

Metabolites Alteration and Antioxidant Activity of *Gracilaria verrucosa* after Fermentation using *Aureobasidium melanogenum* MTGK.31

Mada Triandala Sibero^{1,2,3*}, Adella Maulina Savitri^{1,3}, Evan Hansel Frederick^{1,4}, and Sri Sedjati³



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¹ Natural Product Laboratory, Integrated Laboratory for Research and Services, Universitas Diponegoro. Jl. Prof. Soedarto S.H., Tembalang, Semarang 50275, Central Java, Indonesia

² BETA Research, Perum Bintang Regency, Jabungan, Semarang 50266, Central Java, Indonesia

³ Department of Marine Science, Faculty of Fisheries and Marine Science, Universitas Diponegoro. Jl. Prof. Soedarto S.H., Tembalang, Semarang 50275, Central Java, Indonesia

⁴ School of Biosciences, University of Melbourne, Parkville, VIC 3010, Australia

*Corresponding Author:
madatriandala@hotmail.com

Received: 10 October 2022

Accepted: 25 May 2023

Published: 31 May 2023

Academic Editor: Dr. Endar Marraskuranto

© Squalen Bulletin of Marine and Fisheries Postharvest and Biotechnology, 2023. Accreditation Number: 148/M/KPT/2020. ISSN: 2089-5690, e-ISSN: 2406-9272. <https://doi.org/10.15578/squalen.727>

Abstract

Gracilaria verrucosa is a red seaweed that has been widely utilized in the food and pharmaceutical industries due to its biological properties. The utilization of biological agents in obtaining certain bioactive compounds would confront unavoidable issues, particularly its bioactive sustainability. Hence, microbial fermentation has been reported as a practical approach to maintaining bioactive production and boosting its properties. Our study aimed to evaluate the potential of marine yeast *Aureobasidium melanogenum* MTGK.31 as a fermenting agent for *G. verrucosa* and characterize the seaweed metabolite profile and antioxidant activity after fermentation. The seaweed was fermented using *A. melanogenum* MTGK.31 in a medium consisting of yeast extract, peptone, and glucose. The fermentation was done for 24, 48, and 72 hours. Total plate count and pH were measured after each fermentation period. The primary and secondary metabolites of *G. verrucosa* in each fermentation were observed. Antioxidant assay using the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) method was conducted, followed by total phenolic content using the Folin-Ciocalteu method. It was highlighted that yeast colony increased during the fermentation, while the pH level was decreasing. We found that the fermentation not only boosted some elements in primary metabolites, but also increased *G. verrucosa* bioactive groups. After 72 hours of fermentation, the *G. verrucosa* percent radical scavenging activity (%RSA) increased more than two times compared to the fresh *G. verrucosa* with a %RSA value of 16.09 ± 6.57 . Nevertheless, the highest total phenolic content of 5.62 ± 0.00028 mg GAE/g extract was shown by *G. verrucosa* after 48 hours of fermentation.

Keywords: Antioxidant, *aureobasidium*, fermentation, *gracilaria*, metabolites

Introduction

Free radicals are harmful substances that have been abundantly reported to affect human health (Martemucci et al., 2022). Interestingly, these substances are also produced by normal cell metabolism which means that our body unconsciously releases these molecules (Phaniendra et al., 2015). Reactive oxygen species (ROS) and reactive nitrogen species (RON) are types of free radicals and contain a broad of properties. Those agents are capable to damage the biological molecules, therefore they contribute to the occurrence of physiological issues, such as aging, cancer, cellular injury, hepatic, neurodegenerative, cardiovascular, and renal disorders (Galano, 2015; Losada-Barreiro and Bravo-Díaz, 2017). Further, ROS is reported as the type that mainly damages the biological systems and is influenced by unhealthy lifestyles and environmental stress (Aseervatham et al., 2013). Thus,

certain bioactive agent is needed to tackle the effect of free radicals.

The immune systems play a major role in combating free radicals in our body by producing natural antioxidants. Antioxidants protect the cells by delaying the oxidation process of many living molecules (Sehwag & Das, 2014; Sindhi et al., 2013). Based on their sources, antioxidants are classified into endogenous and exogenous. Although our body is equipped with endogenous antioxidants, the utilization of exogenous antioxidants is recommended to act synergistically against free radicals (Martemucci et al., 2022). However, exogenous antioxidants are dominated by synthetic drugs and vitamins that caused toxicity and carcinogenicity (Kornienko et al., 2019; Meziante et al., 2021; Rutkowski & Grzegorzczak, 2012). Hence, it is suggested to utilize the dietary antioxidants present in food since they are safe and contain rich nutritional

and therapeutic effects (Aryal et al., 2019; Lourenço et al., 2019).

