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# Quality Characteristics and Antioxidant Activity of Fermented Symbiotic Beverages Supplemented with Eucheuma Cottonii

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# Quality Characteristics and Antioxidant Activity of Fermented Symbiotic Beverages Supplemented with *Eucheuma Cottonii*

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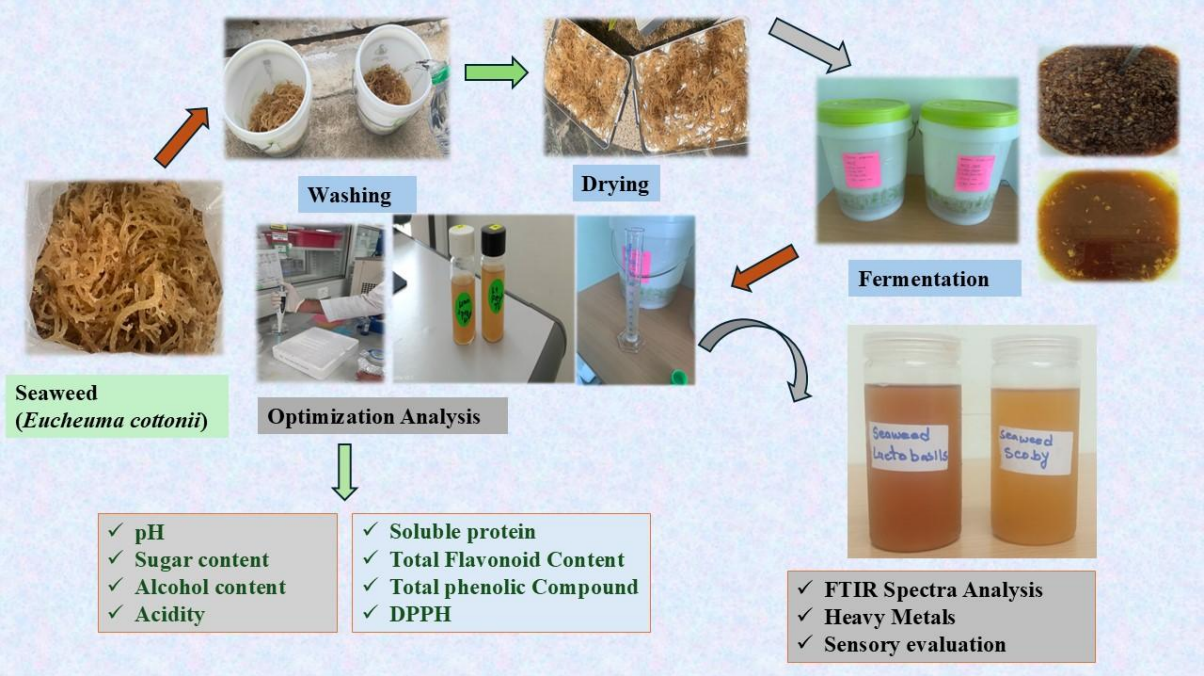
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## Abstract

Seaweeds are an excellent source of nutrients and bioactive compounds associated with health benefits. In this study, symbiotic beverages were prepared by submerged fermentation with Lactobacillus and SCOBY as fermented materials and using *Eucheuma Cottonii* seaweed. During fermentation, the quality characteristics were analyzed and the physicochemical (pH, acidity, sugar, and alcohol content), Total phenolic content (TPC), Total flavonoid content (TFC), antioxidant capacity (DPPH, FRAP) organoleptic properties (9-point hedonic scale) of seaweed beverages. The pH levels of Lacto-based (5.2±1.64) and SCOBY-based beverages reached (4.5±0.12) and acidity for (0.5 ± 0.06) and (0.9±0.07) after 30 days of fermentation. Sugar content decreased, and alcohol content increased with an increase in the number of fermentation days. The protein content increased significantly ($p < 0.05$) during fermentation. However, Lacto-based beverages have a low protein content, and SCOBY-based beverages have a high protein content (0.25 mg/ml). SCOBY-based seaweed beverages have high flavonoids (29.44±0.63) and phenolic contents (27.44 ± 0.02). The DPPH value of Lacto-based beverages is (61.23±1.54 %), SCOBY-based beverages are (61.98±0.22 %), and the FRAP value is (17.53±0.08 %) and (19.98±0.08 %) after 30 days of fermentation. The heavy metal content in seaweed beverages was below the standard value; therefore, it is safe for

consumption. The results showed that SCOBY-based beverages have enhanced nutritional and improved sensory properties, indicating the possibility of commercialization.

**Keywords:** Seaweed fermentation, Symbiotic beverages, Flavonoid and phenolic content, Antioxidant activity, *Eucheuma Cottonii*




**Graphical Abstract**

## 1. Introduction

The increased demand for functional foods and drinks has led to the development of novel products with nutritional properties and health benefits [1]. Consumers are exploring functional beverages containing new ingredients to enhance fermented drinks' quality and antioxidant properties. Incorporating seaweed boosts these nutritional needs and supports sustainable beverage production. Current trends in the beverage industry produce nutritionally significant products with enhanced flavours and appearances owing to the addition of probiotics, prebiotics, or symbiotics [2].

Seaweed consumption has been rising due to the production and marketing of marine products as functional foods and an increasing interest in healthy eating [3]. Food production using

seaweed has expanded due to its rich nutritional profile, which includes polyphenols, carotenoids, polysaccharides, proteins, fatty acids, and minerals [4]. Fermentation of *Pyropia spp*. not only enhances the bioavailability of intracellular compounds but also supports probiotic growth by providing a rich source of polysaccharides, which serve as prebiotic substrates [5]. A novel functional kombucha was developed by fermenting laver red seaweed with kombucha consortia of yeast and bacteria. Tea kombucha exhibited higher total phenols, flavonoid content, FRAP, organic acid, acetic acid, lower pH, and improved DPPH scavenging ability. Thus, laver shows substantial potential as a substrate for new fermented beverages [6].

SCOBY is a symbiotic culture of bacteria and yeast, and SCOBY-based seaweed beverages represent a novel approach in the beverage market due to their enrichment with marine bioactive compounds that provide health benefits. Kombucha, a common fermented tea, has been improved with different herbal and aquatic extracts to produce healthy food products [7]. Furthermore, developed drinks, such as Yakult supplemented with *Lacticaseibacillus casei* and *Lacticaseibacillus rhamnosus*, have enhanced viable microbial count and antioxidant activity [8].

*Eucheuma cottonii* contains valuable bioactive components that enhance the nutritional value of seaweed-based beverages [9]. Seaweed-based food products have bioactive components with antibacterial, anti-inflammatory properties and good antioxidant values [10,11]. Symbiotic beverages, including yogurt, kefir, and kombucha, are gaining popularity for their ability to combine probiotics with prebiotic substrates, promoting gut microbiome health. A study showed that symbiotic yoghurt enhanced with purple-leaf tea demonstrated increased beneficial gut bacteria, such as *Lactobacillales* and *Bifidobacteriales* [12]. *Lactobacillus paracasei* strains can have different properties and health effects to inhibit pathogens and stimulate immune responses; however, *Lactobacillus reuteri* strains are particularly well-researched for their benefits in oral health and infant colic.

Probiotic beverages prepared using seaweed (*Ulva lactuca*) have demonstrated high essential nutrients and bioactive compounds, such as phenols, flavonoids, and antioxidants [13]. During seaweed-based fermentation (*Alaria esculenta*), lactic acid bacteria produce lactic acid, metabolites, and other nutrients [14]. Seaweed increases the accessibility of significant compounds and improves products' organoleptic properties during enzymatic hydrolysis and solid-state fermentation [15–17]. A study showed that *Kappaphycus alvarezii* is a beneficial raw material for nutritionally enriched symbiotic beverages that increase consumer interest and support a healthy diet [11,18].

