

The influence of fermentation using marine yeast *Hortaea werneckii* SUCCY001 on antibacterial and antioxidant activity of *Gracilaria verrucosa*

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Abstract. Fatmawati F, Sibero T, Trianto A, Wijanti DP, Sabdono A, Pronggenies D, Radjasa OK. 2022. The influence of fermentation using marine yeast *Hortaea werneckii* SUCCY001 on antibacterial and antioxidant activity of *Gracilaria verrucosa*. *Biodiversitas* 23: 5258-5266. *Gracilaria verrucosa* is producer of agar, a polysaccharide, that widely used in pharmaceutical and food industries. However, several studies have shown that the secondary metabolite of this seaweed is not potential as an antimicrobial and antioxidant agents. This study aimed to determine the antibacterial and antioxidant properties of methanolic extract of *G. verrucosa* after fermentation using marine yeast *Hortaea werneckii* SUCCY001. The samples were fermented based on different optimization times, specially in three, six and nine days. Samples were extracted using methanol by maceration method. Then the extracts were tested for antimicrobial potential against the several human clinical pathogens namely *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Micrococcus luteus* and *Pseudomonas aeruginosa*. The antioxidant potential was analyzed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-Azinobis 3-ethyl benzothiazoline 6- sulfonic acid (ABTS) method while total phenolic content (TPC) was measured using Folin-Ciocalteu. Secondary metabolites were characterized using thin layer chromatography (TLC) and GC-MS. TLC plate visualization using UV light, DPPH, FeCl₃ and vanillin-sulfuric acid reagents. The results showed that the extract fermented for nine days was able to inhibit the growth of *P. aeruginosa* and *M. luteus* at a concentration of 2 mg/mL. The highest antioxidant activity was found in the nine days fermented extract with an RSA value of 21.9% ± 0.0001 for DPPH and 12.53 % ± 0.002 for ABTS. Furthermore, the highest total phenolic content was found in the nine days fermented extract of 7.57 GAE/mg. The results of characterization using TLC showed the presence of phenolic compounds, terpenoids and antioxidants in fresh seaweed extracts, autoclaved fresh seaweed, three days of fermentation, six days and nine days. Based on this research, it can be seen that the addition of marine yeast starter *H. werneckii* SUCCY001 was able to increase the antibacterial and antioxidant activity of *G. verrucosa*.

Keywords: Antibacterial, antioxidant, fermentation, seaweed, yeast

INTRODUCTION

Nowadays, bacterial infection have become a major challenge in the worldwide since the occurrence of multi-drug resistant organisms (Prestinaci et al. 2015). Hence, it is necessary to explore a new antibacterial agents from natural sources (Wijaya et al. 2022). Besides, synthetic antioxidants including butylated hydroxyl toluene (BHT), and butylated hydroxyl anisole (BHA) have been extensively used to stabilize oils and fats in food products (Asif et al. 2021; Viana da Silva et al. 2021). Regrettably, the overuse of these substances triggers side effects and the emergence of various diseases. Therefore, a safer natural antioxidant and antibiotic agents are urgently required. For instance, natural antioxidants and antibiotic agents are easily found in marine organisms, such as seaweed (Gunathilake et al. 2022).

Seaweeds are marine macroalgae that lives floating or attached to shallow water, which plays an important ecological role. They are divided into three large group based on their pigments, namely Rhodophyta (red

seaweed), Phaeophyta (brown seaweed) and Chlorophyta (green seaweed). Seaweeds have been one of the major commodities in marine and fisheries sector which produce essential primary metabolites such as agar, carrageenan and alginate that are widely used in various industries, such as foods, cosmetics and pharmaceuticals (Naufal et al. 2022; Yao et al. 2022). In addition, seaweeds also contain secondary metabolites that have diverse functions for themselves and can serve as anticancer, anti-aging, antifouling and anti-inflammatory agents for human needs (Widodo et al. 2019; Lomartire et al. 2021).

Gracilaria verrucosa is one type of red seaweeds that is widely cultivated in Indonesia. In the last few decades, coastal communities have extensively consumed them as a source of medicine and food to treat certain diseases (Torres et al. 2014). *G. verrucosa* contains bioactive compounds such as flavonoids, terpenoids, alkaloids and phenols that can be utilized as antibacterial and antioxidant (Maftuch et al. 2016; Rusli et al. 2016; Atta et al. 2017). Many studies have been carried out on the antibacterial and antioxidant activity of *G. verrucosa*. Widodo et al. (2019)

and Setiabudi et al. (2020) showed *G. verrucosa* did not exhibit antibacterial activity and had a weak antioxidant potential. Therefore, a new methodology is needed to optimize the antibacterial and antioxidant activity of *G. verrucosa* by fermentation.

Fermentation is a process of converting a complex organic substances into a smaller and simpler substances by utilizing certain microorganism to increase the added value of the product (Feng et al. 2018). Fermentation is usually carried out by using microorganisms such as bacteria and fungi as fermenting agents (Xiang et al. 2019). Yeast, such as *Saccharomyces cerevisiae*, is the most common fungi that is applied in fermentation (Maicas, 2020). However, the application of other yeast species for fermentation is rarely be conducted. On other studies have shown that *H. werneckii* can be a potential source of bioactive compounds. Rani et al. (2013) and Saleh et al. (2018) reported yeast *H. werneckii* contained melanin pigment, which was known to protect the skin from ultraviolet radiation. Furthermore, Hodhod et al. (2020) also informed about the antibacterial activity of crude extract of *H. werneckii* against *Methicillin-resistant Staphylococcus aureus* (MRSA), *Campylobacter jejuni* and *Salmonella typhimurium*. Interestingly, a marine *H. werneckii* SUCCY001 was isolated as a seaweed-associated marine yeast (Sibero et al. 2022). This study also discovered the ability of *H. werneckii* SUCCY001 to degrade several marine polysaccharides. Therefore, this isolate is expected to be a potential fermenting agent for seaweed fermentation.

In order to increase the antibacterial and antioxidant activities of *G. verrucosa*, a fermentation with the addition of marine *H. werneckii* SUCCY001 was conducted. This research aimed to were to understand the influence of seaweed fermentation using marine *H. werneckii* SUCCY001 to *G. verrucosa*'s antibacterial and antioxidants activities, total phenolic content, and metabolite profile.

MATERIALS AND METHODS

Microbial culture

H. werneckii SUCCY001 is a collection from laboratory of Natural Product, Integrated Laboratory for Research and Service, Universitas Diponegoro. The isolate *H. werneckii* SUCCY001 was cultured in YPG medium (Yeast extract Peptone Glucose) (Rogalski et al. 2021). Then YPG medium containing *H. werneckii* SUCCY001 was agitated for 3×24 hours at room temperature using a shaker at 150 r.p.m (Obata et al. 2016).