As a maritime country, Indonesia possesses unlimited marine resources which have been widely utilized in many industrial sectors, including food (California Environmental Associates, 2018). Marine algae or seaweed are reported with diverse applications as dietary, cosmetics, and pharmaceutical products (Choudhary et al., 2021; Quitral et al., 2022). Red seaweed or Rhodophyta, particularly *Gracilaria* spp., is also considered as the industrially applicable seaweed (Carpena et al., 2021; Hakim & Patel, 2020). Many functional polysaccharides, bioactive compounds, and biological activities have been reported from this seaweed, especially antioxidants (Carpena et al., 2021). Nevertheless, Press et al. (2019) stated that the development of bioactive compounds into commercial products encounters various obstacles, such as the excretion properties and inadequate stability. Considering this fact, new alternatives are needed to maintain sustainability and boost seaweed's biological properties.

Fermentation is a biological process that allows single or multi-biological agent to convert targeted biological molecules into desirable bioproducts (Aslamyiah et al., 2017). This process is not a recently developed methodology as many researchers have utilized seaweed as a fermentation substrate (Chye et al., 2018; Monteiro et al., 2021; Reboleira et al., 2021). However, most of them mainly focused on the lactic acid bacteria (LAB) and baker yeast *Saccharomyces cerevisiae* as the fermenting agents, while the utilization of marine yeast is rarely studied (Sharma et al., 2020). Hence, as the preliminary experiment, our previous study has reported the potential of seaweed-associated yeast *Aureobasidium melanogenum* MTGK.31 as one of the extracellular polysaccharides degrading enzymes (EPEs) producers (Sibero et al., 2022). Considering the outstanding result of microbial fermentation on seaweed biological properties in previous studies, in this work, we aimed to investigate the potential of marine yeast *A. melanogenum* MTGK.31 as the fermenting agent on *Gracilaria verrucosa*'s biological properties and characterize the seaweed metabolites profile and antioxidant activity.

Material and Methods

Sample Preparation

This study utilized red seaweed *G. verrucosa* obtained from the Center for Brackish Water Aquaculture (BBPBAP) Jepara, Central Java, Indonesia on April 2022. The seaweed was washed to eliminate the epiphyte and the salt, then dried. Subsequently, the

seaweed was ground using a hand blender before conducting each experiment. As for the fermentation agent, *A. melanogenum* was revived onto Potato Dextrose Agar (PDA, HiMedia) from our yeast collection and incubated for seven days at 26 °C.

Seaweed Fermentation

Seed culture was prepared by inoculating *A. melanogenum* into a 5 mL liquid medium [0.5% yeast extract (HiMedia), 0.5% peptone (HiMedia), 5% glucose, 5 mL seawater] for seven days at 26 °C (150 r.p.m.). The production culture media were prepared in three replications of each fermentation day by mixing specific compositions, such as 10 g ground *G. verrucosa*, 0.5% yeast extract (HiMedia), 0.5% peptone (HiMedia), and 5% glucose in a 125 mL seawater and sterilized for 20 minutes at 121 °C. In total 10 mL of seed culture was added to the 100 mL of the fermentation flask. The seaweed was fermented with agitation (118 r.p.m.) at room temperature (27 °C). In this work, we applied three different fermentation times for 24 h, 48 h, and 72 h. A pH measurement using a pH meter was conducted before and after fermentation to check the alteration.

Total Plate Count (TPC)

A total colony count was conducted to observe the yeast growth during fermentation. In total 1 mL of the fermented seaweed slurry was transferred into 9 mL of physiological saline solution. A serial dilution was performed until a concentration of 10^{-5} , then in total 100 µL of each concentration was poured onto a PDA plate with two repetitions and incubated at 32 °C for 72 h. Any plate with a number colony between 20-200 was used for the calculation of the colony forming unit (CFU) value (Andrianto et al., 2022).

Extraction

Prior to extraction, the seaweed from the production culture was separated from the liquid content using filter paper. Then, the residue was applied for metabolite extraction. The maceration method with agitation (120 r.p.m) for 24 h was conducted in this study. Furthermore, a food-grade ethanol solvent was utilized to extract the the dried seaweed and the residue of fermented *G. verrucosa* with a ratio of 3:1 (% v/w). The organic solvent was separated and concentrated using rotary evaporator. (Sibero et al., 2020; Fatmawati et al., 2022). The crude extracts were concentrated using a rotary evaporator and stored at -20 °C.

Metabolites Profiling

The fresh and fermented seaweeds were sent to PT Saraswanti Indo Genetech, Bogor, Indonesia to

analyze the primary metabolite contents, such as saturated fat, unsaturated fatty acids (w-3, w-6, and w-9), and amino acids. As a pretreatment for the fermented seaweed, the slurry was filtrated using a cotton filter to remove the liquid. The residue was washed using Aquadest to eliminate the yeast cells. Further, the sample was semi dried at room temperature and then sent for analysis.