The fermentation process is important because of its nutritional advantages and ensures the palatability of the food product. Different factors included in this process, such as temperature, are important for alcoholic fermentation because they affect the flavour profile and acidity of alcoholic beverages [19,20]. pH and sugar are also important for microbial growth and metabolic activity [21]. Controlled fermentation parameters, including temperature (22–27°C) and pH (4.5–6), optimize the bioavailability of phenolic compounds and enhance probiotic viability. This study analyzed the factors and actual yield for fermenting *Eucheuma cottonii* to improve its nutritional properties and produce a symbiotic beverage with good antioxidant activity and sensory properties. The final nutritious product meets consumer acceptability requirements, although promoting the sustainable use of seaweed-based food products.

## 2. Materials and methods

### 2.1 Materials and Sample Collections

Seaweed (*Eucheuma Cottonii)* was bought from Fung Yuan Biotech Sdn Bhd Malaysia. The samples were rinsed with tap water to remove dirt and foreign materials. They were then desalinized by soaking them in two volumes of water for 1 h. Desalinized samples were cut into smaller sizes (1-5 mm) and dried at room temperature for two days. The dried samples

were placed in a small container with other ingredients and kept at 20-23 °C in a tight cap. The sample preparation method was adapted from the study by Tan & Lee [22] with minor modifications.

## 2.2 Fermentation of seaweed

For fermentation, wet seaweed was dried naturally under normal sunlight. One kilogram of seaweed in small pieces was placed in a 6 L barrel. Alkaline water (4 L) was added, and the mixture was stirred continuously until 700 g of sugar was dissolved completely. Separate barrels were inoculated with SCOBY and *Lactobacillus paracasei strains and Lactobacillus reuteri* and fermented for 30 days at room temperature 22-25 °C. At the end of fermentation, the beverage was filtered to remove seaweed residues and stored in capped bottles at 4 °C. Seaweed fermentation was carried out following the method described by Ayed et al. [23] with minor modifications. Fig 1 shows the proper flow chart of the seaweed beverage preparation fermentation process.

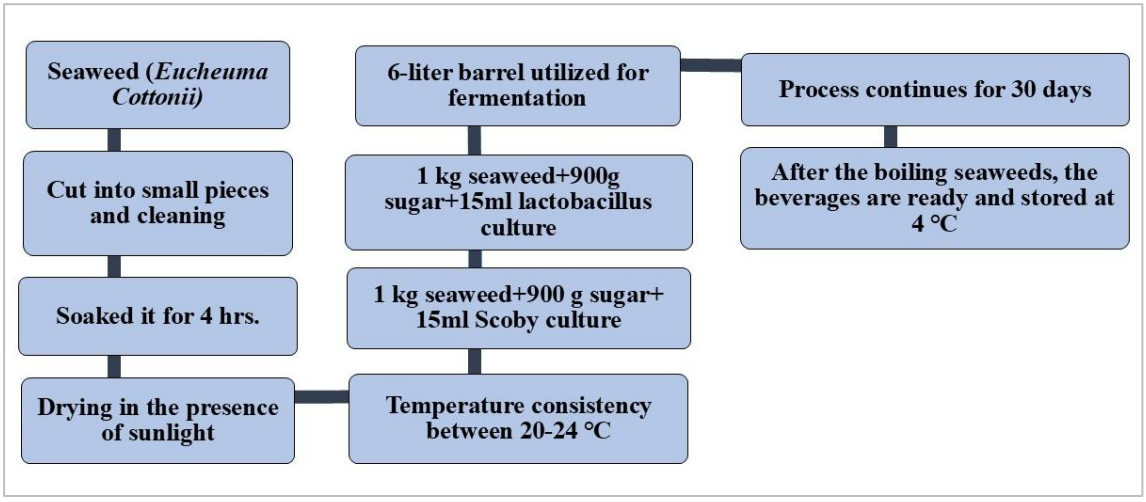



**Figure 1** Flow chart Fermentation process of seaweed beverages

## 2.3 Analytical methods

A pH meter was utilized to determine the pH levels, while the Brix scale (°Br) measures the percentage of soluble solids. Acidity was quantified following the AOAC 925.34 (AOAC, 2000). For this, a 10 ml aliquot of the sample was diluted with 20 mL distilled water. Subsequently, 2-3 drops of Phenolphthalein solution were added as a pH indicator. The mixture was titrated with NaOH solution until a light pink endpoint appeared. Acidity was calculated using Equation (1). The sugar content of seaweed-based beverages was assessed using a handheld refractometer (RHB-62ATC, Brix refractometer, China). An alcoholic hydrometer determined the alcohol content (g/100 ml) of the beverage according to the manufacturer's instructions.

$$\text{Acidity (\%)} \frac{0.009 \text{ x volume of NaOH used (ml)}}{\text{Weight of sample (g)}} \times 100 \quad (1)$$

## 2.4 Total Phenolic and Flavonoid Contents

Total phenolic content (TPC) was measured using the Folin-Ciocalteu (FC) method with slight changes, as stated by Ganesan et al. (2008). In brief, 1 ml of diluted extracts were dissolved with 2.5 ml of Folin–Ciocalteu's reagent (10 %), followed by neutralization with 2 ml sodium carbonate (7.5 %). The reaction mixture was kept in the dark area for 45 min, and absorbance was measured at 720 nm using a spectrophotometer (UV-9200, Biotech Engineering Management Co., UK). So, the Gallic acid was used as the standard for the calibration curve. Furthermore, the total flavonoid content (TFC) was also determined following the method of [26], and catechin was used as the standard for the calibration curve.

## 2.5 Soluble protein content

The estimation of soluble protein content was determined using the Arunima and Satish Verulkar method [27] with minor modifications. The sample of 0.2 mL was taken into a test tube and got to 1 mL by using distilled water. A volume of 4.5 mL of Reagent (I) 48 mL of 2%

sodium carbonate in 0.1 N sodium hydroxide, 1 mL of sodium potassium tartrate in water, and 1 mL of 0.5% copper sulfate pentahydrate in water was added and incubated 10 min. Then, 0.5 mL of Folin's Ciocalteu reagent 1:1 was inserted and incubated for 30 min. The absorbance was measured at 660 nm using a UV-Vis spectrophotometer, and the reference solution used Bovine serum albumin (BSA).

### 2.6 Radical Scavenging Activity

The DPPH assay was performed according to the methodology outlined by Baliyan et al. [28], with minor modifications. Antioxidants help neutralize free radicals in the body, which is thought to improve overall health. Approximately 1 ml of 0.1 mM DPPH solution was added to the 1 ml sample. After thorough mixing, the solution was incubated at ambient temperature for 30 min. A spectrophotometer was used to measure the absorbance at 517 nm. As a positive reference, ascorbic acid was diluted in distilled water, creating 0 to 0.7 mg/ml concentrations. The control was prepared by combining 1 ml of methanol with 1 ml of 0.1 mM DPPH solution. The following formula was used to calculate the percentage of antioxidants:

$$\text{Antioxidant (\%)} = \frac{(\text{Absorbance of Control} - \text{Absorbance of Sample})}{\text{Absorbance of control}} \times 100 \qquad (2)$$

### 2.7 Ferric Reducing Antioxidant Power Assay

The Ferric Reducing Antioxidant Power (FRAP) assay was conducted following the methodology outlined by Abd Razak et al. [29]. A fresh FRAP reagent was created by combining 2.5 mL of 10 mM 2,4,6-tris (1-pyridyl)-5-triazine (TPTZ) solution in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3.6H_2O$, and 25 mL of 0.3 M acetate buffer at pH 3.6 in a 1:1:10 ratio. Acetate buffer consisted of 16 mL glacial acetic acid, 3.1 g sodium acetate trihydrate, and 16 mL distilled water. To perform the FRAP assay, 0.15 mL of the fermented material was mixed with 2.85 mL of FRAP reagent. This mixture was then incubated at 37 °C for 4 min, after which

absorbance was measured at 593 nm using a UV spectrophotometer. A calibration curve was established using a $FeSO_4.7H_2O$ aqueous solution with concentrations ranging from 0.1 to 1.0 mM, following the same procedure applied to the samples. FRAP values were calculated using the following equation:

$$\text{FRAP value} = \frac{(\text{Absorbance value} \times \text{dilution factor} \times \text{volume of solvent})}{\text{weight of mass}} \times 100 \quad (3)$$

