Characteristics of bioactive components in fermented *Sargassum* and *Ulva* Seaweed using SCOBY as potential anti-diabetic candidates

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Abstract. Sargassum and Ulva are abundant seaweeds in Indonesia, renowned for their bioactive compounds with diverse biological activities, including anti-diabetic properties. However, the full utilization of these seaweeds, particularly in the form of functional beverages, has not been achieved. Fermentation using Symbiotic Culture of Bacteria and Yeast (SCOBY) presents a promising method for producing functional seaweed beverages. This study aimed to analyze the chemical composition of Sargassum sp. and Ulva sp., assess their anti-diabetic properties, and investigate the effects of fermentation with SCOBY on their bioactive profile. Hot water extracts of Sargassum sp., Ulva sp., and a combination of both were fermented with SCOBY for 8 days at room temperature. The results revealed that Sargassum sp. contains hydroquinone phenols, steroids, triterpenoids, tannins, and saponins, whereas Ulva sp. contains hydroquinone phenols, steroids, and saponins. The combined seaweed extracts exhibited a higher total phenolic content (69.63 ppm) and demonstrated stronger anti-diabetic activity, with a low IC50 value for alpha-amylase inhibition (4.04±0.09 ppm). LC/MS profiling of extracts and fermented combinations highlighted differences attributed to SCOBY fermentation, suggesting the potential for enhanced anti-diabetic activity.

1 Introduction

Seaweeds are plentiful biological resources found in all Indonesian waters. They have emerged as significant export commodities that serve both the food and non-food sectors. In 2022, seaweed production in Indonesia reached 9,282,433 tons, securing the country in 2nd position in exports [1]. Seaweeds are classified into three main classes: Phaeophyta (brown algae), Rhodophyta (red algae), and Chlorophyta (green algae) [2]. *Sargassum* sp. and *Ulva* sp. are seaweeds widely distributed in Indonesian waters. *Sargassum* sp. are mainly wild and underutilized, whereas *Ulva* sp. have abundant but limited use as abalone feed and chips [3]. These seaweeds are widely distributed in Indonesia, including in the waters of Natuna, Sunda

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Strait, Lampung Bay, the Thousand Islands, Bali Coast, Spermonde, Anambas, Ternate, Ambon, Sumbawa, Kupang, Sumba, Karimunjawa, and Selayar. The production of *Sargassum* sp. reached 3,964 tons in 2013 and increased to 20,221 tons in 2015 in Dompu Regency, West Nusa Tenggara [4]. Studies by Mulyadi *et al*. [5] and Hudaifah *et al*. [6] revealed that *Sargassum* sp. and *Ulva lactuca* contain various bioactive components such as flavonoids, tannins, saponins, β-carotene, alkaloids, phenolics, steroids, and glycosides.

The bioactive compounds in seaweeds play a significant role in health-related biological activities, including antioxidant, antibacterial, anti-inflammatory, antitumour, anticancer, antihypertensive, and antidiabetic effects. Samudra *et al*. [7] demonstrated that *Sargassum* sp. extracts have antidiabetic properties, reducing blood glucose levels by 54.20% and 58.12% in alloxan-treated mice at a dose of 5 mg/20 kg body weight. This reduction was compared to that of the positive control, glybenclamide, which showed a decrease of only 42.86%. Reka *et al*. [8] found that *Ulva lactuca* extract had inhibitory activity against α-amylase (83.43±2.5) and α-glucosidase (61.81±1.83). These studies indicate that *Sargassum* sp. and *Ulva lactuca* seaweeds have potential as antidiabetic agents, a crucial consideration given the high prevalence of diabetes cases in Indonesia, reaching 19.47 million people in 2021, according to the International Diabetes Federation survey. The Ministry of Health Indonesia (KEMENKES) reported a prevalence of diabetes mellitus in Indonesia at 2%, showing an increase from 1.5% in 2013 [9].Among the innovative applications of seaweed is its conversion into food products, such as fermented tea, commonly known as kombucha.

Fermented products offer many advantages in terms of nutrition, nutraceuticals, digestibility, enhanced product flavor, increased nutritional value, elevated active compounds, and reduced anti-nutritional compounds [10]. Fermentation technology using the microbial consortium SCOBY (Symbiotic Colony of Bacteria and Yeast) on tea-produced fermented kombucha [11]. The fermentation process yields other compounds such as ethyl glucuronate, oxalate, lactic acid, 5-ketoglucuronate, water-soluble vitamins (B1, B6, B12, and C), catechins, teaflavins, and flavonols [12]. The fermentation of kombucha can increase the antioxidant activity from 79.30% to 92.85% [13].