As for the secondary metabolites, thin-layer chromatography (TLC) was carried out using chloroform and ethyl acetate with a ratio of 9:1 as the mobile phase. Afterward, the TLC product was stained using Dragendorff to detect alkaloids, vanillin in H_2SO_4 to detect terpene/steroids derivatives, $FeCl_3$ to detect phenol derivatives, ninhydrin to detect free amino acids, and DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) reagents to detect compounds with antioxidant potential (Fatmawati et al., 2022).

Antioxidant Assay and Total Phenolic Content

The DPPH assay was conducted in this study (Benjakul et al., 2018; Sibero et al., 2021). Each crude extract was diluted into a single concentration of 1000 ppm. DPPH solution was prepared to reach a 0.06 mM concentration in methanol. A 900 μ L of DPPH solution was added to a 100 μ L of each sample. Then, the mixture was incubated in the dark condition for an hour. The absorbance was measured using a spectrophotometer (UV-VIS Shimadzu 1800) at 515 nm. The blank solution consisting of methanol and DPPH was used as the negative control. The activity was expressed as percent radical scavenging activity or %RSA.

The Folin-Ciocalteu method was carried out to determine total phenolic content (Sami et al., 2020). Gallic acid was used as the standard solution and

prepared into several concentrations, such as 4, 6, 8, 10, and 12 ppm. On the other hand, each extract was prepared into a 1000 ppm. Each extract was mixed with Folin-Ciocalteu reagent and 1% Na_2CO_3 solutions, then incubated for 30 minutes. The absorbance was determined using a spectrophotometer (UV-VIS Shimadzu 1800) at 680 nm. Total phenolic content was displayed as mg GAE/g.

Data Analysis

Data were analyzed using the SPSS package version for Windows. A 95% ($P < 0.05$) confidence interval was applied in this study.

Results and Discussion

Aureobasidium melanogenum MTGK.31

The selection of *A. melanogenum* MTGK.31 was based on our previous study which reported its ability in producing extracellular polysaccharides-degrading enzymes (EPEs) (Sibero et al., 2022). This yeast had a large colony size, filamentous shape and margin, convex elevation, smooth texture, and white cream color. The macroscopic and microscopic photograph of *A. melanogenum* MTGK.31 is shown in Figure 1.

Aureobasidium melanogenum is a stress-tolerant yeast that can be found in many environmental conditions, including marine (Jiang et al., 2016; Liu et al., 2014; Sibero et al., 2022; Wang et al., 2014; Wu et al., 2022; Yanwisetpakdee et al., 2016). This yeast is equipped with a unique biosynthetic pathway to enhance its resilience by producing exopolysaccharides, enzymes, and bioactive compounds (Chen et al., 2020; Jiang et al., 2016, 2019a; Kang et al., 2022; Liu et al., 2018; Yanwisetpakdee et al., 2016). Those substances are intermediate and final products expressed by *A. melanogenum* biosynthesis encoding genes (L. Chen

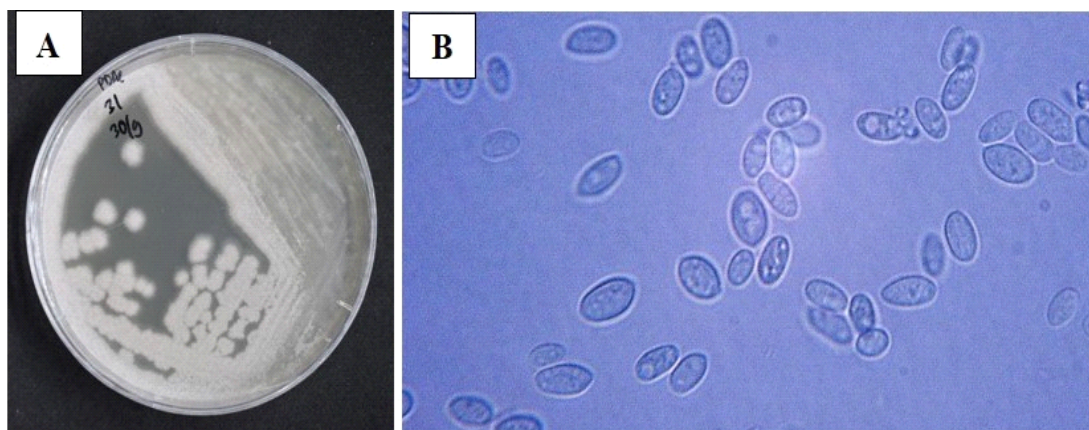


Figure 1. *Aureobasidium melanogenum* MTGK.31 (A. Colonial morphology. B. Cell morphology under microscope with magnification 40 x 10).

et al., 2020; T. J. Chen et al., 2020; Wang et al., 2017; Zhang et al., 2021). Interestingly, many researchers have applied those biological properties to a variety of bioproducts, particularly oligosaccharides, lipids, α -glucan, polymalate, liamocins, siderophores, and melanin (Chi et al., 2016; Liu et al., 2017; Zeng et al., 2019; Zhang et al., 2021). Regardless of its potential, the utilization of this unicellular fungi as a seaweed fermenting agent has never been reported. A prior study reported by Ma et al. (2015) utilized marine *A. melanogenum* to convert inulin into pullulan through fermentation. In addition, previous studies have been conducted to investigate *A. melanogenum* in fermenting beverage materials, such as honey and malt syrup (Hsu et al., 2021; Zeng et al., 2019).