## 2.7 Heavy Metal Analysis of Fermented Beverage

Samples were thoroughly mixed and analyzed directly using an Agilent 5800 ICP-OES without prior digestion. Seaweed beverage samples were diluted 1:1 with 0.2% (v/v) $HNO_3$ and centrifuged for 20 min at 2000 rpm (approximately 630 × g) (Hettich Universal 30F; Tuttlingen, Germany). Sample vials, sampler cups, and glassware materials were cleaned by soaking in 10% (v/v) $HNO_3$ and rinsed with deionized water before use. Appropriate standards for each evaluated element were determined within the concentration range of the elements in the samples. The results were obtained from triplicate measurements. Analytical grade nitric acid (65%) mineralized the samples. The analytical standard solutions used for calibration were prepared by diluting each pure element standard obtained from Merck (Darmstadt, Germany). All aqueous solutions and dilutions were prepared using ultrapure water (Milli-Q; Millipore, Billerica, MA, USA).

## 2.8 Functional Group Characterisation through FTIR Spectra Analysis

Fourier Transform Infrared (FTIR) spectra of samples before and after fermentation were recorded in the wavelength between 4000 and 400 $cm^{-1}$. Free freeze-dried samples were directly measured using FT-IR/FT-NR Spectrometer Spectrum (Perkin Elmer, USA).

## 2.9 Sensory evaluation

A panel of 20 experts conducted sensory evaluation tests and descriptive analysis of three samples: fresh juice and juices fermented for 6 and 12 days. Participants initially engaged in four one-hour training sessions to develop and finalize descriptors. Following the training, duplicate samples were randomly presented at room temperature in plastic cups, with drinking water provided for palate cleansing between samples. To evaluate the aroma intensity, sweetness, flavor, color, and overall acceptability, a scoring scale ranging from 1 to 9 was used.

### 2.10 Statistical analysis

The results are presented as mean ± standard deviation (SD), and all analyses were performed in triplicate. Duncan's multiple range test was conducted to compare mean values. One-way ANOVA was performed to evaluate the statistical significance of the differences between SCOBY and Lactobacillus samples ($p > 0.05$). All Statistical analyses were performed using Origin Lab 2018.

## 3 Results and Discussion

### 3.1 Changes in pH and Acidity during fermentation

The pH of the samples was determined every five days during the fermentation process. **Table 1** shows the effects of fermentation duration on pH and acidity content. The pH in *Lactobacillus*-based fermentation at 0 days is (6.2±0.35), and in SCOBY-based fermentation (6.3±0.05), but at 30 days, pH decreased in *Lactobacillus* (5.2±1.64) and SCOBY-based beverages (4.5±0.12). The Acidity in *Lactobacillus*-based fermentation at 0 days is (0.8±0.05 %), and in SCOBY-based fermentation (0.6±0.04%), but at 30 days, Acidity decreased in *Lactobacillus-based* fermentation (0.5±0.06%) and increased in SCOBY-based fermentation (0.9±0.07%). Acidity and pH decreased and increased during the 30-day fermentation process. Research has demonstrated that the ideal pH and acidity levels for producing bioactive

compounds vary based on the seaweed species, microorganisms involved in fermentation, and specific bioactive compounds being produced.

The initial pH during fermentation plays an important role in spore germination and enzymatic activity. A higher pH has been reported to increase the rates of mycelium growth of spores. However, a low pH inhibits spore germination [30]. During fermentation, pH variations occur because of microbial metabolic activities, influencing the final product quality and microbial growth. In lactic acid bacteria (LAB) fermentation, the Initial stages of pH decrease due to the production of organic, lactic and acetic acid, which are byproducts of carbohydrate metabolism [31].

During macroalgae fermentation, pH typically decreases as LAB produces organic acids, primarily lactic acid [32]. The growth of bacteria, yeasts, and filamentous fungi is inhibited by the lowering of pH caused by organic acid production. It demonstrated the efficiency of pH reduction through LAB fermentation in combating *Salmonella spp* [33]. The pH range of 6.0 to 7.0 is optimal for many LAB strains, and a study suggests that specific strains may exhibit different pH preferences, indicating a need for modified fermentation conditions based on the target LAB species and desired bioactive compound [34].

Acidity in fermentation, influenced by microbial activity, substrate type, and fermentation conditions, affects flavour and stability. Early stages increase as microbes convert sugars into organic acids, like *Lachancea thermotolerans*, which produce lactic acid during fermentation, lower pH and enhance wine freshness [35]. Acidity in waste fermentation can change due to microbial activities, particularly acidogenic bacteria, adapting to different feedstock compositions, affecting volatile fatty acid yields and fermentation environment safety [36]. Research on *Bostrychia* species has shown increased growth and polysaccharide production at pH below 7.2 [37].

**Table 1:** Changes in pH and acidity (%) in *Lactobacillus* strains and SCOBY during fermentation

| Fermentation time (day) | pH | | Acidity (%) | |
|---|---|---|---|---|
| | **Lactobacillus** | **SCOBY** | **Lactobacillus** | **SCOBY** |
| 0 | $6.2\pm0.35^{aA}$ | $6.3\pm0.05^{aB}$ | $0.8\pm0.05^{bA}$ | $0.6\pm0.04^{cB}$ |
| 5 | $5.8\pm0.55^{bA}$ | $5.6\pm0.50^{bB}$ | $0.6\pm0.03^{cB}$ | $0.7\pm0.02^{bA}$ |
| 10 | $5.2\pm0.11^{cA}$ | $5.3\pm0.15^{cB}$ | $0.5\pm0.03^{dB}$ | $0.6\pm0.04^{cA}$ |
| 15 | $5.6\pm0.25^{dA}$ | $5.4\pm0.21^{dB}$ | $0.6\pm0.04^{cB}$ | $0.7\pm0.03^{bA}$ |
| 20 | $5.3\pm0.11^{cA}$ | $4.5\pm0.35^{eB}$ | $0.6\pm0.07^{cB}$ | $0.9\pm0.07^{aA}$ |
| 25 | $4.6\pm0.60^{eB}$ | $5.1\pm0.11^{cA}$ | $1.1\pm0.08^{aA}$ | $0.4\pm0.05^{dB}$ |
| 30 | $5.2\pm1.64^{cA}$ | $4.5\pm0.12^{eB}$ | $0.5\pm0.06^{dB}$ | $0.9\pm0.07^{aA}$ |

Results were expressed as the mean of three replications + standard deviation. Within the same column, means with different letters (a-g) and the same row, means with different letters (A-B) are significantly different at $p < 0.05$.

### 3.2 Effect of Sugar and Alcohol Content during Fermentation

Sugar content measurements for *Lactobacillus* and SCOBY-based fermentation are shown in Table 2. At the start of fermentation (day 0), *Lactobacillus*-based fermentation sugar levels were (11.23± 0.25) and SCOBY-based fermentation (10.23±0.25). The initial fermentation acceleration procedure can explain this sudden increase in the sugar content on the first day. Due to the increase in the fermentation period, sugar content decreased, and alcohol content increased because microorganisms used sugar and produced alcohol. After 30 days, the sugar content decreased in *Lactobacillus* (7.16±0.05) and SCOBY (6.41±0.21). The alcohol content in Lactobacillus and SCOBY-based fermentation at 0 days is the same (0.01±0.01), but at 30 days, alcohol content increased in *Lactobacillus* (1.43±0.11) and SCOBY-based fermentation (1.93±0.15).