Seaweed preparation

Fresh seaweed was purchased from Center for Brackish Water Aquaculture (BBPBAP), Jepara to the laboratory then washed with fresh water (Muthukrishnan and Raja, 2021). Afterwards, the seaweed was resized using blender machine and weighed 50 grams then put into erlenmeyer containing 100 mL YPG medium. As a control, fresh seaweed and autoclaved seaweed (121°C, 1 atm, 15 mins)

without YPG medium were prepared for metabolite extraction.

Fermentation

Fermentation was carried out based on Obata et al. (2016) with slight modification. Seaweed was fermented by adding *H. werneckii* SUCCY001 which has been cultured for 3×24 hours as the seed starter. *G. verrucosa* first sterilized with autoclave at 121°C for 20 minutes. Furthermore, *H. werneckii* SUCCY001 was transferred aseptically into Erlenmeyer flask containing *G. verrucosa*. Then the sample was fermented at room temperature using a shaker at 150 r.p.m with three different periods for three, six and nine days. In addition, total plate count (TPC) and pH value were measured for each period of fermentation to determine the growth curve and pH value during the fermentation process.

Metabolite extraction

Sample extraction was determined using maceration method (Sibero et al. 2019; Gori et al. 2021). In this step, fresh and autoclaved seaweed without fermentation were extracted as a control. The fermented seaweeds were separated using filter paper to obtain the biomass. Afterwards, the biomass was extracted using methanol to a ratio 1:2 (seaweed residue volume:solvent volume) for 30 minutes. Then the macerates were evaporated using a rotary evaporator in 40°C. The crude extracts were weighed and stored at -4°C before used (Pina-Perez et al. 2017).

Metabolite characterization

Thin Layer Chromatography (TLC)

Thin Layer Chromatography was carried out based on Sibero et al. (2020) with some modifications. The analysis was used TLC plates (TLC silica gel 60 F₂₅₄) as the stationary phase and ethyl acetate:chloroform (1:9) as the mobile phase. The extracts were dissolved in methanol and deposited as spots onto the TLC plat using a capillary tube. Then, the TLC plate was put in a TLC chamber and allowed to stand until the solvent reached the upper limit of the plate. The spots on the TLC plates were visualized using UV lamp (366 nm) and addition of several reagents such us DPPH (1,1-diphenyl-2-picrylhydrazyl), FeCl₃ and vanillin-sulfuric acid to detect the presence of several bioactive compounds.

The *R_f* values for each spot was determined according to Kumar et al. (2021), with the following formula:

$$R_f = \frac{\text{Distance covered by sample}}{\text{Distance covered by solvent}}$$

GC-MS analysis

The crude extracts were sent to Integrated Laboratory, Universitas Islam Indonesia for GC-MS (Gas Chromatography-Mass Spectrophotometry) analysis. The GC-MS analysis was carried out refer to our previous work with modification (Sibero et al. 2020). The analysis was used column in dimensions of 0.25 mm × 30 m × 0.25 µm. One µL of each extract was injected to the column at a temperature of 250°C with splitless injection mode. Helium

(He) was the carrier gas with a flow of 0.91 mL/min and linear velocity at 34.4 cm/sec. The spectrum obtained was compared with the database spectrum in GC-MS library and the metabolite profiling was identified by comparing the result with database in the computer laboratory and <https://pubchem.ncbi.nlm.nih.gov>.

Bioassay

Determination of total phenolic content

Total phenolic content was calculated using gallic acid as the standard based on Sami et al. (2020) with few modifications. In total 1 mL of the sample was put into a measuring flask then 1 mL of Folin-Ciocalteu reagent and 1 mL of 1% Na₂CO₃ were added. Then the mixture was measured with aquabidest to reach a total volume of 5 mL. Afterwards, the samples were homogenized and incubated for 30 minutes. The absorbance of the sample was measured using UV-Vis spectrophotometer (Shimadzu 1800, Japan) at 680 nm.

Total phenolic content was calculated with the formula:

$$\text{Total Phenolic Content} = \frac{(a.v)/1000}{G} \times Fp$$

Where:

α : concentration of gallic acid in the test sample (ppm)

v : total volume of test solution (mL)

G : weight of extract used (gram)

Fp : dilution factor

1000 : Total volume conversion factor for the solution

Antibacterial activity

Antibacterial activity was conducted according to Balouiri et al. (2016) using agar well diffusion method with little modification. Dimethyl sulfoxide (DMSO) was used to dissolve the extract to reach a concentration of 2 mg/mL. Five strains of bacteria were used to discover the antibacterial activity, namely *Bacillus subtilis*, *Bacillus cereus*, *Micrococcus luteus* as the representative of gram positive, while *Escherichia coli* and *Pseudomonas aeruginosa* as the representative of gram negative. All bacterial strains were incubated on Mueller Hinton Agar (MHA) for 24 hours at 37°C. Each bacterium was suspended in Mueller Hilton Broth (MHB, HiMedia) to reach 0.5 McFarland standard. Furthermore, it was inoculated onto MHA using a sterile cotton swab. Each agar well was made using a cork borer (diameter 6 mm) then 50 μ L of the extract, positive control and negative control were added to the well. DMSO was used as negative control, while Amoxicillin was used as positive control. Afterwards, all plates were incubated for 24 hours at 37°C then the inhibition zone was observed (Kristiana et al. 2019). The clear zone indicated the positive result of antibacterial activity (Sibero et al. 2019).

Antioxidant activity

DPPH

The DPPH method was conducted based on de Torre et al. (2019) with several modifications. Seaweed extracts

were weighed for 10 mg and dissolved with 1 mL of methanol as a sample with a concentration of 10.000 ppm. Subsequently, the dilutions were made with concentrations of 1000 ppm in three replications. Each replication of sample was added with DPPH solution in a ratio of 1:1 then incubated in the dark at 27 °C for 30 minutes. The absorbance of the sample was measured by UV-Vis spectrophotometer (Shimadzu 1800, Japan) at 517 nm (Sibero et al. 2021).

ABTS

The ABTS^{•+} method was done according to Sridhar and Charles (2019) with slight modifications. ABTS^{•+} stock solution consists of ABTS solution and potassium persulfate solution. Stock solutions were prepared by dissolving 7.4 mM ABTS reagent and 2.6 mM potassium persulfate using deionized water. Then, the two solutions were mixed in a ratio of 1:1 and incubated in the dark for 16-24 hours. Subsequently, 1 mL of the stock solution was diluted into 50 mL of deionized water to obtain an absorbance of 1.1 ± 0.002 at 734 nm. Sample (150 μ L) was mixed with 2850 μ L of ABTS^{•+} solution and incubated for 10 minutes in the dark. The absorbance of the sample was read using UV-Vis spectrophotometer (Shimadzu 1800, Japan) at 734 nm. Blank was made with the same manner by using deionized water instead of the sample.