Gracilaria verrucosa Fermentation

Figure 2 represents the texture alteration of *G. verrucosa* using marine yeast *A. melanogenum* MTGK.31 as the fermenting agent. It is shown that the structure of seaweed was slowly deconstructed during the fermentation period. As for the TPC and pH, it is highlighted in Figure 3 that the yeast colony increases during the fermentation interval, whereas the pH slightly descended from time to time. Interestingly, there is only a slight difference in total colony between 24 h and 72 h fermentation. Then, the value rose considerably in the 72 hours of fermentation.

Alike lactic acid bacteria (LAB), it has been reported that yeasts also rely on sugars for growth (Gao et al., 2019; Huang et al., 2021). In our case, we proposed that *A. melanogenum* MTGK.31 utilized sugars from the growth medium and *G. verrucosa* acted as the additional substrate. Due to its capability in producing extracellular polysaccharides-degrading enzymes (EPEs) (Sibero et al., 2022), we suspected that our isolate employed these hydrolysis enzymes to deconstruct the seaweed structure and utilized the seaweed molecules for its growth during the fermentation period (Gurpilhares et al., 2019). Hence, the composition of the fermentation medium should be investigated to confirm this suggestion (Obata et al., 2016; Sørensen et al., 2021).

According to Figure 3, yeast colonization is initiated with 7.64 log CFU/mL. Then, the graph increases to 9.26 log CFU/mL after 72 hours of fermentation. Our result is supported by Jiang et al. (2019b) who reported that the log phase of *A. melanogenum* growth starts within 24 to 72 hours of cultivation. Nevertheless, the utilization of growth medium and substrates might be the major factor that influenced this result (Jiang et al., 2019b; Xi-Lin et al., 2014). As for the pH level, it is stated that $\text{pH} > 5$ is an unsafe pH level and is classified as an unsuccessful fermentation (Sørensen et al., 2021). Although the pH level reduced moderately, it can be concluded that our work was a successful

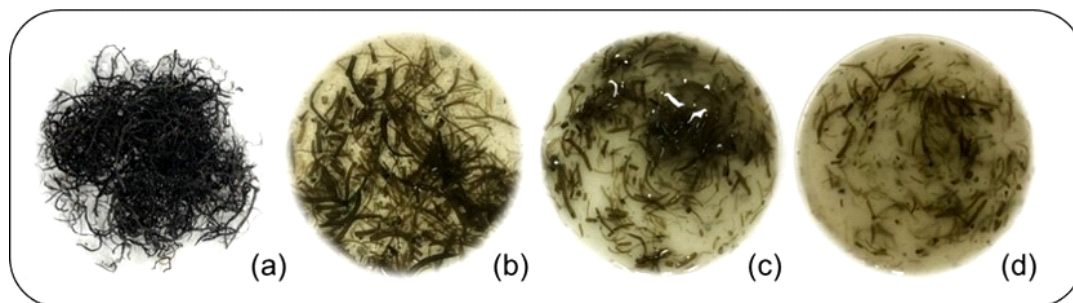


Figure 2. The texture alteration of *G. verrucosa* (a) fresh, (b) Fr 24 h, (c) Fr 48 h, and (d) Fr 72h.