A study determined that sugar content increased during fermentation's first and second days. Makgeolli's sweetness is primarily maltose and glucose, which decreases sugar content after yeast consumes sugar, while alcohol content increases [38]. During fermentation, sugar content decreases and alcohol content increases. Sugar content reduces due to saccharification amylase and yeast's nutrient source, leading to the decomposition of starch into sugar [39]. A significant decrease in glucose content reflects the conversion of yeast to ethanol [40]. Studies have shown that *K. alvarezii*, same spp. *Ecumma cattonii* is rich in fermentable sugars, making it a potential substrate for producing lactic acid and various bioprocesses. *K. alvarezii* pretreated with 1% H2SO4 and yielded 0.71 g of reducing sugar of dry weight, higher than other seaweed species [41].

The sugar content differs among seaweed species, with *Laminaria digitata* primarily containing glucose and *Ascophylum nodosum* consisting of rhamnose; with this fermentation process, sugar content decreased, and alcohol increased [42]. During sugarcane fermentation, the available sugars are metabolized, reducing sugar content and Brix and influencing ethanol production [43]. The hydrolysis of complex polysaccharides in macroalgae, such as laminarin, mannitol, and alginate, releases fermentable sugars [44].

Sugar content decreases as fermenting microorganisms instantly metabolize these sugars. The specific macroalgal species and the selected pretreatment method can influence the initial sugar concentration [45]. The different macroalgal species contain varying amounts of carbohydrates, directly affecting the initial sugar concentration. On a dry weight basis, *Ulva prolifera*, a green macroalga, contains 34.98 ± 3.30% carbohydrates [46]. Moreover, species-specific differences in their chemical makeup are highlighted by the fact that brown macroalgae are distinguished by their fucoxanthin content, red macroalgae by phycobiliproteins, and green macroalgae by chlorophylls [47].

The relationship between Brix and individual sugar components was weaker than that between total sugars and sucrose, indicating that other factors contribute to the Brix values. Studies on sorghum juice found that Brix values correlated with total sugar and sucrose concentrations (r=0.900 and r= 0.894), but no significant correlation with Brix and individual sugars such as glucose and fructose content [48]. This indicates that the decrease in Brix during macroalgal fermentation may be due to reduced total sugars and sucrose.

**Table 2:** Changes in sugar and alcohol content in *Lactobacillus* strains and SCOBY during fermentation

| **Fermentation time (day)** | **Sugar content (°Brix)** | | **Alcohol content (%)** | |
|---|---|---|---|---|
| | **Lactobacillus** | **SCOBY** | **Lactobacillus** | **SCOBY** |
| 0 | $11.23\pm 0.25^{aA}$ | $10.23\pm0.25^{aB}$ | $0.01\pm0.01^{fA}$ | $0.01\pm0.01^{fA}$ |
| 5 | $10.76\pm0.23^{bA}$ | $9.76\pm0.23^{bB}$ | $0.63\pm0.11^{eB}$ | $0.73\pm0.11^{eA}$ |
| 10 | $10.3\pm0.11^{cA}$ | $9.13\pm0.30^{cB}$ | $0.66\pm0.15^{eB}$ | $0.86\pm0.05^{eA}$ |
| 15 | $9.56\pm0.25^{dA}$ | $9.1\pm0.62^{dB}$ | $0.76\pm0.15^{dB}$ | $1.03\pm0.06^{dA}$ |
| 20 | $9.36\pm0.25^{dA}$ | $7.36\pm0.25^{eB}$ | $0.81\pm0.1^{cB}$ | $1.33\pm0.15^{cA}$ |
| 25 | $8.30\pm0.17^{eA}$ | $6.53\pm0.35^{fB}$ | $0.96\pm0.2^{bB}$ | $1.81\pm0.17^{bA}$ |
| 30 | $7.16\pm0.05^{dA}$ | $6.41\pm0.21^{fB}$ | $1.43\pm0.11^{aB}$ | $1.93\pm0.15^{aA}$ |

Results were expressed as the mean of three replications + standard deviation. Within the same column, means with different letters (a-g) and the same row, means with different letters (A-B) are significantly different at $p < 0.05$.

### 3.3 Effect of Soluble protein content during fermentation

Fermentation can change seaweed's stability and make protein content more immediately extractible by solvents. Researchers have observed comparable phenolic and protein content increases during fermentation and other biologically active substances. Protein content measurements for *Lactobacillus* and SCOBY-based fermentation are shown in **Figure 2**. Protein content during *Lactobacillus* and SCOBY-based fermentation increased with the duration of the fermentation process. As the study progressed, the protein content increased

significantly ($p < 0.05$) during fermentation. However, Lactobacillus has low protein content, as shown in Fig (A), and SCOBY has high (0.25 mg/ml) protein content, as shown in Fig (B), at the end of 30 days of fermentation.

Different studies have shown that the protein content increased during the fermentation process. Microbial activity during fermentation leads to the breakdown of complex proteins into simpler forms, making them more bioavailable and easier to detect. This process, known as proteolysis, increases the protein content [49,50]. The fermentation process can reduce other components, such as carbohydrates and ash, thereby increasing the relative proportion of proteins in the fermented product [49,51].

Protein content increased during seaweed fermentation is a breakdown of complex proteins, reduction of other components, and potential contribution of microbial biomass. The type of seaweed, fermentation conditions, and microorganisms used can influence the extent of the protein increase [52]. In contrast, a significant increase in protein content (up to 141%) has been observed in fermented *Macrocystis pyrifera* and seaweed waste [53]. A solid-state fermentation (SSF) of different seaweed species, such as *Ulva rigida*, with fungi, such as *Aspergillus ibericus*, resulted in significant enhancements in protein content as well as increased production of enzymes; it suggested that the fermentation process improved both the nutritional and functional properties of seaweeds [54].

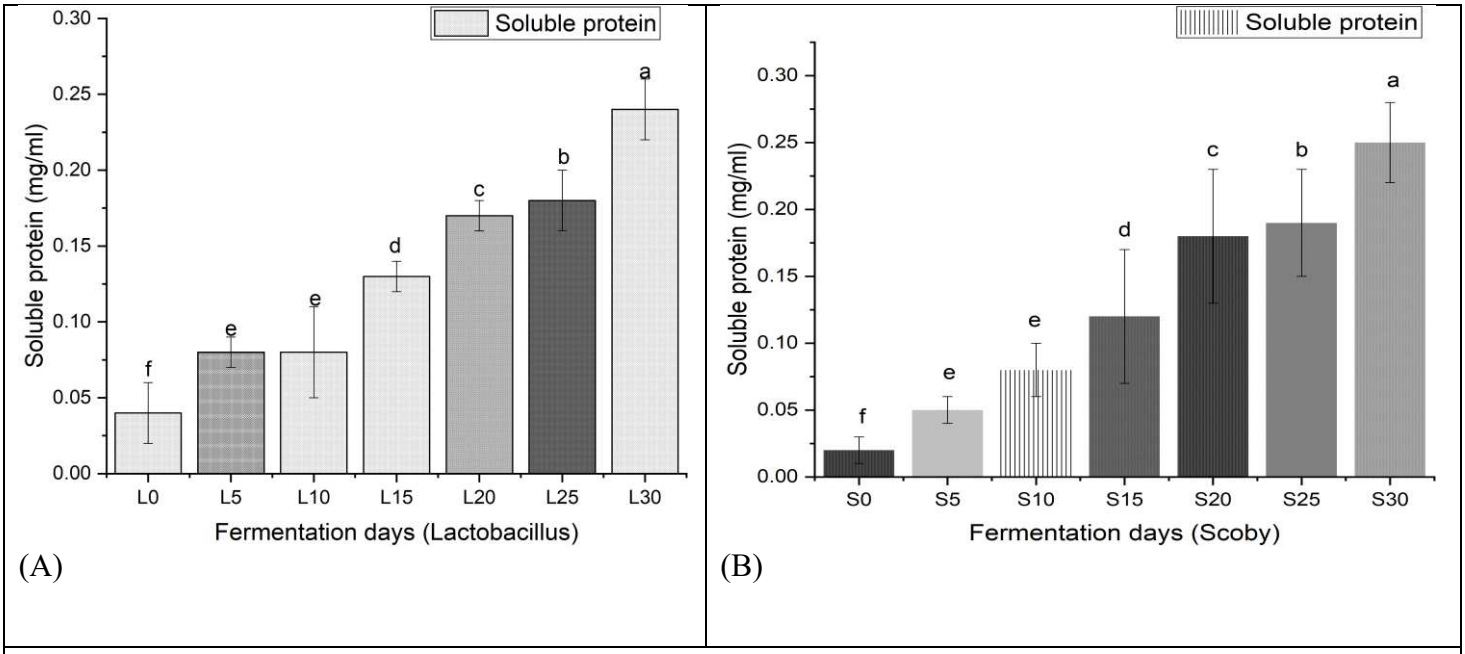



**Figure 2** Soluble protein content of *Lactobacillus* and SCOBY fermentation. Data are presented as the mean ± SE. The means observed with different letters are significantly different on different fermentation days ($P < 0.05$), and similar letters are not significantly different.