Radical scavenging activity was calculated with the following formula (Nazaruddin et al. 2021):

$$\%RSA = \frac{A_0 - A_1}{A_0} \times 100\%$$

Where:

A₀ : Absorbance of control

A₁ : Absorbance of sample

RESULTS AND DISCUSSION

TPC and pH of fermentation

Total plate count and pH of fermentation was carried out to determine the growth curve of *H. werneckii* SUCCY001 during the fermentation. The results showed that the growth curve of *H. werneckii* SUCCY001 on 0D was 9.6×10^6 CFU/mL and increase in 3D to 17.5×10^6 CFU/mL. Furthermore, the number of cells of *H. werneckii* SUCCY001 increased in 6D and 9D to 87.85×10^6 CFU/mL and 111×10^6 CFU/mL, respectively. The slow growth of yeast from 0D to 3D indicated that *H. werneckii* SUCCY001 was in the lag phase, which is the phase of a microbe adapting to a new environment (Vermeersch et al. 2019). In 6D and 9D, yeast growth was increased quite fast indicating that *H. werneckii* SUCCY001 was in the log phase, where the yeast in the growth phase (Achinas et al. 2019).

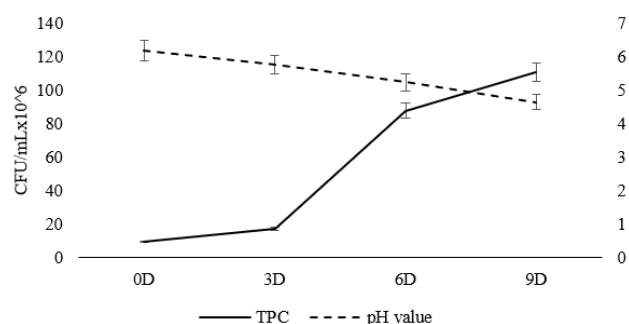


Figure 1. TPC and pH value of fermentation. Note: 0D (0 day fermentation), (3D) three days fermentation, (6D) six days fermentation, (9D) nine days fermentation

Figure 1 showed that the pH value of 0D was 6.2 and decreased in 3D, 6D and 9D to 5.77, 5.24, 4.65, respectively. A decrease in pH, which becomes more acidic during fermentation, can result in an increase in the number of phenolic compounds (Kwaw et al. 2018). This is due to the substrate degradation process by *H. werneckii* SUCCY001 enzymes, which are capable of breaking down conjugated phenolic compounds into free phenolic compounds. According to Zhao et al. (2021), carbohydrate-degrading enzymes produced by yeast during the fermentation process can increase the content of phenolic compounds, increasing their ability as antioxidants.

Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) is the initial analysis to identify bioactive compounds of *G. verrucosa* fermented marine yeast *H. werneckii* SUCCY001 with separation technique. In this study, TLC analysis was done using ethyl acetate and chloroform (1:9) as mobile phase. The selection of this eluent was based on the results of the best compound separation from the extract obtained through visualization of UV light 366 nm which can be seen in figure 2. Bioactive compounds were detected using DPPH, FeCl₃ and vanillin-sulfuric acid reagents to discover the presence of antioxidant property, phenolic and terpenoids, respectively, followed by the calculation of R_f value for each extract.

The R_f value of *G. verrucosa* extracts which visualized under UV light 366 nm is presented in Table 1. The data showed that fermented *G. verrucosa* using marine yeast *H. werneckii* UCCY001 produced different metabolites with fresh and autoclaved seaweed which was characterized by the appearance of spots with different R_f values. Spot with R_f 0.18 and 0.28 was discovered in 3D, 6D and 9D extracts. Besides, spot with R_f 0.4 was found in 6D and 9D extracts. Moreover, spot with R_f 0.66 was present in F, 6D and 9D extracts. The present spot in fermentation extracts (3D, 6D and 9D) expected that this spot was a converted compound which caused by heating process. Gasecka et al. (2020) stated that temperature strongly affected the content of bioactive compound.

The result in Figure 3 convey that the presence of yellowish to colorless on the TLC plate after spraying by DPPH reagent was suspected as an antioxidant agent. This

is in line with previous study by Gogoi et al. (2016) that potential antioxidant was detected by the existence of yellow or colorless spot on TLC plate.

The ability of antioxidant activity was known by the spot abundance of each extract (Wang et al. 2012). Spot with R_f 0.12 only presence in extracts 6D and 9D. In addition, the colorless spot at R_f 0.83 in extract 6D and 9D were more intensive rather in other extracts. While spot with R_f 0.95 was observed in all extracts of *G. verrucosa* (F, A, 3D, 6D and 9D). It can be seen that extracts of 6D and 9D have the higher antioxidant activity than other extracts which directly confirmed with the result of DPPH and ABTS⁺⁺ assays in Figure 6.

Figure 3 showed the presence of blue-dark on TLC plate after spraying with FeCl₃ reagent followed by heating. This result indicated as phenolic compound which consistent with Simamora et al. (2020) that the appearance of blue-dark spot on TLC plate supposed to be phenolic. The existence spot with R_f 0.75 and 0.83 was only found in extracts 6D and 9D of *G. verrucosa*, while in extracts F, A and 3D was not found. Furthermore, spot with R_f 0.33, 0.6 and 0.95 was found in all extracts of *G. verrucosa* (F, A, 3D, 6D and 9D).

TLC analysis using vanillin-sulfuric acid reagent performed the positive result of terpenoids for all extracts of *G. verrucosa* (F, A, 3D, 6D and 9D) which indicated by the presence of purple spot on TLC plate. Akpalo et al. (2020) express that purple spot detected on TLC plate was expected as terpenoids.

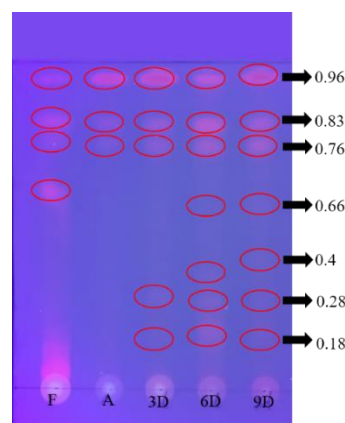


Figure 2. TLC visualized under UV light 366 nm. Note: F: fresh seaweed extract, A: extract with autoclave, 3D: extract three days fermentation, 6D: extract six days fermentation, 9D: extract nine days fermentation

Table 1. R_f value under UV light 366 nm

Extract	Retention (R_f) value						
	0.18	0.28	0.4	0.66	0.76	0.83	0.96
F	×	×	×	✓	✓	✓	✓
A	×	×	×	×	✓	✓	✓
3D	✓	✓	×	×	✓	✓	✓
6D	✓	✓	✓	✓	✓	✓	✓
9D	✓	✓	✓	✓	✓	✓	✓

Note: F: fresh seaweed extract, A: extract with autoclave, 3D: extract three days fermentation, 6D: extract six days fermentation, 9D: extract nine days fermentation, ✓: present, ×: absent