Fermented seaweeds made from *Sargassum* sp. and *Ulva* sp. were predicted to have better bioactivity than non-fermented seaweeds. Research on the impact of seaweed species and fermentation process (seaweed kombucha) on the phytochemical profile and bioactivity is still limited. This study aimed to characterize the chemical components of Sargassum and Ulva seaweed, assess the anti-diabetic activity of seaweed extracts, and evaluate the impact of fermenting these seaweed extracts with a SCOBY (Symbiotic Culture of Bacteria and Yeast) on the bioactive component profile.

2 Methods

2.1 Sampling of seaweed

Sargassum sp. seaweed was obtained from Panganten Beach, Ujung Genteng, Cikangkung Village, Ciracap District, Sukabumi Regency, and West Java. The coordinates of the sampling point are at latitude -7.36759° and longitude 106.45732°. *Ulva* sp. seaweed originates from the waters of Tual, Rumaat Village, Kei Kecil Timur District, Southeast Maluku Regency, and Maluku. The sampling point coordinates at latitude -5.825854° and longitude 132.810874°. Samples of *Sargassum* sp. seaweed were collected fresh and stored in bags, whereas *Ulva* sp. samples were collected under dry conditions after being sun-dried for 3-5 days. Fresh *Sargassum* sp. seaweed was air-dried after reaching the laboratory to prevent decomposition. The dried *Sargassum* sp. and *Ulva* sp. seaweeds were then rinsed with fresh water to remove impurities and residual sand. Seaweeds that remain attached to

corals or rocks and other attached seaweeds are removed during the cleaning process. The cleaned seaweed was dehydrated using a dehydrator at 60 °C for 5-6 hours. Once dried, the seaweed was pulverized using a blender until a coarse powder was formed.

2.2 Phytochemical assay of dried seaweed

Phytochemical analysis was used to determine the active components of dried *Sargassum* sp. and *Ulva* sp. seaweeds. Qualitative phytochemical tests included assessment of alkaloids, flavonoids, hydroquinone phenols, tannins, saponins, steroids, and triterpenoids.

2.3 Extraction and fermentation of seaweed

Seaweed extraction was performed using the hot-water method. The treatments consisted of 120 g of dried *Sargassum* sp., 120 g of dried *Ulva* sp., and a combination of dried *Sargassum* sp. and *Ulva* sp. seaweed weighing 60 g. Boiling water (1 L) at a temperature of 100 °C extracted 120 g of dry seaweed in 5 min. The extraction results were filtered using filter paper no.1. A total of 28 g of stevia was added to 1 liter of extraction filtrate [14]. One piece of SCOBY culture and 10% (v/v of the total fermented seaweed) starter liquid was added to the seaweed tea. Fermentation was performed in a covered (anaerobic) container at room temperature (18-25 °C) for eight days. Successful fermentation was indicated by the formation of a new SCOBY layer on its surface. We assumed that the fermentation process proceeds at a specified time. In this case, it was halted by removing the parent and offspring SCOBY cultures and then filtering the liquid to remove any remnants of the SCOBY culture. pH, total titratable acidity, and alcohol content were measured during fermentation.

2.4 Evaluation of total phenol production

Quantitative analysis of the total phenols was performed on the extraction filtrate of seaweed using the AOAC method [15]. A 2.5 mL mixture of 10% Folin-Ciocalteu reagent and 7.5% $NaHCO_3$ was added to the 1 mL sample. In the blank solution, 0.5 ml of methanol, 10% Folin-Ciocalteu reagent, and 7.5% $NaHCO_3$ (2.5 mL) were added. The samples were then incubated for 1 h at 45 °C. Then, the absorbance of the samples was determined using UV-Vis spectrophotometry at a 725 nm. Repetitions were performed for up to three times. Total phenol content was calculated using the following formula:

$$Total\ phenolic\ content = c \times \left(\frac{V}{m}\right) \quad (1)$$

Explanation:
cConcentration of total phenol from gallic acid standard curve (mg/L).
V : sample volume (L)
m : sample weight (g)

2.5 Evaluation of inhibition α-amylase activity

α-Amylase enzyme inhibition was evaluated on the concentrated extract following a previously established method [16]. The samples underwent dilution with phosphoric acid buffer to achieve at least five distinct concentrations. Then, 200 µl of the sample was combined with 200 µl of α-amylase enzyme (2 units/mL). This mixture was allowed to incubate for 15 minutes at 37 °C. Following this, 200 µl of starch substrate dissolved in buffer was introduced, and the mixture underwent an additional 10-minute incubation at 37 °C.