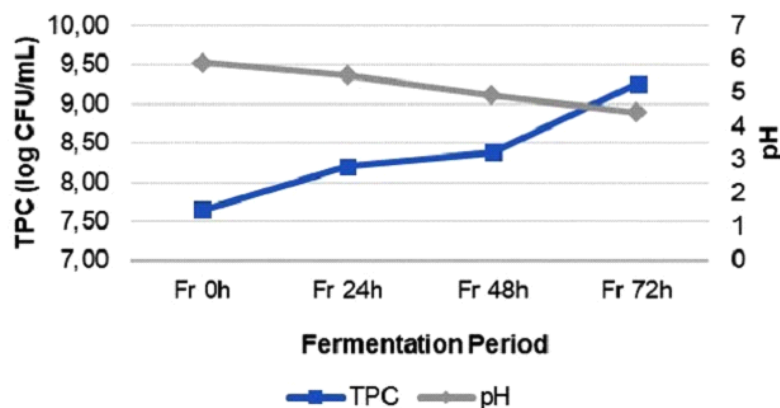


Figure 3. Total plate count (TPC) and pH alteration during the fermentation

fermentation. The decrease in pH value after fermentation is thought to be due to the conversion of glucose to alcohol, organic acids, phenolic derivatives, and CO₂. Kamassah et al. (2013) stated that the production of these components would lower the pH value during fermentation. This assumption is in accordance with the results of the total phenolic content values presented in Table 2. It can be seen that the total phenolic content of seaweed increases as the pH value decreases. Furthermore, many previous studies have suggested conducting a pre-treatment step to promote the isolate colonization and obtain a desirable pH level (Abo et al., 2019; Soùowski et al., 2020; Sørensen et al., 2021).

Metabolites Characterization

Our current study observed the influence of yeast fermentation on *G. verrucosa*'s primary metabolites such as amino and fatty acids (Suppl. 1). Figure 4 and Table 1 represent the effect of yeast fermentation on *G. verrucosa* primary metabolites. We failed to detect the presence of omega-3 fatty acids from both fresh and fermented seaweed. However, it is highlighted that the fermentation increases the production of omega-6 as shown in Figure 4. Nonetheless, the production decreased drastically after 72 h of fermentation. The production of omega-9 also increased after 24 h of fermentation but then decreased in 48 h of

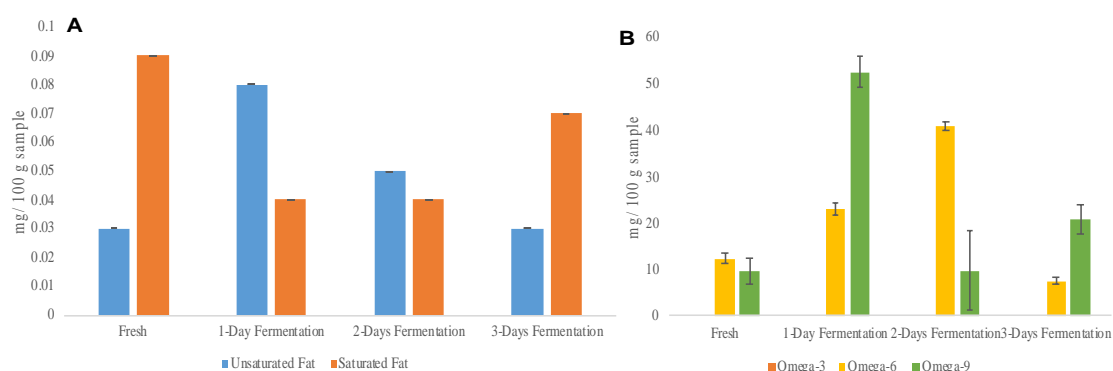


Figure 4. The alteration of (A) unsaturated and saturated fat; and (B) unsaturated fatty acids content in fresh and fermented *G. verrucosa*.

Table 1. Alteration of amino acid composition on *G. verrucosa* during fermentation

No	Amino Acid	Amino Acids Composition (mg/ kg sample)			
		Fresh	Fr 24 h	Fr 48 h	Fr 72 h
1	L-Serine	788.11 ± 11.10	830.17 ± 0.00	585.57 ± 12.34	684.70 ± 17.15
2	L-Glutamic acid	860.88 ± 76.60	1332.55 ± 16.72	1052.35 ± 13.06	1083.52 ± 15.63
3	L-Phenylalanine	850.10 ± 97.65	629.5 ± 8.49	564.32 ± 3.63	678.45 ± 7.60
4	L-Isoleucine	361.93 ± 5.30	381.57 ± 5.61	273.50 ± 2.71	346.50 ± 3.72
5	L-Valine	474.22 ± 3.59	515.14 ± 7.30	394.13 ± 7.01	471.00 ± 6.47
6	L-Alanine	587.13 ± 2.11	870.85 ± 2.43	740.13 ± 3.95	749.35 ± 1.59
7	L-Arginine	599.1 ± 5.12	611.23 ± 6.72	521.61 ± 5.40	657.85 ± 7.47
8	Glycine	679.61 ± 2.34	1386.83 ± 3.41	1458.31 ± 0.73	1629.51 ± 0.93
9	L-Lysine	236.75 ± 1.88	410.35 ± 6.51	284.28 ± 3.95	313.58 ± 1.92
10	L-Aspartic Acid	723.41 ± 6.52	918.32 ± 12.86	653.41 ± 6.18	708.84 ± 9.51
11	L-Leucine	662.67 ± 3.67	680.58 ± 3.21	496.21 ± 3.02	621.29 ± 3.97
12	L-Tyrosine	403.09 ± 8.05	382.86 ± 10.11	321.36 ± 7.12	408.97 ± 3.65
13	L-Proline	399.15 ± 5.64	818.40 ± 0.78	784.92 ± 11.53	856.92 ± 6.51
14	T-Threonine	663.31 ± 2.65	544.95 ± 8.08	401.96 ± 3.27	555.25 ± 8.56
15	L-Histidine	284.94 ± 9.62	250.05 ± 4.98	211.77 ± 0.71	256.31 ± 4.32

Note: Data is average ± standard deviation

fermentation. For the production of amino acids, we noticed that there were also declining results in some aspects, particularly in amino acids, such as L-Glutamic acid, L-Phenylalanine, T-Threonine, and L-Histidine.