### 3.4 Effect of Total Flavonoid and Phenolic Content during Fermentation

The fermentation of seaweeds can significantly affect their flavonoid content, enhancing their bioactive properties and antioxidant potential. Sugar content measurements for Lactobacillus and SCOBY-based fermentation are shown in Table 2. At the start of fermentation (day 0), Lactobacillus-based fermentation had a TFC content of (4.17±0.05 mg CE/g), and SCOBY-based fermentation had (5.53±0.61 mg CE/g). After 30 days, the TFC content increased in lactobacillus-based (23.9±0.07mg CE/g) and SCOBY-based fermentation (29.44±0.63 mg CE/g). The TPC content in lactobacillus-based fermentation at 0 days was (2.70±0.22 mg GAE/g) and SCOBY-based fermentation (1.08±0.01 mg GAE/g). However, at 30 days, TPC content increased in lactobacillus (24.4±0.02 mg GAE/g) and SCOBY-based fermentation process (27.44±0.02 mg GAE/g). The total phenolic and flavonoid content for lactobacillus and SCOBY-based fermentation increased significantly ($p < 0.05$) during fermentation.

| **Table 3. Changes in TFC and TPC content in *Lactobacillus* strains and SCOBY during fermentation** | | | | |
|---|---|---|---|---|
| **Fermentation time (day)** | **Total Flavonoid Content (mg CE/g)** | | **Total Phenolic Content (mg GAE/g)** | |
| | **Lactobacillus** | **SCOBY** | **Lactobacillus** | **SCOBY** |
| 0 | $4.17\pm0.05^{gB}$ | $5.53\pm0.61^{gA}$ | $2.70\pm0.22^{gA}$ | $1.08\pm0.01^{gB}$ |
| 5 | $6.81\pm0.15^{fB}$ | $7.81\pm0.16^{fA}$ | $3.97\pm0.66^{fB}$ | $4.45\pm0.04^{fA}$ |
| 10 | $7.74\pm0.44^{eB}$ | $9.77\pm0.47^{eA}$ | $8.64\pm0.36^{eB}$ | $9.49\pm0.02^{eA}$ |
| 15 | $10.9\pm0.65^{dB}$ | $12.58\pm1.03^{dA}$ | $12.9\pm0.38^{dB}$ | $14.06\pm0.55^{dA}$ |
| 20 | $12.7\pm0.14^{cB}$ | $14.76\pm1.14^{cA}$ | $14.21\pm0.30^{cB}$ | $17.42\pm0.03^{cA}$ |
| 25 | $20.3\pm1.25^{bB}$ | $25.01\pm0.58^{bA}$ | $22.3\pm0.51^{bB}$ | $23.23\pm0.02^{bA}$ |
| 30 | $23.9\pm0.07^{aB}$ | $29.44\pm0.63^{aA}$ | $24.4\pm0.02^{aB}$ | $27.44\pm0.02^{aA}$ |
| Results were expressed as the mean of three replications + standard deviation. Within the same column, means with different letters (a-g) and the same row, means with different letters (A-B) are significantly different at $p < 0.05$. | | | | |

Different studies show that phenolic and flavonoid contents increase during fermentation. Australian beach-cast seaweeds showed that the species *Durvillaea sp*. displayed a significant release of flavonoids (0.35 mg QE/g) during the intestinal digestion phase, flavonoid levels increased during colonic fermentation, indicating enhanced bioavailability during fermentation [55]. Irish brown seaweeds, *Saccharina latissima*, and *Laminaria digitata*, demonstrated that probiotic fermentation with *Lactobacillus rhamnosus* led to a 1.18- to 2.04 TFC increase [56]. In study phenolic content in brown seaweed *Sargassum wightii* (16.2±2.3) and *S. marginatum* (16.4±2.1 mg GAE/100g), *Enteromorpha compressa* (15.9±1.5) and green seaweeds *Ulva lactuca* (16.0±1.0 mg GAE/100g) and red seaweed *Gracilaria corticata* (12.5 ±2.0) and *Gracilaria verrucosa* (16.1 ±1.5) mg GAE/100g [57].

Fermentation of *Kappaphycus alvarezii*, through solid substrate fermentation with *Aspergillus oryzae,* resulted in significant enhancement of phenolic and flavonoid content, reaching up to 10.022 mg GAE/g, particularly on the fourth day of fermentation [58]. The effects of fermentation on the flavonoid and phenolic content of seaweeds demonstrate significant increases in bioactive compounds and antioxidant properties. *Gracilaria verrucosa*, a red seaweed fermented using the marine yeast *Aureobasidium melanogenic*, showed an increase in total phenolic content after 48 h of fermentation (5.62 mg GAE/g extract), along with improved radical scavenging activity, thus indicating a doubling in antioxidant potential compared with unfermented samples [59].

Fermentation of *Ulva sp*. and *Laminaria sp*. with *Saccharomyces cerevisiae* reduced aldehyde and increased aromatic compounds and nutritional properties [60]. Solid-state fermentation of different seaweed species, such as *Ulva rigida*, with fungi, such as *Aspergillus ibericus*, resulted in seaweeds' nutritional and functional properties [54]. When *Saccharina japonica* and *Undaria pinnatifida* were fermented with *Monascus purpureus*, their bioactive properties improved and increased the flavonoid content [61].

Fermentation of freshwater macroalgae like *Spirogyra sp*. using lactic acid bacteria resulted in a flavonoid content increase to 21.48 QEmg/g, proving that this method is effective in macroalgae fermentation [62]. Lactic acid bacteria and fungi (*Monascus)* fermentation mostly has increased flavonoid content, such as *Cladophora sp*. and *Microspora sp*. from different regions, increase in flavonoid content (11.55 QEmg/g and 13.48 QEmg/g).

Flavonoid content increased in brown seaweed *S. wightii* (4.1±1.2) and *S. marginatum* (4.3 ±1.0 mg RE/100g), green seaweeds *Ulva lactuca* (4.0 ±0.5 mg RE/100g) and red seaweed *Gracilaria corticata* (3.7 ±1.4) [57]. These conclusions demonstrate that the fermentation of seaweeds increases the availability and levels of flavonoids, thus improving their potential as functional

foods rich in antioxidants. This innovation could significantly impact their utilization in developing nutraceuticals and functional food products.

### 3.5 Changes in Antioxidant Activity (DPPH, FRAP) during Fermentation

During seaweed fermentation, antioxidant properties are significantly influenced by different factors such as time duration, temperature, type of bacteria, and acidity levels. *Lactobacillus plantarum* species have valuable potential for enhancing antioxidants due to their enzyme production. Increasing fermentation can increase DPPH and FRAP values but decrease over time due to extreme fermentation and the breakdown of beneficial compounds [63].