Furthermore, 1 ml of DNS color reagent was added to the incubation mixture and allowed to react for 10 minutes before being heated in a water bath at 85 °C until a color change occurred. Once the color changed, the reaction tubes were taken out and cooled to room temperature for absorbance measurement using a UV-Vis spectrophotometer at 540 nm. A blank was prepared using 100% enzyme and the sample was replaced with a buffer. A reaction blank was created using the sample at each concentration without enzyme addition. The positive control was treated with acarbose, using the same procedure.
The inhibitory activity of α-amylase was presented as a percentage of inhibition and was determined using the formula:

$$\%Inhibition = \left(1 - \frac{(Abs\ B - Abs\ C)}{Abs\ A}\right) \times 100 \quad \text{.....(2)}$$

Explanation:
Abs A : Absorbance with α-amylase enzyme and without sample
Abs B : Absorbance with α-amylase enzyme and sample
Abs C : Absorbance with sample and without α-amylase enzyme

2.6 Profiling bioactive compound using LC-MS/MS

The analysis was conducted using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The samples were first prepared by Solid-Phase Extraction (SPE). The prepared samples were then drawn using a microsyringe (5 µL each) and injected into the sample inlet, entering the UPLC column. A single replicate was used. Chromatograms of polar compounds first appeared, followed by those of compounds with lower polarity levels. The separation results were read using a QToF-MS detector, which generated chromatogram peaks and were interpreted using Masslynx software.

2.7 Data analysis

In this study, a Completely Randomized Design (CRD) was utilized, incorporating various types of seaweed extracts as treatments. Data were evaluated for both normality and homogeneity. If the data met the criteria of normal distribution and homogeneity, further analysis was carried out using Analysis of Variance (ANOVA) at a 95% confidence interval (α=0.05). If significant effects were detected ($p < 0.05$), Duncan's test was applied with a confidence level of 95%.

3 Results and discussion

3.1 Phytochemical compound on dried seaweed of *Sargassum* sp. and *Ulva* sp.

The composition of the active compounds in *Sargassum* sp. and *Ulva* sp. can be identified through qualitative phytochemical screening. Dried *Sargassum* sp. contain hydroquinone phenols, steroids, triterpenoids, tannins, and saponins as its active components. In contrast, dried *Ulva* sp. contain hydroquinone, phenol, steroids, and saponins as its bioactive components. Steroids were dominant in *Sargassum* sp., whereas *Ulva* sp. contained a higher concentration of saponins (**Table 1**).

Table 1. Phytochemical compound of dried seaweed.

Phytochemical	*Sargassum* sp.	*Ulva* sp.
Alkaloids	-	-
Flavonoids	-	-
Phenol hydroquinone	+	+
Steroids	+	+
Triterpenoids	+	-
Tannins	+	-
Saponins	+	+

Note : (+): detected; (-): not detected

The findings of this study exhibit some variance from previous research that examined the phytochemical compositions of *Sargassum* sp. and *Ulva* sp. after organic solvent extraction. Nurjanah *et al*. [17] reported the presence of flavonoids, tannins, hydroquinone phenols, and steroids in *Sargassum* sp. The ethanol and n-hexane extracts of *Sargassum* sp. exhibited positive identification of alkaloids and triterpenoids, whereas the ethyl acetate extract of *Sargassum* sp. was positive for hydroquinone phenols [18]. According to an investigation by Bensy *et al*. [19], an ethanol extract of *Ulva* sp. contained alkaloids, tannins, and saponins. The ethyl acetate extract was positive for alkaloids, flavonoids, and terpenoids. The chloroform extract tested positive for alkaloids, flavonoids, saponins, and steroids. The methanol extract exhibited positive results across all test parameters, whereas the acetone extract only showed negative results for flavonoids. Variations in phytochemical composition are influenced by several factors such as the specific type of seaweed, habitat, season, extraction methods, and solvents [20]. Dried *Sargasum* sp. and *Ulva* sp. underwent hot water extraction prior to fermentation with SCOBY. The extracts were concentrated using an evaporator and subsequently analyzed for total phenolic content and alpha-amylase inhibitory activity.

