 Introduction

Indonesia has great potential in seaweed production, especially the *Gracilaria* sp**.** species, a significant commodity in the food, pharmaceutical, and cosmetic industries (1). The production of this seaweed is very high and contributes significantly to the total global production. According to (2), *Gracilaria* sp. is a red seaweed widely distributed in tropical and subtropical waters. *Gracilaria* sp. contains nutrients like polysaccharides, proteins, vitamins, and minerals. One of its main components is galactose polysaccharide, which acts

* Corresponding author: septiana.sulistiawati@fpik.unmul.ac.id

as a prebiotic, excellent for promoting the growth of good bacteria in the digestive system (3). Furthermore, *Gracilaria* sp. also contains abundant antioxidant compounds, such as polyphenols, flavonoids, and vitamin C, which play a crucial role in neutralizing free radicals and reducing oxidative stress(4).

Yoghurt is a fermented milk product made with lactic acid bacteria (LAB) such as *Streptococcus thermophilus* and *Lactobacillus plantarum* (5). There is a growing trend in developing plant-based yoghurts, as plant-based ingredients are often more affordable, easily accessible, and have comparable nutritional value to dairy-based yoghurts (6). Using plant-based alternatives in yoghurt production, as demonstrated by Hiola et al. (7) using dragon fruit extract, shows the potential to increase the product's economic value. *Gracilaria* sp. has the potential to be used as an additive in yogurt production because its nutritional content is suitable for the growth of LAB, with dietary fiber that can enhance the activity of these bacteria (8).

Previous studies have shown that adding seaweed to yogurt can improve quality characteristics. For example, the addition of *Eucheuma spinosum* seaweed can increase viscosity(9), while adding 30% *Gracilaria* sp. can increase the total number of lactic acid bacteria (10). However, research specifically investigating the effect of adding *Gracilaria* sp. on increasing the antioxidant content in yogurt is still limited. Therefore, this study aims to explore the potential of *Gracilaria* sp. as an additive that can enhance the antioxidant content, physical characteristics, and consumer acceptance of yoghurt products.

2 Material and Methods

2.1 Material

The materials used in this study include those for making the seaweed yogurt drink and for analysis. We used dried *Gracilaria* sp. seaweed, yoghurt starter (Biokul), water, and UHT milk (Ultra Milk) for the yogurt production. The chemical reagents for analysis consisted of MRS Agar medium, 97% alcohol, DPPH solution, NaOH, N-hexane, bromocresol green and methyl red (BCG-MR) indicators, $CuSO_4$, concentrated H_2SO_4, distilled water, HCl, 1% phenolphthalein indicator, and H_3BO_4. The equipment for the research was divided into two groups. The yogurt preparation tools included a blender, spoons, basins, a stove, scales, Styrofoam boxes, glasses, a thermometer, and a spatula. For the analysis, we utilized a pH meter, a hot plate, a stirrer, test tubes, various pipettes (volumetric, micro, and droppers), petri dishes, a spectrophotometer, a Kjeldahl flask, a desiccator, an oven, a furnace, a burette, a digital scale, beakers, Erlenmeyer flasks, porcelain crucibles, an analytical balance, a food dehydrator, filter paper, a Soxhlet extractor, and an electric stove.

2.2 Preparation of *Gracilaria* sp.

The preparation of *Gracilaria* sp. was based on the method by Fauziah et al. (10), with some modifications. Dried *Gracilaria* sp. seaweed was first washed to remove any dirt. It was then soaked in water for 6 hours with added lime juice. The seaweed was rewashed until its pH returned to 7. Next, it was heated at 75°C for 5 minutes with a 1:1 water mixture. After heating, the seaweed was pureed in a food processor to obtain a smooth paste.