Unsaturated fatty acids (UFAs) are essential biological properties that are ubiquitous in marine organisms, including seaweeds. According to Lu et al. (2019), the production of UFAs from seaweeds is 21% on par with mollusks at 33%. Moreover, Ayu et al. (2019) stated that *G. verrucosa* contains omega-3 and omega-6 as major polyunsaturated fatty acids (PUFAs). In contrast, our study only detected the presence of omega-6 in the seaweed. As shown in Figure 4, the fermented *G. verrucosa* demonstrated an improvement of omega-6 composition nearly four times higher than the fresh sample. Unfortunately, the value decreased drastically from 41 to 7.55 mg/100 g at 72 hours of fermentation. This change is supported by Yue et al. (2022) who reported the increasing of seaweed *Laminaria japonica* UFAs content after fermentation using *S. cerevisiae* AMnb091, *Lactiplantibacillus plantarum* LP1406, and *L. rhamnosus* F-B19-1. It is suspected that our fermenting agent, *A. melanogenum* MTGK.31, released lipase during the fermentation (Gupta et al., 2011; Peeters et al., 2018). Hence, it is highly recommended to observe the saturated fatty acids contents and conduct the screening of lipase activity to confirm this hypothesis.

Besides fatty acids, amino acid content was also observed in this study. Terriente-Palacios and Castellari (2022) reported that *Gracilaria* spp. accommodate the highest protein content up to 35 g/100 dry weight and 1.20 of essential to the non-essential amino acid ratio (EAA/NEAA) compared to other red seaweeds. Interestingly, Milinovic et al. (2020) mentioned that several amino acids, like L-Glutamic acid and L-Aspartic acid, play an important role in the umami taste in seaweeds. Considering this fact, our work successfully described the influence of *A. melanogenum* MTGK.31 on *G. verrucosa* amino acid contents as most of them underwent moderate improvement levels during the fermentation. Besides measuring the amino acids content (Table 1), the changes were also detected through TLC-ninhydrin analysis as shown in Table 2 where the Fr 72 h extract gives six spots compared to the fresh extract with four spots. However, it can be concluded from the graph that the improvement could not be maintained over 24 hours of fermentation as the level started decreasing at 48 hours of fermentation. We proposed that this phenomenon occurred due to the thermolabile and pH-sensitive characteristics of the amino acids (Banki et al., 2021; Kato et al., 2017). Furthermore, it is also suspected that the amino acids were utilized as growth-

promoting materials for our fermenting agent (Ferreira & Guido, 2018). Eventually, only glycine showed constant improvement levels during the fermentation. To characterize the secondary metabolites, a further analysis using thin-layer chromatography (TLC) plate successfully detected 11 spots under the UV-illumination at 366 nm. Moreover, the visualization of TLC plate with several reagents aimed to detect the presence of particular bioactive groups thoroughly. For instance, the Dragendorff reagent was used for detecting alkaloids, vanillin in H_2SO_4 for terpenes/steroids derivatives, $FeCl_3$ for phenol, ninhydrin for free amino acids, and DPPH for antioxidant compounds (de Moura et al., 2016; Singh Rana & Saklani, 2017; Sinhababu et al., 2015; Zhang et al., 2021).

In general, we found that the fermentation using *A. melanogenum* MTGK.31 successfully enhanced the number of secondary metabolites in *G. verrucosa*. Figure 5 and Table 2 show that the number of spots was increased by the fermentation time. It was also noted that some of the spots had different colors after UV-illumination visualization and/or reagent addition. It means the metabolites profile in *G. verrucosa* also changed during the fermentation (Fatmawati et al., 2022). These alterations were obtained during the fermentation as the yeast *A. melanogenum* MTGK.31