3.2 Total phenol and inhibition α-amylase activity of seaweed extracts

The results of the total phenol test of the liquid extract of seaweed showed that the combined extract of *Sargassum* sp. and *Ulva* sp. had the highest total phenol content (69.63 ppm). The lowest total phenol content was found in the *Ulva* sp. extract, with a total of 27.26 ppm. The total phenol content in the combined extract of both seaweed species was higher than that in the extracts of each material. This indicates a synergistic effect between the active components present in both types of seaweed, which can enhance the composition of the active compounds. The analysis of variance results showed that the composition of seaweed significantly affected the total phenol content ($p<0.05$). The interaction between the active compound components can result in antagonistic and synergistic effects. Antagonistic effects confer resistance, whereas synergistic effects provide benefits [21]. Synergistic effects are characterized by the higher activity of combination extracts than single extracts [22]. Better effects occur when combining compounds such as alkaloids, flavonoids, tannins, and saponins, which work synergistically.

Fig. 2 demonstrates significant differences in the IC_{50} values of the extracts against the alpha-amylase enzyme among the different extract types ($p<0.05$). The best result, based on the smallest IC_{50} value, was obtained from the *Sargassum* sp. and *Ulva* sp. combination

extracts, with an average value of 4.04±0.09 ppm. As the positive control, acarbose obtained an IC_{50} value of 44.79±5.14 ppm. The IC_{50} value of the combination extract of *Sargassum* sp. and *Ulva* sp. lower than that of the individual extracts of *Sargassum* sp. and *Ulva* sp. The inhibitory capability of seaweed extracts was speculated to be associated with the phenolic content, wherein the combined extracts of *Sargassum* sp. and *Ulva* sp. exhibited the highest phenolic content (**Fig. 1**). Additionally, it is suspected that the diversity of bioactive components in the extracts also influenced α-amylase inhibitory ability. Polyphenols are one type of phytochemical components that can bind to the active sites of α-amylase [23]. Polyphenols can act as natural inhibitors that hydrolyze carbohydrates from enzymes, thus inhibiting the increase in blood glucose levels. The inhibition of α-amylase by polyphenols is similar to the principle of acarbose inhibition, which delays carbohydrate hydrolysis and glucose absorption and inhibits the conversion of sucrose to glucose [24].

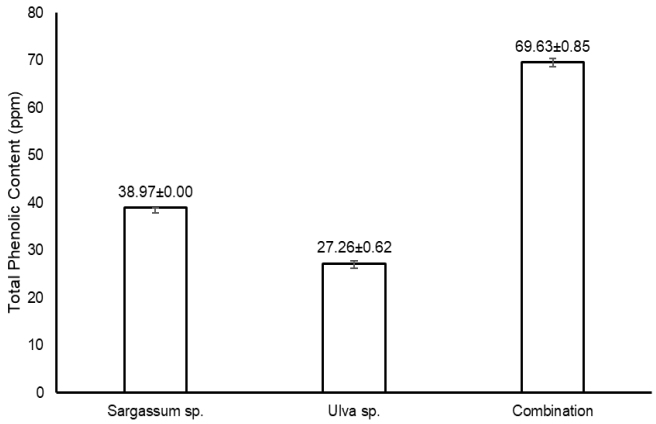

Fig. 1. Total phenolic content of seaweed extracts.

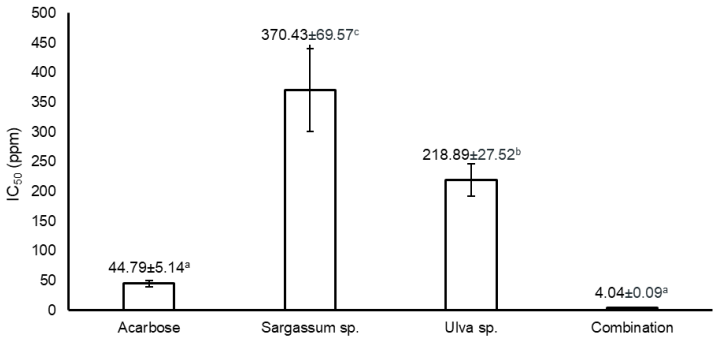

Fig. 2. Inhibition α-amylase activity of seaweed extracts.