2.3 Seaweed Yoghurt Production

The yogurt was made using a modified method based on Wahyu (9), with four treatments. For each treatment, 500 mL of milk was mixed with varying amounts of seaweed

Gracilaria sp.—puree: 0%, 10%, 20%, and 30%. Then, 10 grams of granulated sugar (2% of the milk volume) was added. This mixture was heated to 70-80°C for 5 minutes, then cooled to 40-45 °C at room temperature to 40-45°C. Next, a 5% starter culture of Biokul plain yogurt was added. This starter contains bacteria such as *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, and *Bifidobacterium*. The mixture was then poured into sterilized glass jars, sealed tightly, and left to ferment for 12 hours at room temperature.

2.4 Viscosity Analysis

Viscosity testing using a viscometer refers to Wibawanti and Widiastuti (11). The viscometer was turned on, and then the spindle was installed. The yoghurt sample was tested using a VT-04F Rion viscometer with rotor number 1. The yoghurt sample was placed in a container under the spindle, and then the spindle was dipped into the yoghurt. After that, it was left for a while, and the number that appeared was recorded.

2.4 pH Analysis

pH (acidity) testing using a pH meter refers to the pH measurement method SNI 06-6989-11-2004. The pH of yoghurt is measured using a digital pH meter. The cathode is dipped into the yoghurt and allowed to wait until the pH meter scale stabilises to obtain an accurate pH value. The measurement results are read on the pH meter. pH measurements are performed in duplicate to ensure consistency and accuracy of results.

2.5 Lactic Acid Analysis

The lactic acid content was analysed using the method of Abdul et al. (12). First, a 10 mL yoghurt sample was placed into an Erlenmeyer flask. The sample was then titrated with 0.1 N NaOH, using 5 drops of phenolphthalein indicator. The titration endpoint was reached when the solution changed from colourless to a faint pink. The resulting data were then used in the following formula to calculate the lactic acid content:

$$\text{Lactic acid content (\%)} = \frac{\text{mLNaOHx } 0.009 \text{ X } 100\%}{\text{G Sampe}l}$$

2.6 Antioxidant Activity Analysis

The antioxidant activity was tested using a DPPH free radical scavenging assay via spectrophotometry (Eppendorf BioSpectrometer Basic), following a method by Farhan et al. (13). To begin, 1 mL of the diluted sample extract in ethanol was mixed with 1 mL of 0.15 mM DPPH in ethanol. At the same time, a control sample was prepared, consisting of 1 mL of DPPH mixed with 1 mL of ethanol. Both the reaction mixture and the control were thoroughly mixed by hand and then incubated in the dark at room temperature for 30 minutes. After incubation, the absorbance of each mixture was measured at 515 nm, using ethanol as a blank. The percentage of antioxidant capacity was then calculated using the following formula:

$$\%\ Inhibition = \frac{\text{A } contol - \text{A } sample}{\text{A } sample} \text{ x } 100$$

Where:

A *control* = The absorbance of the control sample (DPPH + ethanol).

A *sample* = The absorbance of the sample reaction mixture (DPPH radical + sample).

2.7 Hedonic Analysis

The hedonic test was conducted using Suryono et al. 's method(14). Thirty untrained panellists were asked to evaluate the yogurt samples with added *Gracilaria* sp. seaweed. The samples were presented according to standard procedures and labeled with a specific code. The panellists evaluated the samples based on several attributes: color, aroma, taste, and texture. Their preferences were recorded on a score sheet using a 9-point hedonic scale: 1: extremely disliked, 2: very much disliked, 3: disliked, 4: slightly disliked, 5: neutral, 6: liked somewhat, 7: liked, 8: very much liked, 9: extremely liked.

2.8 Data Analysis

This research utilized a Completely Randomized Design (CRD) with four distinct treatment groups: A0 (without *Gracilaria* sp.), A1 (with 10% *Gracilaria* sp.), A2 (with 20% *Gracilaria sp.*), and A3 (with 30% *Gracilaria* sp.). Each treatment was performed in triplicate. The data were analyzed using ANOVA (Analysis of Variance). If a significant difference was found ($p<0.05$), a DMRT (Duncan's New Multiple Range Test) was conducted as a post-hoc test to determine the most effective treatment. A Kruskal-Wallis Test was applied for the hedonic test data, and a Mann-Whitney Test was applied to identify specific differences between the treatments. All statistical analyses were carried out using SPSS software.