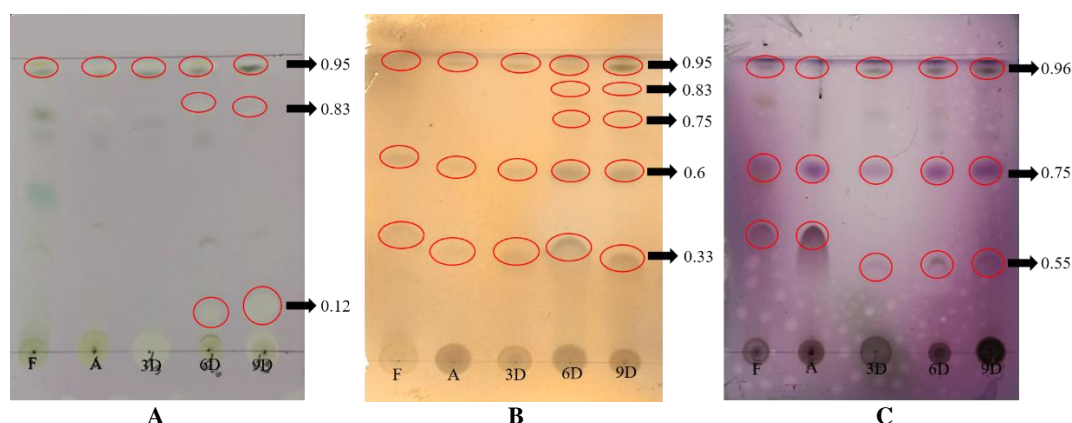


Figure 3. TLC visualized: A. using DPPH reagent, B. using FeCl_3 reagent, C. using vanillin reagent. Note: F: fresh seaweed extract, A: extract with autoclave, 3D: extract three days fermentation, 6D: extract six days fermentation, 9D: extract nine days fermentation, ✓: present, ×: absent

Table 2. R_f value with DPPH reagent

Extract	Retention (R_f) value		
	0.12	0.83	0.95
F	×	×	✓
A	×	×	✓
3D	×	×	✓
6D	✓	✓	✓
9D	✓	✓	✓

Note: F: fresh seaweed extract, A: extract with autoclave, 3D: extract three days fermentation, 6D: extract six days fermentation, 9D: extract nine days fermentation, ✓: present, ×: absent

Table 3. R_f value with FeCl_3 reagent

Extract	Retention (R_f) Value				
	0.33	0.6	0.75	0.83	0.95
F	✓	✓	×	×	✓
A	✓	✓	×	×	✓
3D	✓	✓	×	×	✓
6D	✓	✓	✓	✓	✓
9D	✓	✓	✓	✓	✓

Note: F: fresh seaweed extract, A: extract with autoclave, 3D: extract three days fermentation, 6D: extract six days fermentation, 9D: extract nine days fermentation, ✓: present, ×: absent

Total phenolic content

Total phenolic content was analyzed using Folin-Ciocalteu reagent and calculated based on the gallic acid standard calibration curve. Total phenolic content of *G. verrucosa* fermented marine yeast *H. werneckii* SUCCY001 in this study can be seen in Table 5.

The highest total phenolic content was found in extract 9D (7.57 ± 0.0017 mg GAE/g sample) followed by extract 6D (3.49 ± 0.0052 mg GAE/g sample). The result showed that phenolic compound of *G. verrucosa* fermented marine yeast *H. werneckii* SUCCY001 increased during fermentation process. The increased of phenolic compound expected caused by the enzymatic reaction from metabolic process of *H. werneckii* SUCCY001 which produced phenolic, thereby this caused the total amount of phenolic content of the extracts increased. Suhardini and Zubaidah

Table 4. R_f value with vanillin-sulfuric acid reagent

Extract	Retention (R_f) Value		
	0.55	0.75	0.96
F	✓	✓	✓
A	✓	✓	✓
3D	✓	✓	✓
6D	✓	✓	✓
9D	✓	✓	✓

Note: F: fresh seaweed extract, A: extract with autoclave, 3D: extract three days fermentation, 6D: extract six days fermentation, 9D: extract nine days fermentation, ✓: present, ×: absent

Table 5. Total phenolic content of *Gracilaria verrucosa* fermented marine yeast *H. werneckii* SUCCY001

Extract	Total Phenolic Content (mg GAE/g sample) \pm SD
F	2.78 ± 0.0041
A	1.09 ± 0.0007
3D	3.43 ± 0.005
6D	3.49 ± 0.0052
9D	7.57 ± 0.0017

Note: F: fresh seaweed extract, A: extract with autoclave, 3D: extract three days fermentation, 6D: extract six days fermentation, 9D: extract nine days fermentation, SD: standard deviation

(2016) revealed that microorganism such as yeast and bacteria can affect total phenolic content through enzymatic reaction during fermentation.

GC-MS analysis

GC-MS analysis was conducted to observe the metabolite alteration due to fermentation. The result of GC-MS analysis in this study presented in Table 6. There are five compounds were identified, namely methyl palmitate, methyl linoleate, methyl oleate, methyl arachidate and methyl behenate. It can be seen that there is no significant difference of metabolite profiling for all extracts (Figure 4).

Table 6 indicated metabolite profiling in *G. verrucosa* extracts are fatty acid derivatives. This is expected that GC-MS can only detect volatile and heat-resistant compounds. Hence, other compounds which are actually different from

these five extracts was not detected. This is confirmed by Beale et al. (2018) that GC-MS have limitation in its application which only able to separate volatile and recognize low molecular weight compounds.

Antibacterial activity

Antibacterial activity of *G. verrucosa* fermented marine yeast *H. werneckii* SUCCY001 was conducted with agar well diffusion using extract at a concentration of 2 mg/mL. The results of antibacterial activity assay was presented in Table 7 and Figure 5.

Figure 5 showed that extracts of 6D and 9D have antibacterial activity against *M. luteus* and *P. aeruginosa* which characterized by the formation zone of inhibition. Extract of 6D has weak antibacterial activity (diameter 4

mm zone of inhibition) and extract 9D has moderate antibacterial activity (diameter 9 mm zone of inhibition). Amatulloh et al. (2021) state the strength of antibacterial activity was divided into four categories based on the formation of inhibition zone: weak (diameter <5 mm), moderate (diameter 5-10 mm), strong (10-20 mm) and very strong (>20 mm). Widodo et al. (2019) have evaluated antibacterial activity of methanolic extract *G. verrucosa* from BBPBAP, Jepara and the studied showed that extract of *G. verrucosa* has no antibacterial activity against *P. aeruginosa*. Therefore, fermentation of *G. verrucosa* using yeast *H. werneckii* was able to increase antibacterial activity.