3.3 Profile of fermented seaweed extracts

The pH of the fermented seaweed significantly decreased during the 8-day fermentation period. The pH values of seaweed-fermented seaweed during the 8-day fermentation range from 3.06 to 3.44, with the lowest value observed in the combination of *Sargassum* sp. and *Ulva* sp. fermented seaweed on the eighth day of fermentation, whereas the highest value was found in *Sargassum* sp. fermented seaweed on the 0th day of fermentation. Fermented seaweeds with different compositions exhibited different pH values. Fermented seaweed has

a pH value ranging from 3.06 to 3.16 after 8-days fermentation. On the 0th day of fermentation, the samples already exhibited acidic pH (pH<7) due to the addition of a fermented seaweed starter, which facilitated the fermentation process with its initial acidic properties. The obtained pH values of fermented seaweed fall within the range of pH values recommended for fermented seaweed by Kombucha Brewers International (2.20-3.80). Analysis of variance (ANOVA) indicated that fermentation time significantly influenced the total acid content of the fermented seaweed (p<0.05). At the same time, seaweed composition did not have a significant effect (p>0.05) on the total acid content of the fermented seaweed. No interaction between fermentation time and seaweed composition significantly affected the total acid content of fermented seaweed (p>0.05).

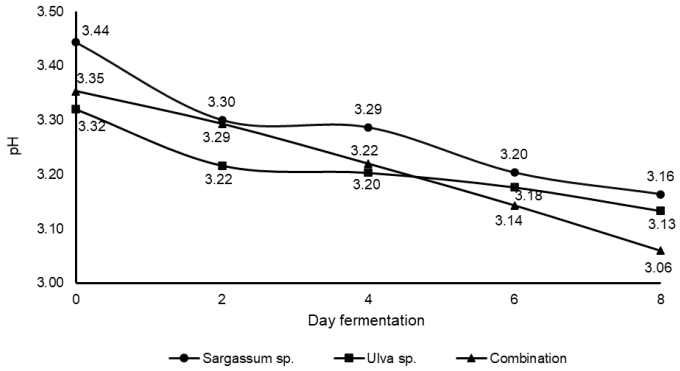

Fig. 3. Profile acidity (pH) of fermented seaweed extracts: : *Sargassum* sp. (circle legend), *Ulva* sp (square legend), and combination (triangle legend)

The total acid content of the fermented seaweed significantly increased during fermentation. The total acid content of the fermented seaweed ranged from 0.75% to 0.98% after 8-days of fermentation. The increase in total acid content during fermentation indicates an inverse relationship with the decrease in the pH of the fermented seaweed during fermentation. The total acid content of fermented seaweed still falls within the range of total acid values determined by Kombucha Brewers International (0.27-2.03%). Analysis of variance (ANOVA) indicated that fermentation time significantly influenced the total acid content of the fermented seaweed (p<0.05). At the same time, seaweed composition did not have a significant effect (p>0.05) on the total acid content of the fermented seaweed. The interaction between fermentation time and seaweed composition significantly affected the total acid content of fermented seaweed (p<0.05).

The change in total acidity during the fermentation period was caused by the production of organic acids generated by SCOBY. According to Zubaidah *et al*. [25], the accumulation of organic acids increases the total acid content and decreases the pH of kombucha. *Acetobacter xylinum* produces primary metabolites, such as acetic acid, gluconic acid, guluronic acid, malic acid, tartaric acid, citric acid, butyric acid, and lactic acid. *Saccharomyces cerevisiae* converts glucose and fructose into alcohols and CO_2 through glycolysis during fermentation. The CO_2 produced reacts with water to form carbonic acid, while the alcohol is oxidized by *Acetobacter xylinum* into acetaldehyde, which then becomes acetic acid.