DPPH, a free radical, is commonly utilized to evaluate reducing agents. Due to its nitrogen-free radical composition, a free radical scavenger can readily neutralize DPPH. This study assessed the ability of the antioxidative substance to neutralize proton radicals. **Figures 3 (A)** and **(B)** show the DPPH value of *lactobacillus-based* (61.23±1.54%) and SCOBY-based (61.98±0.22 %) at 30 days of fermentation. **Figures 3 (C)** and **(D)** show the FRAP value of *lactobacillus-based* (17.53±0.08%) and SCOBY-based (19.98±0.08%) at the end of fermentation 30 days. Antioxidant activity was enhanced during the increase in fermentation time.

The fermentation process leads to the breakdown of complex compounds, the release of bioactive substances, and enhancing overall antioxidant capacity. In sorghum fermentation, DPPH and FRAP values increase with longer fermentation times [64]. Similarly, quinoa fermentation significantly increased total polyphenol content and antioxidant capacity measured by FRAP [65]. A study showed that the antioxidant capacity does not always increase smoothly with fermentation time. During garlic clove storage, antioxidant capacities measured by DPPH and FRAP reached maximum values at 8 weeks and then decreased [66]. Similarly,

in tef injera fermentation, no clear pattern was observed in the DPPH and FRAP values across different fermentation times [67].

Fermentation enhances antioxidant properties, as evidenced by increased DPPH radical scavenging and FRAP values. A notable example is the cheonggukjang fermentation, during which DPPH and ABTS radical scavenging activities and FRAP assay results show progressive improvement throughout fermentation [68]. Similarly, the fermentation of barnyard millet under optimized conditions resulted in improvements in DPPH% by up to 13.13% and FRAP by 13% individually [69]. Fermentation can enhance the antioxidant properties of various substrates. Fermentation of *Caulerpa racemosa*, a green alga, resulted in significantly higher total phenolic content, DPPH radical scavenging activity, and FRAP values compared to the unfermented sample [70].

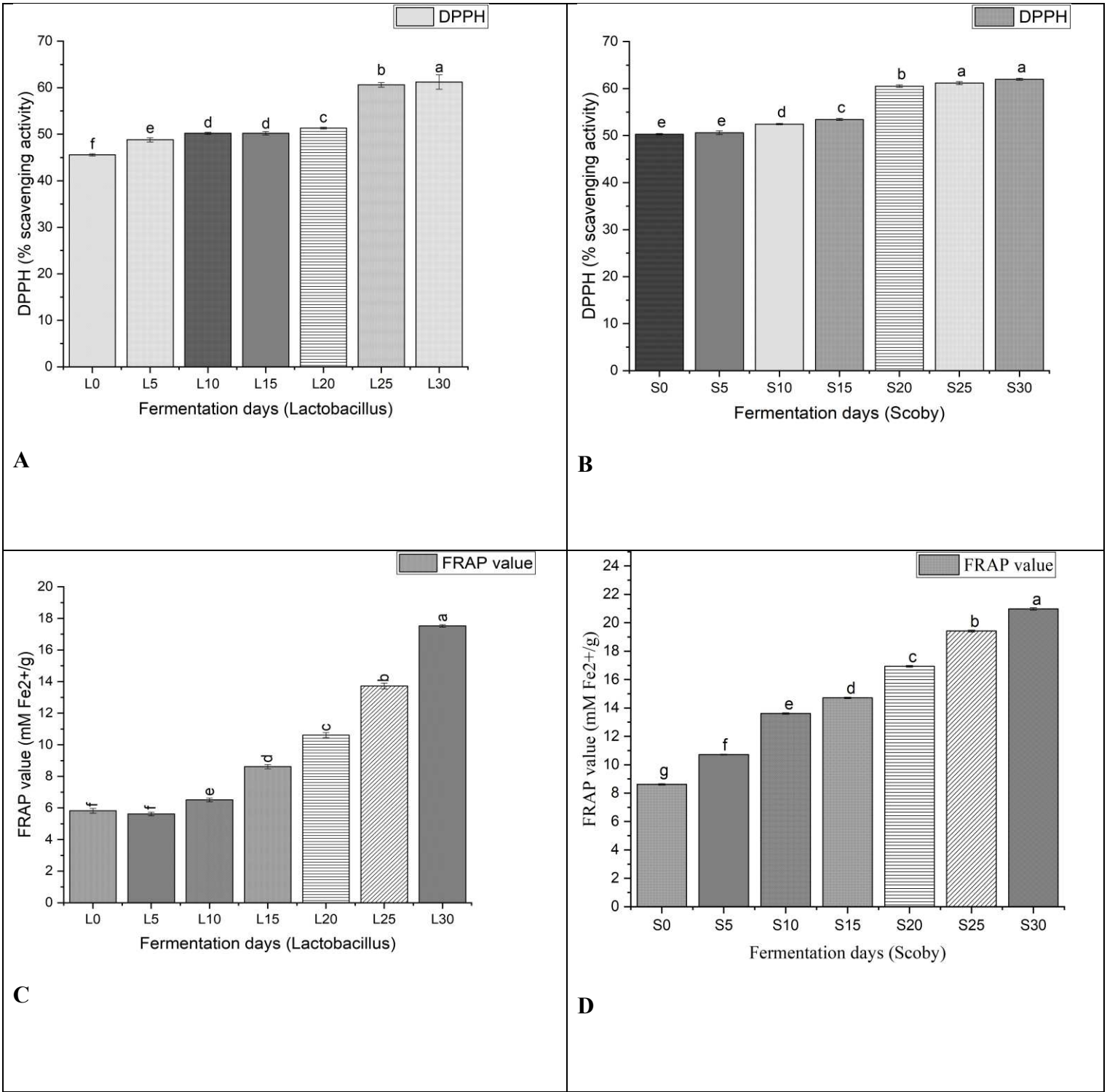



**Figure 3** DPPH and FRAP values of SCOBY and lactobacillus fermentation. Data are presented as the mean ± SE. The means marked with different letters are significantly different on different fermentation days ($P < 0.05$), and similar letters are not significantly different.

In conclusion, it was observed that increased fermentation times frequently correlate with increased DPPH and FRAP values due to the conversion of bioactive compounds. The optimum

fermentation time for extending the antioxidant properties of macroalgae and seaweed depends upon the substrate and fermentation conditions [71,72].

### 3.6 Heavy Metal Analysis of Fermented Seaweed Beverage

Human consumption of heavy metals can cause potential health risks, and most seaweeds get heavy metals from water and other environmental sources [73,74]. The fermentation and bio-adsorption techniques used in fermenting seaweed products define their capacity to adsorb metals, including Cd, Pb, and Cu, thereby affecting their heavy metal absorption. Since the quantity of metals exceeds the permissible limits set by food safety authorities, more observation of safety criteria is essential [75].

**Table 4** shows the heavy metal content in fermented seaweed drinks.

| **Seaweed drink** | **Arsenic mg/100g** | **Mercury mg/100g** | **Cadmium mg/100g** | **Lead mg/100g** | **Antimony mg/100g** |
|---|---|---|---|---|---|
| **Lactobacillus** | <0.001 | <0.001 | 0.015 | 0.001 | 0.002 |
| **SCOBY** | <0.001 | 0.003 | 0.002 | <0.001 | <0.001 |
| **Standard value** (mg/litre) | 0.010 | 0.002 | 0.005 | 0.005 | 0.006 |

**Reference standard value:** [76]

**Table 4** shows that the heavy metal content in seaweed beverages is low compared to the standard values and is safe for drinking. The value of metal contents in *Lactobacillus-based* fermentation Arsenic (<0.001), cadmium (0.015 mg/100g), lead (0.001 mg/100g), and SCOBY-based fermentation have Mercury (0.003 mg/100g), and cadmium (0.002 mg/100g) highest contents.