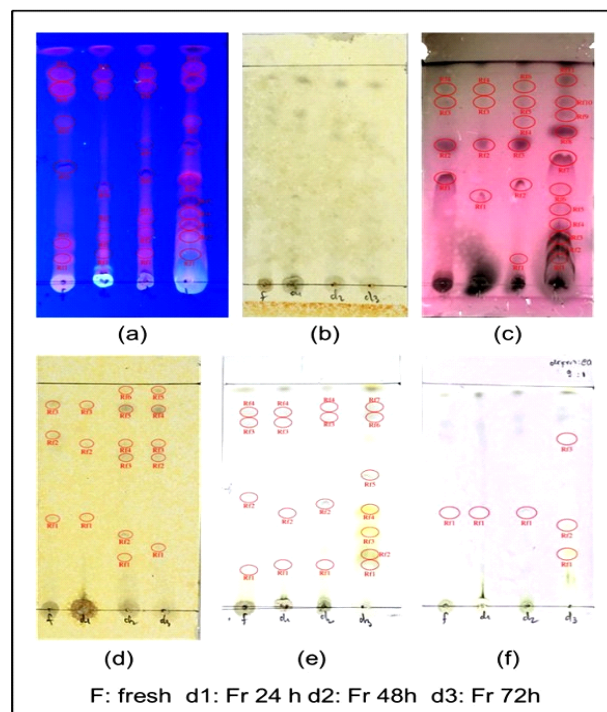


Figure 5. Detected spot on TLC plates; (a) Under UV light illumination at 366 nm; (b) Sprayed with Dragendorff; (c) Stained with vanillin; (d) Dyed with $FeCl_3$; (e) Sprayed with ninhydrin; (f) Stained with DPPH reagent

Table 2. Retention factor (R_f) of TLC analysis before and after reagents addition

UV light illumination												
Extract	Retention factor (R_f)											
	0.12	0.16	0.21	0.26	0.33	0.41	0.48	0.6	0.66	0.85	0.9	
Fresh	✓	✓	✗	✗	✗	✗	✓	✗	✓	✓	✓	
Fr 24 h	✓	✓	✗	✓	✗	✓	✗	✗	✗	✓	✓	
Fr 48 h	✓	✓	✗	✓	✗	✗	✓	✓	✗	✓	✓	
Fr 72 h	✓	✓	✓	✓	✓	✓	✗	✓	✓	✓	✓	
Vanillin												
Extract	Retention factor (R_f)											
	0.12	0.16	0.21	0.26	0.33	0.41	0.48	0.6	0.66	0.75	0.81	0.9
Fresh	✗	✗	✗	✗	✗	✗	✓	✓	✗	✗	✓	✓
Fr 24 h	✗	✗	✗	✗	✗	✓	✗	✓	✗	✗	✓	✓
Fr 48 h	✓	✗	✗	✗	✗	✗	✓	✓	✗	✓	✓	✓
Fr 72 h	✓	✓	✓	✓	✓	✓	✗	✓	✓	✓	✓	✓
FeCl ₃												
Extract	Retention factor (R_f)											
	0.23	0.26	0.33	0.41	0.66	0.73	0.78	0.9	0.96			
Fresh	✗	✗	✗	✓	✗	✗	✓	✓	✗			
Fr 24 h	✗	✗	✗	✓	✗	✓	✗	✓	✗			
Fr 48 h	✓	✗	✓	✗	✓	✓	✗	✓	✓			
Fr 72 h	✗	✓	✗	✗	✓	✓	✗	✓	✓			
DPPH												
Extract	Retention factor (R_f)											
	0.25	0.36	0.43	0.77								
Fresh	✗	✗	✓	✗								
Fr 24 h	✗	✗	✓	✗								
Fr 48 h	✗	✗	✓	✗								
Fr 72 h	✓	✓	✗	✓								
Ninhydrin												
Extract	Retention factor (R_f)											
	0.15	0.2	0.25	0.33	0.48	0.5	0.58	0.83	0.88			
Fresh	✓	✗	✗	✗	✗	✓	✗	✓	✓			
Fr 24 h	✗	✓	✗	✗	✓	✗	✗	✓	✓			
Fr 48 h	✗	✓	✗	✗	✓	✗	✗	✓	✓			
Fr 72 h	✗	✓	✓	✓	✓	✗	✓	✗	✓			

Note: Fr 24 h: 24 h fermentation; Fr 48 h: 48 h fermentation; Fr 72 h: 72 h fermentation; ✓: positive; ✗: negative

hydrolyzed the algal cell wall that composed of polysaccharides, leading to optimal secretion of biological molecules (Gurpilhares et al., 2019; Kalasariya et al., 2021). Due to the cell's breakdown, the organic solvent was able to extract more bioactive from the fermented seaweed.