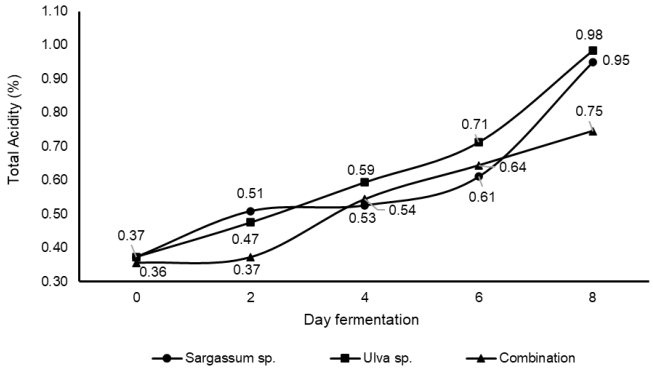

Fig. 4. Profile titratable acidity of fermented seaweed extracts: *Sargassum* sp. (circle legend), *Ulva* sp (square legend), and combination (triangle legend)

3.4 Chemical compound profiling of extract and fermented extract

The profiling of bioactive components was conducted by LC-MS/MS analysis. Analysis was performed on the extracts and fermented seaweed extract combinations of *Sargassum* sp. and *Ulva* sp., which showed the best results in the α-amylase enzyme activity test. The analysis resulted in 2 (two) chromatograms, as shown in **Fig. 5**. Peaks with greater heights indicate the presence of more compounds (dominant). Both chromatograms showed differences in the peaks produced by the seaweed extracts and fermented seaweed. Identical peaks were detected in both chromatograms, such as the peak at a retention time of 17.00 minutes, which had a greater height in the fermented seaweed chromatogram. This indicates the presence of additional or larger compounds in the fermented seaweed sample than in the seaweed extract. The quantity of this bioactive component is believed to affect the bioactivity of seaweed and kombucha extract. However, in this study, we were unable to assess the bioactivity of kombucha as an alpha-amylase inhibitor because of inappropriate methods. According to Suhardini and Zubaidah [13], the fermentation process of kombucha has been demonstrated to increase antioxidant activity from 79.30% to 92.85%.

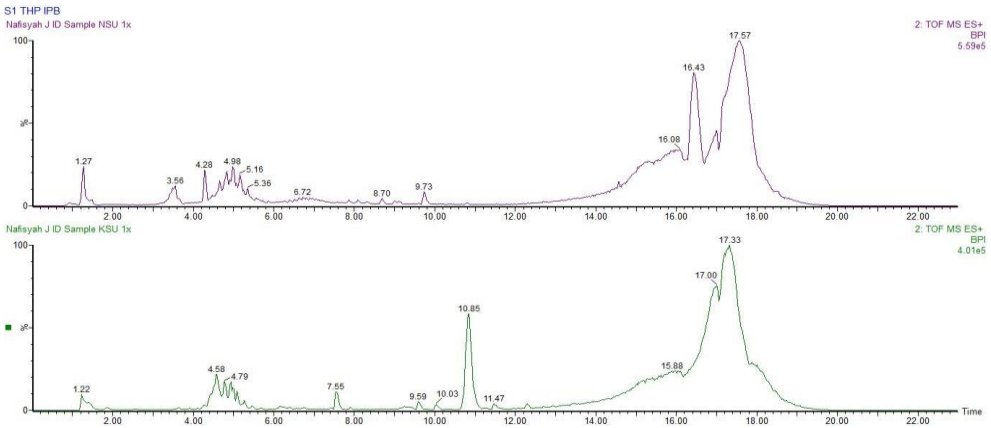

Fig. 5. LC-MS/MS chromatogram seaweed extract (up) and fermented seaweed extract (down).

Table 2. Chemical compound profiling of extract and fermented extract from a combination of *Sargassum* sp. and *Ulva* sp.