3 Results and Discussion

3.1 pH Value

pH value is one of the important factors that affect the growth of microorganisms and the formation of fermentation products, because each organism has an optimal pH range for its living environment (15). pH measurement will show whether the solution is acidic or alkaline. The results of pH analysis of yogurt with the addition of different *Gracilaria* sp. can be seen in Figure 1.

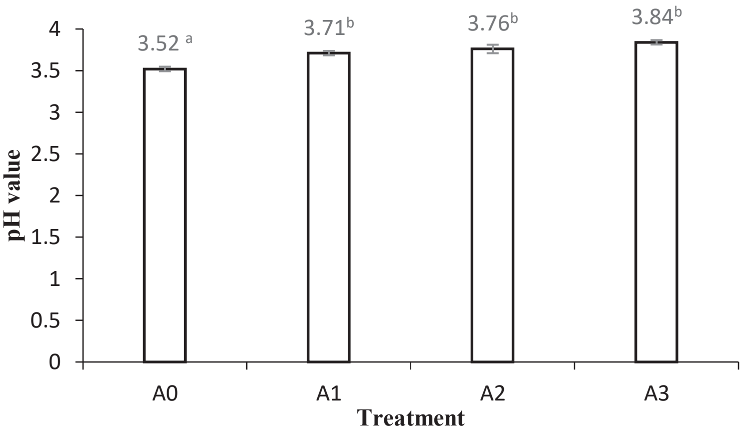


Fig. 1. pH values of yogurt with the addition of different amounts of *Gracilaria* sp. A0 (Control: without *Gracilaria* sp.), A1 (10% *Gracilaria* sp.), A2 (20% *Gracilaria* sp.), A3 (30% *Gracilaria* sp.)

The pH of the yogurt increased as the concentration of *Gracilaria sp.* increased. This reduces the production of lactic acid, leading to a higher pH. ANOVA results showed a significant effect ($p<0.05$). The highest pH (3.84) was found in the A3 treatment (30% seaweed), while the lowest (3.52) was in the control. All pH values were within the acceptable range of < 4.5 (16).This is likely due to the simple sugars (sucrose) in the seaweed, which can create osmotic pressure that inhibits the growth of lactic acid bacteria (LAB) (17).

3.2 Viscosity

Viscosity is defined as the measure of a fluid's resistance to flow, resulting from the internal friction of its moving particles. While water behaves as a simple Newtonian fluid, yogurt displays more intricate properties; it is characterized as viscoelastic, shear-thinning, and thixotropic (18). The results of the analysis of the viscosity value of yogurt with the addition of *Gracilaria* sp. can be seen in Figure 2.

Fig. 2. Viscosity values of yogurt with the addition of different amounts of *Gracilaria* sp. A0 (Control: without *Gracilaria* sp.), A1 (10% *Gracilaria* sp.), A2 (20% *Gracilaria* sp.), A3 (30% *Gracilaria* sp.)

The viscosity of the yogurt was significantly affected by the addition of *Gracilaria sp.* ($p<0.05$). The highest viscosity was observed in the A1 treatment (10% seaweed), with a value of 300 cP. The increase in viscosity is attributed to the galactose polysaccharide in *Gracilaria* sp., which has water-binding properties. However, at higher concentrations (A3), the viscosity decreased. This may be because an excess of polysaccharides can disrupt the gel structure and increase free water, making the yogurt less viscous (19)

3.3 Total Lactic Acid

Lactic acid is one of the important factors that can determine the quality of fermented products. Lactic acid is an organic acid produced from the fermentation process of lactic acid bacteria. During fermentation, LAB can break down lactose into lactic acid (20). Total lactic acid is the amount of lactic acid formed during fermentation. The results of the analysis of the total acid content of yogurt with the addition of *Gracilaria* sp. can be seen in Figure 3.