Several studies show that heavy metal concentrations in seaweeds change depending on the species, location, and type of metal. Brown macroalgae show higher levels of contamination

compared to green and red macroalgae [74] and Mixed seaweed products Mercury (Hg) have 0.02–0.15 mg/kg [77], Arsenic (As) content 0.5–3.0 mg/kg in *Gelidium*, *Himanthalia*, *Laminaria* species [78]. For heavy and toxic metals, acceptable concentrations were found in both seaweeds. The concentration ranges for the most undesirable heavy metals were as follows: Pb (0.39–0.51 mg/kg), As (0.11–0.40 mg/kg), Cd (0.01–0.02 mg/kg), and Hg (0.00–0.02 mg/kg) [79].

The studies showed that seaweeds contained the highest trace elements and metals levels, specifically iron, zinc, and manganese. The research highlights the need to constantly monitor toxic metals in food products, such as aluminum, cadmium, arsenic, and iodine, causing health risks. The Hazard Index for Al in green seaweed exceeded one unit for children, indicating a normal to high risk of adverse health effects [80]. Different processing and handling techniques influence the heavy metal content in seaweed. Heating, boiling, steaming, and microwaving can decrease the intensity of toxic metals in seaweeds and seaweed-based products. After that, proper heavy metal detection enhances the safety of seaweeds for consumption [81].

### 3.7 FTIR analysis

In Fourier Transfer Infrared spectra analysis, the major functional groups present in the seaweeds were carboxyl, hydroxyl, sulfate, and phosphate, which have excellent binding sites for metal retention [79]. FTIR spectroscopy observed that the variations in functional groups, before and after fermentation (using *Lactobacillus* and SCOBY), were recorded on an FTIR spectrometer across a wavenumber range of 400-4000 $cm^{-1}$. The Results showed only minimal changes in peak positions, representing that fermentation had an inadequate influence on the functional groups present in the seaweed.

The main chemical groups present in unfermented seaweed and fermented with SCOBY and *Lactobacillus* are illustrated in **Figure 4** and **Figure 5**. Based on the figures, the major chemical

constituents indicated that the highest O-H absorption ranging from 3400 and 3300 $cm^{-}1$ related to the main phenols chemical group, namely hydroxyl amide I, amide II, and primary amine groups present in fermented seaweed beverages, to which this larger inhibitory effect is observed [82]. Higher absorption was observed for both *Lactobacillus*-based beverages ranging between 3339 $cm^{-1}$ (Fig. 4), respectively, whereas, in SCOBY-based drinks, the highest hydroxyl peak was read at 3278 $cm^{-1}$ (Fig. 5).

Absorption ranging from 1100 to 1000 cm-1 was also observed, indicating C-H bending or C-O or C-C stretching vibration of carbohydrates and polysaccharides. Meanwhile, absorption from 1654-1700 $cm^{-1}$ showed the ester group's C-O stretches and N-O asymmetric stretching. C-C stretching vibration of aromatic ring compounds was also observed at 1300 $cm^{-1}$. Also, weak absorption at 877 and 882 $cm^{-1}$ indicated C-H bending vibration, confirming the presence of carbohydrates.

FTIR analysis has shown the presence of different key functional groups in seaweed-based fermented drinks. Alginates are over 33% of seaweeds' dry weight and have been identified through FTIR spectroscopy [83]. Moreover, findings on kombucha made from different ingredients showed the presence of functional groups such as C-N amine, C=C alkene, C≡C alkyne, and O-H alcohol [84,85]. These results show the flexibility of FTIR in detecting a wide range of functional groups in fermented seaweed drinks.

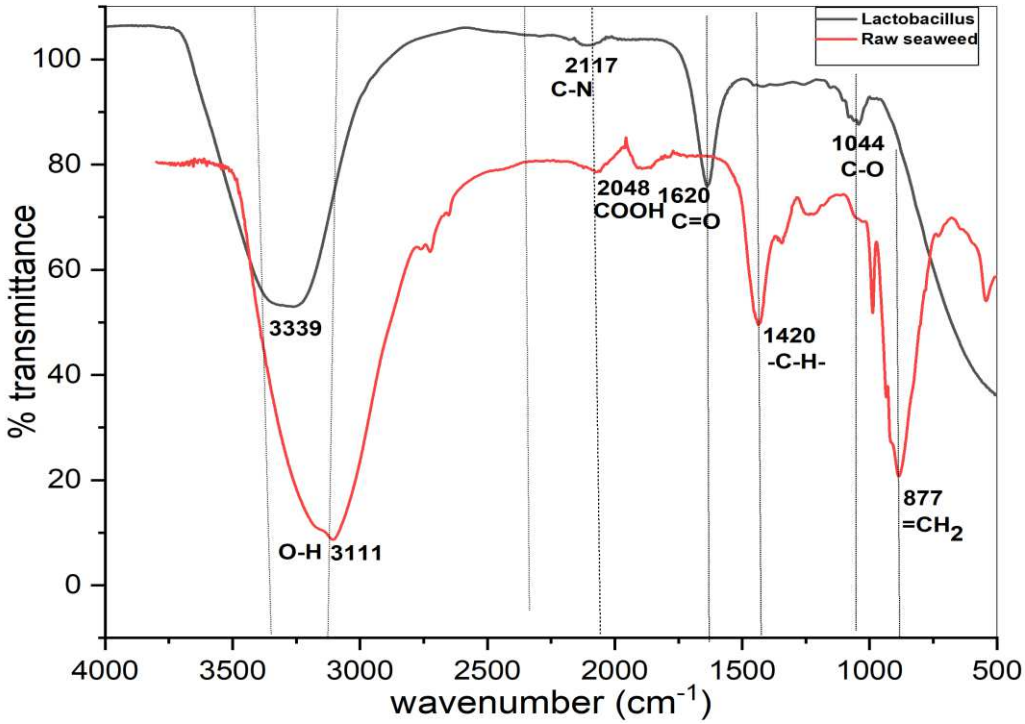



**Fig 4**: FTIR Spectra for Unfermented raw seaweed and fermented with *lactobacillus.*

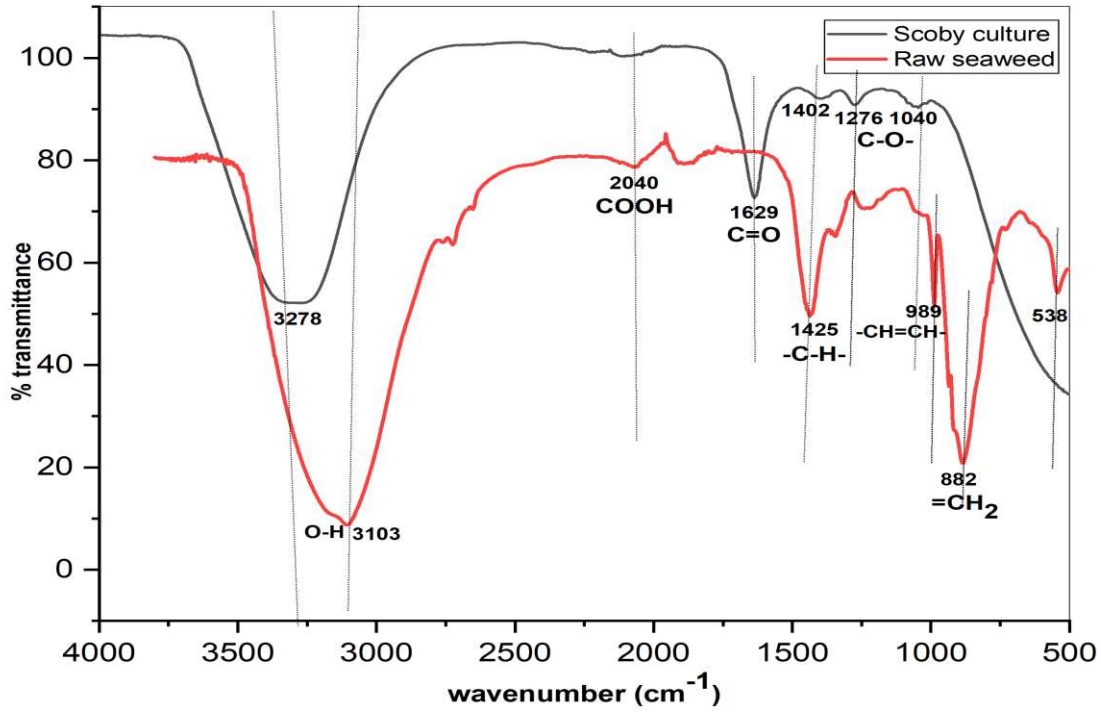



**Fig 5**: FTIR Spectra for Unfermented raw seaweed and fermented with SCOBY.

The study showed that Fermentation can significantly improve the extraction of flavonoids and phenolic compounds by using FTIR-ATR to characterize these compounds in plant-based