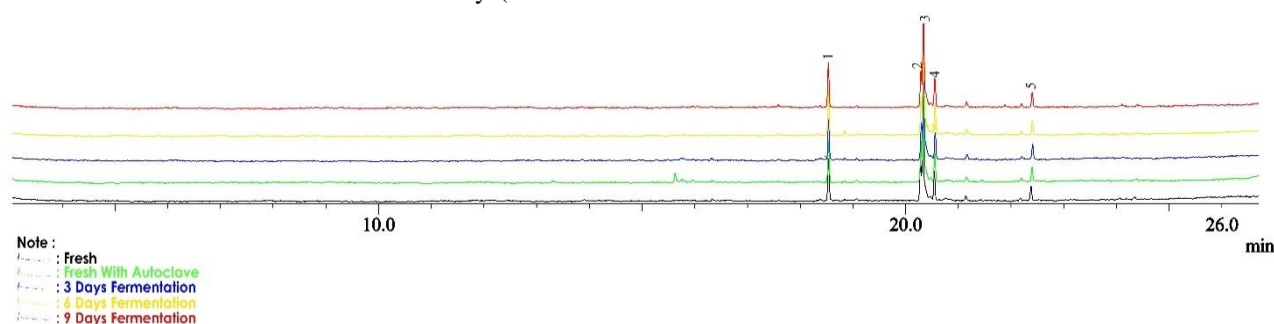


Figure 4. GC-MS Chromatogram of *G. verrucosa* extracts

Table 6. Identified metabolite profiling in *G. verrucosa* extracts using GC-MS

Peak no.	Mass	Compounds	% area
1	371	Methyl palmitate	17.12
2	286	Methyl linoleate	14.84
3	346	Methyl oleate	41.65
4	312	Methyl arachidate	11.04
5	319	Methyl behenate	6.02

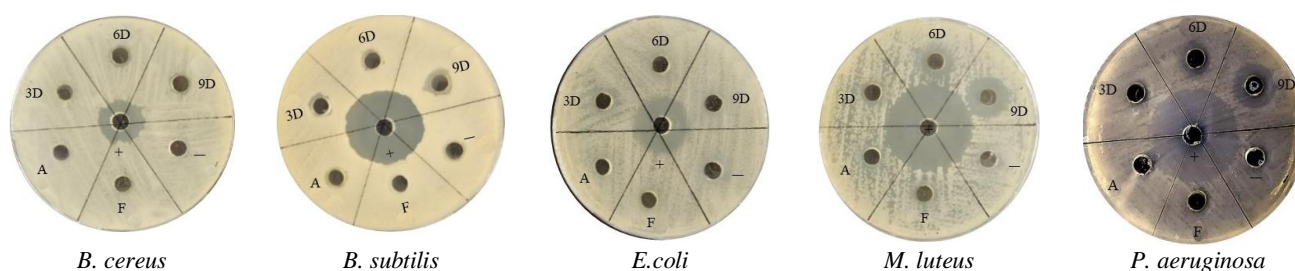


Figure 5. Antibacterial activity of *G. verrucosa* fermented marine yeast *H. werneckii* SUCCY001. Note: F: fresh seaweed extract, A: extract with autoclave, 3D: extract three days fermentation, 6D: extract six days fermentation, 9D: extract nine days fermentation, -: negativ control, +: positive control

Table 7. Antibacterial activity of *G. verrucosa* fermented marine yeast *H. werneckii* SUCCY001

Pathogens	Antibacterial activity (mm)					Control (mm)	
	F	A	3D	6D	9D	Positive	Negative
<i>B. cereus</i>	-	-	-	-	-	15	-
<i>B. subtilis</i>	-	-	-	-	-	24	-
<i>E. coli</i>	-	-	-	-	-	19	-
<i>M. luteus</i>	-	-	-	4	9	26	-
<i>P. aeruginosa</i>	-	-	-	4	9	26	-

Note: F: Fresh extract, A: Extract with autoclave, 3D: Extract three days, 6D: Extract six days, 9D: Extract nine days

The antibacterial activity expected positively correlates with the presence of fatty acid derivatives of the extract according to the result of GC-MS analysis. Toshkova-Yotova et al. (2022) mentioned that fatty acid was associated with antibacterial activity which are responsible to inhibit the growth of gram-positive and gram-negative bacteria. Besides, the presence of terpenoids and phenolic compounds are known as antibacterial activity (Daud et al. 2021). Furthermore, the antibacterial activity also related to total phenolic content, where the highest phenolic content was found in 9D extract and followed by 6D extract. Phenolic compounds destroy the growth of bacteria by denaturation of cell protein. The hydrogen bonds formed between protein and phenolic caused the damage of protein structure and affected the permeability of cell wall and cytoplasmic membrane (Mailoa et al. 2014). Permeability of cell wall and cytoplasmic membrane caused an imbalance of macromolecules and ions in the cell, thus led to bacterial death. Therefore, other extracts did not have antibacterial activity expected due to the phenolic compounds in this extract were still low and insufficient to denature protein cells of bacteria.

Antioxidant activity

Antioxidants are compounds that are useful to prevent and inhibit cell damage in the body (Borges et al 2019). Antioxidant activity in this study was determined by DPPH and ABTS^{•+} methods to obtain the total antioxidant contained in a sample. DPPH and ABTS^{•+} have a different mechanism in free radical scavenging. DPPH radical is reduced by single electron transfer of antioxidant and the reaction was characterized by a reduction of DPPH radical from purple to yellow or colorless (Shahidi and Zhong 2015). In ABTS^{•+}, the blue-green ABTS radical is reduced by hydrogen-donating antioxidant which characterized by discoloration from blue-green to colorless (Ilyasov et al. 2020). The data of these assays are shown in Figure 6.

The result of antioxidant activity showed that both DPPH and ABTS^{•+} have the same trend of the data. Extract 9D was the highest percentage of the scavenging activity either DPPH or ABTS^{•+} (21.9 % \pm 0.0001 and 12.5 % \pm 0.002), while extract A was the lowest radical percentage of scavenging activity (4.6 % \pm 0.0004 and 6.5 \pm 0.003). This is allegedly influenced by high temperature during sterilization thus some bioactive compounds, especially from phenolic group, are damaged. This is due to thermolabile phenolic compounds which may be denatured by heating (Cascais et al. 2021). The previous study by Chakraborty et al. (2015) found that total phenolic content, DPPH and ABTS^{•+} scavenging activity have positive correlation in red seaweed extracts.

The percentage of scavenging activity both DPPH and ABTS^{•+} was in line with the total phenolic content of the extracts and the number of yeast during fermentation. This is due to phenolic are the major group of compounds that play an important role as natural antioxidants of plant including seaweed (Gomes et al. 2022). In addition, during fermentation process phenolic compounds of seaweed degraded by enzyme activity of microbes which releasing the C chain and phenolic acid, namely phlorenic acid and phenylacetic acid chain therefore the total phenolic content increases (Huynh et al. 2014).