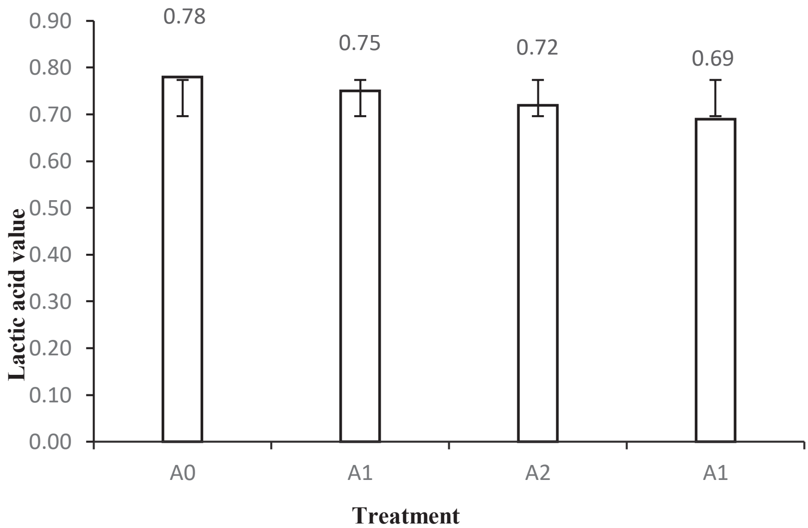
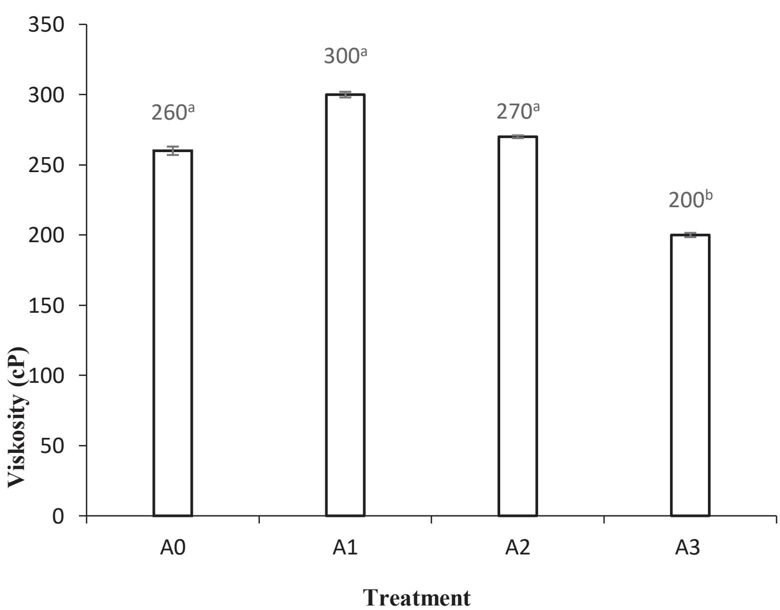


Fig. 3. Lactic acid values of yogurt with the addition of different amounts of *Gracilaria* sp. A0



(Control: without *Gracilaria* sp.), A1 (10% *Gracilaria* sp.), A2 (20% *Gracilaria* sp.), A3 (30% *Gracilaria* sp.)

The concentration of *Gracilaria* sp. did not significantly affect the total lactic acid content (p>0.05). As seaweed concentration increased, the total lactic acid content tended to decrease. This could be due to the LAB's inability to fully break down the complex nutrients in the seaweed or the presence of minerals like potassium and calcium, which can react with the acid to form salts, thereby reducing the overall acidity (21). All samples still met the SNI (2981:2009) standard of 0.5-2.0%.