Combination Seaweed Extract			
RT (min)	**MW (m/z)**	**Formula**	**Chemical compound**
1.27	191.0917	$C_8H_{14}O_5$	1,2-O-Isopropylidene-α-D-xylofuranose
3.56	191.0918	$C_8H_{14}O_5$	1,2-O-Isopropylidene-α-D-xylofuranose
4.28	120.0805	C_8H_9N	Indoline
4.98	103.0377	$C_4H_6O_3$	2-Oxobutyric acid
6.72	89.0593	$C_4H_8O_2$	Butyric acid
8.70	120.0819	$C_5H_{13}NS$	3-(Dimethylamino)-1-propanethiol
16.43	685.4384	$C_{36}H_{52}N_{12}O_2$	N,N'-2,7-Naphthalenediylbis[4-({2-[(diaminomethylene)amino]ethyl}amino)-1-(3-methylbutyl)-1H-pyrrole-2-carboxamide]
17,57	355.0719	$C_5H_{11}N_{12}O_5Cl$	N/A
Fermented Combination Seaweed Extract			
RT (min)	**MW (m/z)**	**Formula**	**Chemical compound**
1.22	85.0293	$C_4H_4O_2$	2(5H)-Furanone
4.79	89.0597	$C_4H_8O_2$	Butyric acid
7.55	163.0596	$C_2H_6N_6O_3$	1H-1,2,4-Triazole-3,5-diamine nitrate (1:1)
9.59	105.0332	C_7H_6O	Benzaldehyde
10.03	240.2308	$C_{15}H_{29}NO$	N-Dodecylacrylamide
10.85	171.1364	$C_{10}H_{18}O_2$	γ-Decalactone
11.47	268.2640	$C_{17}H_{33}NO$	Hexadecyl isocyanate
15.88	184.0736	$C_3H_5N_9O$	N/A
17.00	355.0695	$C_{20}H_{18}S_3$	1,1',1''-(1,1,1-Ethanetriyltrisulfanediyl)tribenzene
17.33	355.0713	$C_5H_{11}N_{12}O_5Cl$	N/A

Analysis of the chromatogram of the combined extract of *Sargassum* sp. and *Ulva* sp. revealed several bioactive compounds. The highest peak at a retention time of 17.57 minutes detected a compound with the molecular formula $C_5H_{11}N_{12}O_5Cl$. Other detected compound components included butyric acid, 2-oxobutyric acid ketone, a derivative of monosaccharide α-D-xylofuranose in the form of 1,2-O-Isopropylidene-α-D-xylofuranose, and indoline, an organic heterocyclic compound with an aromatic ring. Butyric acid compounds can be obtained from brown seaweeds such as *Padina australis* and *Turbinaria conoides* [26]. The highest peak in the chromatogram of the fermented seaweed combination of *Sargassum* sp. and *Ulva* sp. was detected at a retention time of 17.33 minutes, and it contained $C_5H_{11}N_{12}O_5Cl$, which was the same as the highest peak in the extract chromatogram. Butyric acid has also been detected in fermented seaweed. Butyric acid also serves as a product of fermentation, and butyric acid fermentation is carried out by *Clostridium acetobutylicum*. Glycolysis involves the oxidation of sugars into pyruvic acid, which is subsequently oxidized by the enzyme pyruvate-ferredoxin oxidoreductase into acetyl-CoA. Some of the acetyl-CoA is converted to acetic acid and acetoacetyl-CoA. Acetoacetyl-CoA is then reduced to butyryl-CoA, which is transformed into butyric acid. Carbon dioxide is generated during butyrate fermentation, which leads to an increase in pH [27]. Butyric acid has several benefits for

treating gastrointestinal diseases such as diarrhea, intestinal inflammation, functional disorders, and dysbiosis, as well as postoperative and chemotherapy conditions [28].

Moreover, organic compounds and fermentation byproducts were detected in the fermented seaweed profile. The detected fermentation by-products included 2(5H)-furanone as a flavor component, γ-decalactone as an aromatic compound, and benzaldehyde as a volatile flavor compound. γ-Decalactone was synthesized by yeast *Yarrowia lipolytica* through oxidative degradation of fatty acids [29]. A similar metabolic process yields γ-decalactone in kombucha through the activity of the bacterium *Acetobacter indonesiensis* [30]. Furthermore, benzaldehyde levels demonstrated an increasing trend during fermentation. This carbonyl component is formed from the oxidative degradation of sugars and fatty acids, which reacts with amino groups in amino acids [31]. Suffys *et al*. [32] detected benzaldehyde compounds on days 7^{th} and 9^{th} days of kombucha fermentation. The yeast *Zygosaccharomyces* sp. can induce the synthesis of volatile organic compounds such as benzaldehyde, isoamyl alcohol, and phenethyl acetate [33].

4 Conclusion

The phytochemical content of the seaweed species influences its α-amylase inhibitory activity. The combination of *Sargassum* sp. and *Ulva* sp. seaweeds exhibited synergistic properties among their phytochemical components, resulting in improved α-amylase activity. The seaweed kombucha was successfully obtained after an 8-day fermentation, exhibited acidic properties. Fermentation of seaweed with SCOBY produces several compounds that are believed to contribute to the flavor and biological activity of kombucha.

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