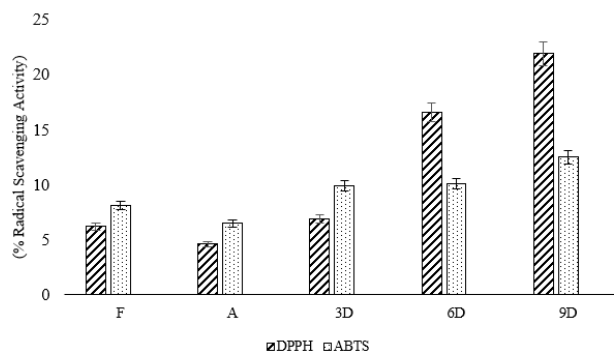


Figure 6. Antioxidant activity of *G. verrucosa*. Note: F: fresh seaweed extract, A: extract with autoclave, 3D: extract three days fermentation, 6D: extract six days fermentation, 9D: extract nine days fermentation

In conclusion, extract of fermented seaweed *G. verrucosa* from Center for Brackish Water Aquaculture (BBPBAP), Jepara present antibacterial activity on *M. luteus* and *P. aeruginosa* in 6D and 9D. Moreover, the highest antioxidant activity and total phenolic content was found in extract of 9D. Secondary metabolites of fermented seaweed *G. verrucosa* were detected as antioxidant agent, phenolic and terpenoids.

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REFERENCES

- Achinas S, Charalampogiannis N, Euverink GJW. 2019. Brief Recap for Bacteria Adhesion. *Applied Sciences* 9: 1-15.
- Akpalo, AE, Saloufou IK, Eloh K, Kpegba K. 2020. Wound healing biomolecules present in four proposed soft aqueous extractions of *Ageratum conyzoides* Linn. *Int J Biol Chem Sci* 14 (2): 638-651. DOI: 10.4314/ijbcs.v14i2.26
- Amatulloh Z, Mulyani Y, Dewanti P, Haetami K. 2021. Growth Characteristics and Tracing Antagonistic Properties of *Bacillus flexus* and *Bacillus subtilis* as Antibacterials to Overcome the Attack of *Aeromonas hydrophila* Bacteria on Fish. *World Scientific News* 158: 91-104.
- Asif HM, Ishfaq U, Sohail S, Izhar M, Rafeeq H, Hayder MA, Sabtain T, Hussain A. 2021. A Review on Natural Antioxidants in Foods and New Insights on Animal Body Compounds, Role, Production and Future Perspectives. *Saudi J. Med. Pharm. Sci* 7(1): 20-25. DOI: 10.36348/sjmps.2021.v07i01.004
- Atta EM, Mohamed NH, Abdelgawad AAM. 2017. Antioxidants: an Overview on the Natural and Synthetic Types. *European Chemical Bulletin* 6(8): 365. DOI: 10.17628/ecb.2017.6.365-375
- Azizah N, Al-baarri AN, Mulyani S. 2012. Pengaruh Lama Fermentasi Terhadap Kadar Alkohol, pH, dan Produksi Gas pada Proses Fermentasi Bioetanol dari Whey dengan Substitusi Kulit Nanas. *Jurnal Aplikasi Teknologi Pangan* 1 (2): 72-77. [Indonesian]
- Balouiri M, Sadiki M, Ibsouda SK. 2016. Methods for in vitro evaluating antimicrobial activity: A review. *J Pharm Anal* 6 (2): 71-79. DOI: 10.1016/j.jpba.2015.11.005