3.4 Antioxidant Activity

Antioxidants play a major role as free radical scavengers in the metabolic process (22). Antioxidant activity testing is needed to determine the antioxidant activity in yogurt. This study used the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. The DPPH method works based on oxidation-reduction reactions, where DPPH is a synthetic free radical soluble in polar compounds such as ethanol and methanol (23). The results of the antioxidant activity test using the DPPH method can be seen in Table 1.

Table 1. Antioxidant activity of Gracilaria yoghurt

Treatment	Inhibition (%)
A0	$30{,}98 \pm 0{,}13^{a}$
A1	$40{,}14 \pm 0{,}46^{b}$
A2	$42{,}74 \pm 0{,}06^{c}$
A3	$45{,}89 \pm 0{,}36^{d}$

Description: A0 (Control), A1 (*Gracilaria* sp. 10%), A2 (*Gracilaria* sp. 20%), A3 (*Gracilaria* sp. 30%)

The DPPH free radical scavenging activity test results showed that the antioxidant value produced was relatively high. The lowest antioxidant activity was obtained in yogurt with treatment A0, with a value of 30.98%, while the highest was obtained in yogurt with treatment A3, with a value of 45.89%. The higher the concentration of *Gracilaria* sp., the higher the antioxidant activity of the yogurt. This increase in antioxidant activity may be due to the flavonoid content in *Gracilaria* sp., which has antioxidant effects, as these compounds can inhibit the formation of free radicals. In addition, cow's milk is also a source of antioxidants, where β-lactoglobulin in cow's milk acts as an antioxidant with an activity of 26.41%(24). *Gracilaria* sp. contains various secondary metabolites, such as alkaloids, flavonoids, terpenoids, and other bioactive compounds that function as antifungals and antioxidants. (25). Research conducted by Loho et al. (26)This shows that *Gracilaria* sp. extract has significant antioxidant activity and a low IC_{50} value, which can effectively reduce free radicals. In addition, the fermentation process also plays a role in increasing antioxidant activity. Nisak (2023) states that during the fermentation process, organic acids are formed, which increase the concentration of phenolic compounds.

The ANOVA test results showed that the difference in *Gracilaria* sp. concentration had a significant effect ($p<0.05$) on the antioxidant activity of yogurt, so the DMRT test was continued. The further test results showed that each treatment in this study differed significantly. The antioxidant activity of yogurt had a DPPH inhibition value ranging from 30.98% to 45.89%. Antioxidant activity was expressed quantitatively by IC_{50} (Inhibitor Concentration 50%). IC_{50} is the concentration of the test solution that provides 50% inhibition of DPPH. A compound is considered a powerful antioxidant if the IC_{50} value is less than 50 ppm, strong (50-100 ppm), moderate (100-150 ppm), and weak (151-1000 ppm) (Edison et al., 2020). The IC50 value is inversely proportional to the antioxidant capacity, where the smaller the IC50 value, the higher the antioxidant capacity of the sample (Susanto, 2019).

3.5 Consumer acceptance

Table 2. Average hedonic test scores for seaweed yogurt

Hedonic parameter	Treatment			
	A0	A1	A2	A3
Color	6,23 ±1,10^{a}	6,20 ± 1,21^{a}	6,70±1,34^{a}	5,10±1,60^{b}
Taste	4,83 ± 1,46^{a}	4,80 ± 1,67^{a}	5,73 ± 1,89^{a}	4,40 ± 1,63^{a}
Aroma	5,60 ± 0,93^{a}	5,83 ± 1,21^{a}	6,03 ± 1,63^{a}	5,60 ± 1,57^{a}
Texture	4,97 ± 0,96^{a}	5,63 ± 1,45^{b}	6,30 ± 1,62^{b}	4,67 ± 1,60^{a}

Description: A0 (Control), A1 (*Gracilaria* sp. 10%) A2 (*Gracilaria* sp. 20%), A3 (*Gracilaria* sp. 30%)