products. Specific functional groups related to these compounds include hydroxyl (OH) groups often seen in the range of 3200-3500 $cm^{-1}$, indicative of phenolic structure, and carbonyl (C=O) stretching at 1600-1650 $cm^{-1}$ associated with flavonoids [86]. FTIR-ATR and FT-Raman spectroscopy were utilized to identify seaweed polysaccharides, hydrocolloids, carrageenan, and agar. These compounds substantially conclude seaweed's gelling and emulsifying properties, which are preserved and modified through fermentation. The characteristic vibrational bands, especially in the 500-1500 $cm^{-1}$ range, help recognize different polysaccharides in fermented seaweed drinks [87].

FTIR spectroscopy was applied in the antioxidant profiling of seaweed and other Sea Fern (*Acrostichum aureum*) extracts, highlighting key functional groups like the C=C and C=O stretches. It demonstrated how spectroscopic methods can confirm phenolics and flavonoids' antioxidant potential in such extracts [88]. In a study, FTIR is used to analyze the lactic acid production from seaweed fermentation. Its detected characteristic hydroxyl (OH) bands around 3000-3500 $cm^{-1}$ and carbonyl (C=O) stretching at 1656 $cm^{-1}$ reveal the occurrence of alcohols and acids produced during fermentation [89].

### 3.8 Sensory evaluation

The sensory evaluation is evaluated by the judges' responses to the product and their liking on a scale. It plays a fundamental role in assessing the quality and acceptability of seaweed-based beverages and other food products. Studies have shown that different factors affect the sensory properties of these beverages, including processing methods, storage conditions, and different ingredient combinations. The results of the sensory evaluation for the *lactobacillus* and SCOBY-based drinks are presented in **Table 5**. After 30 days of fermentation, seaweed beverages were stored at 4 °C for 24 h, and the colour, flavour, taste, appearance, and overall

preference were assessed. Regarding overall sensory preference, SCOBY-based beverages were favoured over lactobacillus-based beverages ($P < 0.05$).

SCOBY culture used in the fermentation of seaweed-based drinks can change their sensory characteristics, increasing the density of flavours, acidity, and bioactive compounds. The fermentation of *Alaria esculenta* using *Lactiplantibacillus plantarum* and SCOBY has organic acids and volatile compounds, developing in sour and mild beverages. [90]. Brown seaweed tea, after a 1-minute blanching process, when compared its flavour to other products that are available commercially that have good sensory properties [91]. After the seaweed (*Ulva lactuca* and *Gracilaria*) blend, fermentation with anchovy flour enhanced sensory qualities, improving visual appeal, smell, thickness, taste, and overall consumer acceptability [92].

**Table 5:** Sensory evaluation of seaweed beverages after 2 days of storage at 4 °C. The content results evaluate the hedonic1-9 rating scale.

| **Seaweed beverages** | **Color** | **Flavor** | **Taste** | **Appearance** | **Overall acceptability** |
|---|---|---|---|---|---|
| **Lactobacillus** | $5.4 \pm 0.52^{b}$ | $5.5 \pm 0.53^{b}$ | $5.5 \pm 0.53^{b}$ | $5.4 \pm 0.52^{b}$ | $5.6 \pm 0.53^{b}$ |
| **SCOBY** | $6.4 \pm 0.97^{a}$ | $6.6 \pm 1.07^{a}$ | $6.6 \pm 0.84^{a}$ | $6.6 \pm 1.07^{a}$ | $6.7 \pm 0.67^{a}$ |

The sensory data shows mean with standard deviations (three samples/treatment). Mean sharing similar letters in a column (a-b) is statically non-significant ($p>0.05$)

The assessment of physicochemical quality and shelf life of *U. rigida* during storage. The result shows that changes in sensory properties were more significant than changes in physicochemical properties [93]. The macroalga *Hydropuntia eucheumatoides* seaweed fermentation from *Lacticaseibacillus casei* and *Saccharomyces boulardii* share similar color, density, smoothness, and viscosity but differ in smell and taste. The *Lacticaseibacillus casei* product tastes sour, whereas *Saccharomyces boulardii* is slightly sour and sweet [94]. The

fermentation processes of coffee processing that showed time duration significantly affect the sensory characteristics and overall quality of the beverage [95].

During the Fermentation process, the breakdown of compounds produces metabolites with different sensory profiles and affects the final product's characteristics. Adding hibiscus and tamarind to Kombucha can enhance the nutritional quality and phytochemical composition [96]. Fermentation in oat drinks produces volatile components such as diacetyl and acetoin, even though the overall taste and Odor are still correlated with oats rather than dairy [97]. Fermentation provides new components, such as ketones and alcohols, resulting in a distinct flavour profile from traditional seaweed drinks. This approach increases acidity while decreasing tropomyosin, a protein in seaweed that can influence flavour and safety when present in excessive concentrations.

## Conclusion

Seaweed fermentation enhances the bioavailability of nutritional and bioactive compounds, improving its functional properties for beverage development. In this study, fermented *Lactobacillus* and SCOBY were used to prepare good and nutritional seaweed-based beverage products. Throughout the 30-day fermentation, pH and acidity fluctuated. By the end of fermentation (day 30), Lactobacillus-based fermentation resulted in a higher pH ($5.2 \pm 1.64$), while SCOBY-based fermentation led to increased acidity ($0.9 \pm 0.07$). During fermentation, sugar content decreased as it was metabolized by microorganisms, leading to an increase in alcohol content over time. The protein content increased significantly ($p < 0.05$) during fermentation. However, Lactobacillus has a low protein content, and SCOBY has a high protein content (0.25 mg/ml). SCOBY-based seaweed beverages have high flavonoids and phenolic contents and smoothly increase during fermentation. Heavy metal analysis showed that cadmium levels in Lactobacillus-fermented beverages (0.015 mg/L) and mercury levels in SCOBY-based beverages (0.003 mg/L) were detected. However, all values remained within safe limits as per US FDA standards. Antioxidant activity was enhanced during the increase in fermentation time. Do emphasise that the results indicate that SCOBY-based seaweed beverages possess enhanced bioactive compounds, higher antioxidant activity, and improved sensory attributes, supporting their potential for commercialization in the functional beverage market.

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**Authors' Contributions**

Muhammad Qasim Ali: writing original draft, graph, Table, Nur Fathin Ruslan: graph and table: Noormazlinah Ahmad, Mohd Akmal Azhar, Mimi Sakinah Abdul Munim, Luay M Alsubhi, Nour Hamid Abdurahman, Abeer Essam Noman: Editing and Review of the Article.

**Ethics approval and consent to participate.**

The university's Ethics Committee Malaysia Pahang Alsultan Abdullah ethically approved the research. Prior to participation, all participants (faculty members and postgraduate students) were duly informed of their rights and responsibilities and provided explicit informed consent. The study was conducted in agreement with the guidelines governing research involving human participants, as outlined by the Ethics Committee of the Faculty of Chemical and Process Engineering Technology.

**Competing interests**

There is no conflict of interest.

**Data availability statement**

Not

**Funding Declaration**

No funding

**Consent to Publish Declaration**

Not Applicable

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