- Beale DJ, Pinu FR, Kouremenos KA, Poojary MM, Narayana VK, Boughton BA, Kanojia K, Dayalan S, Jones OAH, Dias DA. 2018. Review of recent developments in GC-MS approaches to metabolomics-based research. *Metabolomics* 14 (11). DOI: 10.1007/s11306-018-1449-2
- Borges L, Da S, Lima GPP, Artés F, de Souza ME, Freitas, L de S, de Jesus HI, Santos, N de FA, Melo MR da S. 2019. Efficiency of DPPH and FRAP assays for estimating antioxidant activity and separation of organic acids and phenolic compounds by liquid chromatography in fresh-cut nectarine. *Aust J Crop Sci* 13 (7): 1053-1060. DOI: 10.21475/ajcs.19.13.07.p1368
- Cardona F, Andrés-Lacueva C, Tulipani S, Tinahones FJ, Queipo-Ortuño MI. 2013. Benefits of polyphenols on gut microbiota and implications in human health. *J Nutr Biochem* 24 (8): 1415-1422. DOI: 10.1016/j.jnutbio.2013.05.001
- Cascais M, Monteiro P, Pacheco D, Cotas J, Pereira L, Marques JC, Gonçalves AMM. 2021. Effects of heat treatment processes: Health benefits and risks to the consumer. *Appl Sci* 11 (18): 8740. DOI: 10.3390/app11188740
- Chakraborty K, Joseph D, Praveen NK. 2015. Antioxidant activities and phenolic contents of three red seaweeds (Division: Rhodophyta) harvested from the Gulf of Mannar of Peninsular India. *J Food Sci. Technol* 52(4): 1924-1935. DOI: 10.1007/s13197-013-1189-2
- Daud AD, Azman NS, Alias NIA, Syaifudin N, Harun A, Aziz NA. 2021. Phytochemical and antibacterial properties on three medicinal plants for potent anti-soft rot agents. *Malaysian J. Anal. Sci* 25(1): 119-128.
- de Torre MP, Caverio RY, Calvo MI, Vizmanos JLW. 2019. A simple and a reliable method to quantify antioxidant activity in vivo. *Antioxidants* 8(5): 1-11. DOI: 10.3390/antiox8050142
- Feng R, Chen L, Chen K. 2018. Fermentation trip: amazing microbes, amazing metabolisms. *Annals of Microbiology* 68(11): 717-729. DOI: 10.1007/s13213-018-1384-5
- Gąsecka M, Siwulski M, Magdziak Z, Budzyńska S, Stuper-Szablewska K, Niedzielski P, Mleczek M. 2020. The effect of drying temperature on bioactive compounds and antioxidant activity of *Leccinum scabrum* (Bull.) Gray and *Hericium erinaceus* (Bull.) Pers. *Journal of Food Science and Technology* 57(2): 513-525. DOI: 10.1007/s13197-019-04081-1
- Gogoi J, Nakhuru KS, Policegoudra RS, Chattopadhyay P, Rai AK, Veer V. 2016. Isolation and characterization of bioactive components from *Mirabilis jalapa L. radix*. *Journal of Traditional and Complementary Medicine* 6(1): 41-47. DOI: 10.1016/j.jtcme.2014.11.028
- Gomes L, Monteiro P, Cotas J, Gonçalves AMM, Fernandes C, Gonçalves T, Pereira L. 2022. Seaweeds' pigments and phenolic compounds with antimicrobial potential. *Biomolecular Concepts* 13 (1): 89-102. DOI: 10.1515/bmc-2022-0003
- Gori A, Boucherle B, Rey A, Rome M, Fuzzati N, Peuchmaur M. 2021. Development of an innovative maceration technique to optimize extraction and phase partition of natural products. *Fitoterapia* 148. DOI: 10.1016/j.fitote.2020.104798
- Gunathilake T, Akanbi TO, Suleria HAR, Nalder TD, Francis DS, Barrow CJ. 2022. Seaweed Phenolics as Natural Antioxidants, Aquafeed Additives, Veterinary Treatments and Cross-Linkers for Microencapsulation. 1-50.
- Hodhod MSED, Gaafar ARZ, Alshameri A, Qahtan AA, Noor A, Abdel-Wahab M. 2020. Molecular characterization and bioactive potential of newly identified strains of the extremophilic black yeast *Hortaea werneckii* isolated from Red Sea mangrove. *Biotechnol Biotechnol Equip* 34(1): 1288-1298. DOI: 10.1080/13102818.2020.1835535
- Huynh NT, Van CJ, Smaghe G, Raes K. 2014. Improved release and metabolism of flavonoids by steered fermentation processes: A review. *International Journal of Molecular Sciences* 15(11): 19369-19388. DOI: 10.3390/ijms151119369
- Ilyasov IR, Beloborodov VL, Selivanova IA, Terekhov RP. 2020. ABTS/PP decolorization assay of antioxidant capacity reaction pathways. *Intl J Mol Sci* 21 (3). DOI: 10.3390/ijms21031131
- Kristiana R, Sibero MT, Farisa MY, Ayuningrum D, Dirgantara D, Hanafi M, Radjasa OK, Sabdono A, Trianto A. 2019. Antibacterial potential of nudibranch-associated bacteria from Saparua and Nusa Laut Islands, Indonesia. *Biodiversitas* 20(7): 1811-1819. DOI: 10.13057/biodiv/d200704
- Kumar M, Prakash S, Radha, Kumari N, Pundir A, Punia S, Saurabh V, Choudhary P, Changan S, Dhumal S, Pradhan PC, Alajil O, Singh S, Sharma N, Ilakiya T, Singh S, Mekhemar M. 2021. Beneficial role of antioxidant secondary metabolites from medicinal plants in maintaining oral health. *Antioxidants* 10(7): 1-32. DOI: 10.3390/antiox10071061
- Kwaw E, Ma Y, Tchabo W, Apaliya MT, Wu M, Sackey AS, Xiao L, Tahir HE. 2018. Effect of lactobacillus strains on phenolic profile, color attributes and antioxidant activities of lactic-acid-fermented mulberry juice. *Food Chemistry* (250): 148-154. DOI: 10.1016/j.foodchem.2018.01.009.
- Lomartire S, Cotas J, Pacheco D, Marques JC, Pereira L, Gonçalves AMM. 2021. Environmental impact on seaweed phenolic production and activity: An important step for compound exploitation. *Marine Drugs* 19(5): 1-20. DOI: 10.3390/md19050245
- Maftuch, Kurniawati I, Adam A, Zamzami I. 2016. Antibacterial effect of *Gracilaria verrucosa* bioactive on fish pathogenic bacteria. *Egypt J Aquat Res* 42 (4): 405-410. DOI: 10.1016/j.ejar.2016.10.005
- Maicas S. 2020. The role of yeasts in fermentation processes. *Microorganisms* 8(8): 1-8. DOI: 10.3390/microorganisms8081142
- Mailoa MN, Mahendradatta M, Djide N. 2014. Antimicrobial activities of tannins extract from guava leaves on pathogens microbial. *The International Asian Research Journal* 2(1): 43-50.
- Muthukrishnan S, Raja P. 2021. Immunostimulant Effect of Seaweeds in *Channa punctatus* Challenged by *Aeromonas hydrophila*. *J Drug Deliv Ther* 11 (4): 20-23. DOI: 10.22270/jddt.v11i4.4948
- Naufal I, Nurhayati A, Rizal A, Maulina I, Handaka SAA. 2022. Feasibility Analysis of Seaweed, *Gracilaria* sp., Cultivation in Polyculture System In Ponds: A Case Study From Domas Village, Pontang Serang Banten, Indonesia. *Asian Journal of Fisheries and Aquatic Research* 16(1): 1-11. DOI: 0.9734/ajfar/2022/v16i130361
- Nazarudin MF, Alias NH, Sharifuddin N, Abidin AZ, Ahmad MI, Mazli NAIN, Natrah I, Aliyu-Paiko M, Isha A. 2021. Preliminary evaluation of the biochemical and antioxidant properties of seaweed species predominantly distributed in peninsular malaysia. *J Fish. Environ* 45(2): 119-133.
- Obata O, Akunna J, Bockhorn H, Walker G. 2016. Ethanol production from brown seaweed using non-conventional yeasts. *Bioethanol* 2(1): 134-145. DOI: 10.1515/bioeth-2016-0010
- Pina-Pérez MC, Rivas A, Martínez A, Rodrigo D. 2017. Antimicrobial potential of macro and microalgae against pathogenic and spoilage microorganisms in food. *Food Chemistry* 235: 34-44. DOI: 10.1016/j.foodchem.2017.05.033
- Prestinaci F, Pezzotti P, Pantosti A. 2015. Antimicrobial resistance: A global multifaceted phenomenon. *Pathogens and Global Health* 109 (7): 309-318. DOI: 10.1179/2047773215Y.0000000030
- Rani MHS, Ramesh T, Subramanian J, Kalaiselvam M. 2013. Production and Characterization of Melanin Pigment from Halophilic Black Yeast *Hortaea werneckii*. *Intl J Pharm. Sci. Rev. Res* 2(8): 9-17.
- Rogalski E, Ehrmann MA, Vogel RF. 2021. Strain-specific interaction of *Fructilactobacillus sanfranciscensis* with yeasts in the sourdough fermentation. *European Food Research and Technology* 247(6): 1437-1447. DOI: 10.1007/s00217-021-03722-0
- Rusli A, Metusalach, Tahir MM, Salengke, Syamsuar. 2016. Analysis of bioactive compounds of *Caulerpa racemosa*, sargassum sp. and *gracilaria verrucosa* using different solvents. *Jurnal Teknologi* 78(4-2): 15-19. DOI: 10.11113/jt.v78.8146
- Saleh H, Abdelrazak A, Elsayed A, El-Shishtawy H, Osman Y. 2018. Optimizing production of a bioprotective by black yeast. *Egypt J Biol Pest Control* 28 (1): 1-12. DOI: 10.1186/s41938-018-0078-4
- Sami FJ, Soekamto NH, Firdaus, Latip J, Nur S. 2020. Antibacterial activity and total phenol contents of *Sargassum polycystum* extract against *Escherichia coli* and *Staphylococcus aureus*. *Plant Cell Biotechnol Mol Biol* 21 (43-44): 1-7.
- Setiabudi A, Pringgenies D, Ridlo A. 2020. Aktivitas Penangkapan Radikal Bebas DPPH dan Daya Reduksi Ekstrak *Gracilaria verrucosa*. *JRST (Jurnal Riset Sains Dan Teknologi)* 4(2): 47. DOI: 10.30595/jrst.v4i2.5761 [Indonesian]
- Shahidi F, Zhong Y. 2015. Measurement of antioxidant activity. *Journal of Functional Foods* 18: 757-781. DOI: 10.1016/j.jff.2015.01.047
- Sibero MT, Bachtiarini TU, Trianto A, Lupita AH, Sari DP, Igarashi Y, Harunari E, Sharma AR, Radjasa OK, Sabdono A. 2019. Characterization of a yellow pigmented coral-associated bacterium exhibiting anti-Bacterial Activity Against Multidrug Resistant (MDR) Organism. *Egypt J Aquat Res* 45 (1): 81-87. DOI: 10.1016/j.ejar.2018.11.007
- Sibero MT, Frederick EH, Sabdono A, Wijayanti DP, Pringgenies D, Radjasa OK, Zilda DS, Murwani R. 2022. First report of seaweed-associated yeast from Indonesia: Species composition and screening