Color is the first parameter consumers assess before other parameters such as aroma, texture, and taste. The results of the hedonic can be seen in Table 1. The Kruskal-Wallis test results for the color parameter of yogurt with the addition of *Gracilaria* sp. showed a significant effect ($p<0.05$), so the Mann-Whitney test was continued. The highest level of preference for the color of yogurt with the addition of *Gracilaria* sp. was found in treatment A2, which was 6 (somewhat like), and the lowest was in treatment A3, which was 5 (neutral). The color produced by yogurt without the addition of *Gracilaria* sp. was milky white, while yogurt with the addition of *Gracilaria* sp. showed a color change to brownish white. The color change increased with increasing concentrations of *Gracilaria* sp. This was due to the brownish color of *Gracilaria* sp., which affected the final color of the yoghurt. The fiber content in seaweed affects the ash content, indirectly affecting the product's color. In some cases, adding seaweed reduces consumer sensory acceptance, such as in fish sticks, where appearance and aroma are reduced (27).

Taste plays a significant role in consumers' decisions to accept or reject food. The results of the Kruskal-Wallis test showed that adding *Gracilaria* sp. had no significant effect ($p>0.05$) on the taste parameters of yogurt. The highest level of liking for yogurt with the addition of *Gracilaria* sp. was found in treatment A2, which was 5 (neutral), and the lowest was in treatment A3, which was 4 (somewhat dislike). The results showed that the higher the concentration of *Gracilaria* sp., the lower the panelists' liking for the yogurt. The panelists' dislike of yogurt with the addition of *Gracilaria* sp. was thought to be due to its sour taste. This is because the addition of seaweed can increase viscosity, which can affect the taste, and the addition of seaweed can cause a reduction in the sweetness of the product (28). Consumers prefer yogurt with a sweet taste and have the highest score in organoleptic assessment (29).

Aroma assessment using the sense of smell. This sense will capture volatile compounds or compounds that easily evaporate found in the product. The Kruskal-Wallis test results show that adding *Gracilaria* sp. has no significant effect ($p>0.05$) on the aroma parameters of yogurt. The liking score for the aroma of yogurt ranged from 5.60 to 6.03, which is neutral to somewhat liked. The highest score was found in yogurt with 20% *Gracilaria* sp., and the lowest was found in yogurt with 30% *Gracilaria* sp., without any addition. Fermented yogurt has a distinctive sour aroma, with a slight fishy aroma that is thought to originate from *Gracilaria* sp, according to Anggraini et al. (30)Seaweed has a distinctive aroma that is slightly fishy but not too strong. Preliminary processes such as washing, soaking, and boiling reduce this aroma by removing seaweed's volatile (easily evaporated) odor compounds.

Yogurt generally has a semi-solid texture, which is not too runny or thick. The Kruskal-Wallis test results show that adding *Gracilaria* sp. significantly affects the texture parameters of yogurt ($p<0.05$). The results of the hedonic texture test showed that the panelists preferred sample A2 (yogurt with 20% *Gracilaria* sp. added) with a score of 6.30 (somewhat like), and the lowest score was for sample A3 (yogurt with 30% *Gracilaria* sp. added) with a score of 4.67 (somewhat dislike). The study results show that the more seaweed is added to yogurt, the less desirable the texture becomes because it is too liquid or watery. This could be because the more seaweed added, the higher the water content produced, resulting in a thinner yogurt texture.

4 Conclusion

Adding different *Gracilaria* sp. to yogurt affects its physical characteristics and consumer acceptance. Higher additions of *Gracilaria* sp. cause an increase in pH and antioxidant activity, as well as a decrease in viscosity and total lactic acid. The yogurt preferred by the panelists was yogurt with 20% *Gracilaria* sp. addition, with a pH of 3.76, viscosity of 270 cP, total lactic acid of 0.72, and antioxidant activity with inhibition value 42,74 ± 0,06 %.

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