The TLC visualization indicated fermentation using *A. menalogenum* MTGK.31 increased the number of TLC spots for terpene/steroids, phenols, free amino acids, and antioxidants. Interestingly, spots with antioxidant potential (R_f 0.25, 0.36, 0.43, and 0.77) were suggested as phenolic derivatives, due to their similar R_f values on TLC + FeCl₃ plates. Fatmawati et al. (2022) also applied a TLC visualization approach to characterize the lead compounds with antioxidant potential from fermented seaweed. They found that the antioxidant compounds belonged to phenolic

derivatives. Previous studies stated that phenolic and tannin are bioactive groups that play a vital role in pigmentation and possess a wide bioactivity range, especially antioxidants (Cotas et al., 2020; Parra-Palma et al., 2020). Reboleira et al. (2021) stated that the fermentation will increase the release of several bioactive compounds such as phenols, phlorotannin, peptides, pigments, carotenoids, etc. This fact supported our finding that the number of spots for phenol, terpene/steroids, and free amino acids also increased after fermentation.

Antioxidant Activity

To determine the antioxidant activity, the DPPH assay was carried out in this study. In Table 3, it can be expected that fermentation boosted the *G. verrucosa*'s percent free radical scavenging activity

Table 3. DPPH free radical scavenging activity and total phenolic content

Extract	DPPH (%RSA)	Total Phenolic Content (mg GAE/g)
Fresh	7.74 ± 4.69 ^a	0.64 ± 0.00014 ^a
Fr 24 h	9.85 ± 0.87 ^a	1.57 ± 0.00014 ^b
Fr 48 h	14.93 ± 3.03 ^b	5.62 ± 0.00028 ^c
Fr 72 h	16.09 ± 6.57 ^b	3.22 ± 0.00021 ^d

Note: DPPH assay was conducted using a single dose of 1000 ppm. The presented data are average ± standard deviation. Fr 24 h: 24 h fermentation; Fr 48 h: 48 h fermentation; Fr 72 h: 72 h fermentation. The different notation means a significantly difference with $P < 0.05$

(%RSA) and the total phenolic content ($P < 0.05$). It is noted that statistically, the antioxidant potential of fresh and fermented seaweed for 24 h was not significantly different. The same understanding is also applied to fermented seaweed for 48 h and 72 h. On the other hand, the fermentation time had a significant effect on the total phenolic content in *G. verrucosa* content value of 5.62 ± 0.00028 mg GAE/g at 48 h before the value decreased to 3.22 ± 0.00021 mg GAE/g at 72 h.

Several studies stated that antioxidant property has a positive correlation to antioxidant activity. Interestingly, in this study, we found that the total phenolic content in Fr 72 h was lower than Fr 48 h, although the %RSA value of Fr 72 h was higher than Fr 48 h. Several other non-phenolic compounds that have potential as antioxidants, such as pigments and carotenoids, are thought to increase during fermentation. It is suspected that *A. melanogenum* MTGK.31 secreted hydrolase to break the seaweed cells and induced the release of pigments and carotenoids from *G. verrucosa* (Reboleira et al., 2021). Therefore, although the phenolic content was decreased in Fr 72 h, the %RSA value was higher than the Fr 48 h. The presence of these compounds was not measured in this study. Yue et al. (2022) supported this enhancement by reporting the antioxidant and hypoglycemic activities of fermented *L. japonica* using the combination of brewer yeast and LAB. Regardless of this improvement, the %RSA value in this study is categorized as low activity as previous studies reported more than 50 %RSA was demonstrated from a 50 µg/mL concentration of *Gracilaria* spp.'s crude extract (Gouda et al., 2013; Sornalakshmi et al., 2021). Subsequently, the total phenolic content also underwent a similar condition, where fresh *G. verrucosa* contains total phenolic content of up to 20 mg GAE/g (Febrianto et al., 2019; Sasadara & Wirawan, 2021). Besides the organic solvent, we assumed that the drying treatment on the seaweed affected this result. The drying treatment is reported to reduce seaweed's total phenolic and flavonoid contents (Gupta et al., 2011; Julião et al., 2021; Norra et al., 2017).

Conclusion

This study found that *A. melanogenum* MTGK.31 was able to increase *G. verrucosa* biological properties, especially its omega-6, omega-9, amino acids, terpenes/steroids, and phenolic contents. Further, the Fr 72 h extract promoted the antioxidant activity of *G. verrucosa* with a percent radical scavenging activity (%RSA) of 16.09 ± 6.57. Nonetheless, the %RSA value of Fr 48 h and Fr 72 h was not significantly different ($P < 0.05$). As for the total phenolic content, the highest value was demonstrated by the Fr 48 h extract with total phenolic content of 5.62 ± 0.00028 mg GAE/g. Our finding appears to be useful for a further study aiming to ensure the safety of *A. melanogenum* MTGK.31 as a fermenting agent, evaluate antioxidant activity with other methodologies, and isolate the lead compounds.

Acknowledgement

This work is the additional outcome of the Riset Publikasi International Bereputasi Tinggi (RPI) scheme granted to the first author by Universitas Diponegoro with contract number 185-96/UN7.6.1/PP/2022.

Supplementary Materials

Supplementary materials are available online at the Journal's website.

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