- of their polysaccharides-degrading enzymes. *Biodiversitas* 23 (3): 1408-1419 DOI: 10.13057/biodiv/d230327
- Sibero MT, Siswanto AP, Murwani R, Frederick EH, Wijaya AP, Syafitri E, Farabi K, Saito S, Igarashi Y. 2020. Antibacterial, cytotoxicity and metabolite profiling of crude methanolic extract from andaliman (*Zanthoxylum acanthopodium*) fruit. *Biodiversitas* 21 (9): 4147-4154. DOI: 10.13057/biodiv/d210928
- Sibero MT, Siswanto AP, Pribadi R, Sabdono A, Radjasa OK, Trianto A, Frederick EH, Wijaya AP, Haryanti D, Triningsih DW, Hayuningrat SJ, Igarashi Y. 2020. The effect of drying treatment to metabolite profile and cytotoxic potential of *Rhizophora apiculata* leaves. *Biodiversitas* 21(5): 2180-2187. DOI: 10.13057/biodiv/d210546
- Sibero MT, Zilda DS, Haryanti D, Igarashi Y. 2021. *Astrogorgia* sp. From Saparua, Maluku: Phytochemical Content, Antimicrobial, Antioxidant, and Cytotoxicity Properties; IOP Conference Series: Earth and Environmental Science 750: 012062. DOI: 10.1088/1755-1315/750/1/012062
- Sibero MT, Zhou T, Fukaya K, Urabe D, Radjasa OK, Sabdono A, Trianto A, Igarashi Y. 2019. Two new aromatic polyketides from a sponge-derived *Fusarium*. *Beilstein Journal of Organic Chemistry* 15: 2941–2947. DOI: 10.3762/bjoc.15.289
- Simamora A, Santoso AW, Rahayu I, Timotius KH. 2020. Chromatographic (TLC) Fingerprinting, Antioxidant, α -Glucosidase, and Lipase Inhibitory Activities of Ethanol Extract of *Muntingia calabura* L. Fruits. *J. HerbMed Pharmacol* 9 (4): 346-354.
- Sridhar K, Charles AL. 2019. In vitro antioxidant activity of Kyoho grape extracts in DPPH [rad] and ABTS [rad] assays: Estimation methods for EC 50 using advanced statistical programs. *Food Chemistry* 275: 41-49. DOI: 10.1016/j.foodchem.2018.09.040
- Suhardini PN, Zubaidah E. 2016. Study of Antioxidant Activity on Various Kombucha Leaves During Fermentation. *Jurnal Pangan dan Agroindustri* 4(1): 221-229.
- Torres FAE, Passalacqua TG, Velásquez AMA, de Souza RA, Colepicolo P, Graminha MAS. 2014. New drugs with antiprotozoal activity from marine Algae: A review. *Rev Bras Farmacogn* 24 (3): 265-276. DOI: 10.1016/j.bjp.2014.07.001
- Toshkova-Yotova T, Georgieva A, Iliev I, Alexandrov S, Ivanova A, Pilarski P, Toshkova R. 2022. Antitumor and antimicrobial activity of fatty acids from green microalga *Coelastrella* sp. BGV. *S Afr J Bot* 000. DOI: 10.1016/j.sajb.2022.04.003
- Vermeersch L, Perez-Samper G, Cerulus B, Jariani A, Gallone B, Voordeckers K, Steensel J, Verstrepen KJ. 2019. On the duration of the microbial lag phase. *Current Genetics* 65(3): 721-727. DOI: 10.1007/s00294-019-00938-2
- Viana da Silva M, Santos MRC, Alves Silva IR, Macedo Viana EB, Dos Anjos DA, Santos IA, Barbosa de Lima NG, Wobeto C, Jorge N, Lannes SCDS. 2021. Synthetic and Natural Antioxidants Used in the Oxidative Stability of Edible Oils: An Overview. *Food Rev Int* 00(00): 1-24. DOI: 10.1080/87559129.2020.1869775
- Wang J, Yue, Y De, Tang F, Sun J. 2012. TLC screening for antioxidant activity of extracts from fifteen bamboo species and identification of antioxidant flavone glycosides from leaves of *bambusa textilis mcclure*. *Molecules* 17(10): 12297-12311. DOI: 10.3390/molecules171012297
- Widodo RW, Subagiyo S, Pramesti R. 2019. Aktivitas Antibakteri Ekstrak Metanol Rumpun Laut *Gracilaria verrucosa*, Greville, 1830 (Florideophyceae : Gracilariaceae) di Balai Besar Perikanan Budidaya Air Payau Jepara. *J Mar Res* 8(3): 285-290. DOI: 10.14710/jmr.v8i3.25271 [Indonesian]
- Wijaya AP, Sabdono A, Sibero MT, Trianto A, Radjasa OK. 2022. Antimicrobial activity of nudibranch *Chromodoris lineolata* associated bacteria against skin diseases pathogens from Jepara Coastal Waters, Indonesia. *Biodiversitas* 23(4): 1911-1919. DOI: 10.13057/biodiv/d230425
- Xiang H, Sun-Waterhouse D, Waterhouse GIN, Cui C, Ruan Z. 2019. Fermentation-enabled wellness foods: A fresh perspective. *Food Science and Human Wellness* 8 (3): 203-243. DOI: 10.1016/j.fshw.2019.08.003
- Yao W, Qiu HM, Cheong KL, Zhong S. 2022. Advances in anti-cancer effects and underlying mechanisms of marine algae polysaccharides. *Intl J Biol Macromol* 221: 472-485. DOI: 10.1016/j.ijbiomac.2022.09.055
- Zhao YS, Eweys AS, Zhang JY, Zhu Y, Bai J, Darwesh OM, Zhang HB, Xiao X. 2021. Fermentation affects the antioxidant activity of plant-based food material through the release and production of bioactive components. *Antioxidants* 10(12): 1-15. DOI: 10.3390/antiox10122004