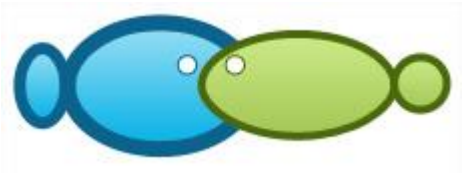

Symbiotic microbes from various seaweeds with antimicrobial and fermentative properties

[1]Delianis Pringgenies, [2]Endang I. Retnowati, [3]Dafit Ariyanto, [1]Kartika Dewi, [1]Muhamad A. S. Viharyo, [1]Ragil Susilowati

[1] Department of Marine Sciences, Faculty of Fisheries and Marine Sciences, Diponegoro University, Semarang, Indonesia; [2] Department of Chemical Engineering, Faculty of Engineering and Informatics, University of PGRI, Semarang, Indonesia; [3] Department of Aquaculture, Faculty of Agriculture, University of Asahan, Kisaran, Indonesia. Corresponding author: D. Pringgenies, delianispringgenies@lecturer.undip.ac.id

Abstract. Symbiotic organisms are viable sources of bioactive compounds. This study aims to determine the microbial species from some seaweeds through molecular identification. The samples were collected from the waters of Bunaken, Northern Sulawesi using purposive sampling method. The spread method was used in microbial isolation and purification, whereas overlay and agar diffusion methods were used in the antimicrobial activity test. Oxidative fermentative (OF) test was used to determine glucose metabolism. The microbes were identified using molecular identification method (PCR 16S rDNA). The isolation process found 88 symbiotic microbes from 3 seaweed species (*Eucheuma spinosum*, *Gracilaria gracilis*, and *Glacilaria verrucosa*). 16 isolates showed antimicrobial activity, whereas 9 isolates showed both fermentative properties and antimicrobial activity. The antimicrobial sensitivity test found 4 microbial isolates from *E. spinosum* with active antimicrobial activity against the pathogenic microbes *Escherichia coli* and *Staphylococcus aureus,* while the 5 remaining isolates from *G. verrucosa* and *G. gracilis* only showed active antimicrobial activity against the pathogenic microbe *S. aureus*. Molecular identification of the 4 isolates with both antimicrobial activity and fermentative properties from isolate ES 2.2 showed 98% homology with *Vibrio parahaemolyticus,* isolate GM 4.4 showed 99% homology with *Vibrio jascida,* 2 strains of *Vibrio hyugaensis,* and 2 strains of *Vibrio alginolyticus.*

Key Words: algae, antimicrobial activity, *Euchema spinosum*, *Gracilaria* sp.

Introduction. Bioethanol is one of the renewable energy sources promoted to replace fossil-based energy sources. The use of ethanol in micro businesses, fisheries, farming, transportation and public service operations (PSO) in Indonesia in 2016 made up 2% of the total energy consumption. This figure is expected to rise to 5% on 2020 and to keep increasing to 20% in January 2025 (Ministry of ESDM 2018). Data of ethanol production in Indonesia indicates that the current production capacity can only meet 50% of the domestic market demand. The lack of data represents one of the major challenges for Indonesia in its effort to provide renewable resources. In order to address this challenge, exploration of natural resources using biotechnology need to be conducted, such as the microbial application of seaweed bacteria in bioindustry.

Symbiotic microbes of seaweed have antimicrobial, anti-fouling and anti-fungal properties (Penesyan et al 2009; Monciardini et al 2014). Many of the discovered microbes have been discovered as anti-microbial agents, yet most of these species are non-fermentative in nature. The fermentative capabilities of anti-microbial microbes are often left out, although a few findings have reported such characteristics for certain microbe species (Behnken & Hertweck 2012).

Fermentative symbiotic microbes have the potential to be used in the fermentation process of bioethanol production, considering that many microbial species found in microalgae have the ability to break down sugars produced by algae such as alginate, cellulose and mannitol (Goecke et al 2010). Symbiotic microbes show promising potential as a renewable resource in bioactive compound production (Penesyan et al 2009; Rocha-Martin et al 2014), as antimicrobial agents, and as fermenters in bioethanol

production, because the bioactive compounds produced by marine organisms can be cultured and manipulated easily in bioreactors. This study aims to determine the microbial species from some seaweeds through molecular identification, as a basis for further bioethanol production.

Material and Method

Sampling and isolation of symbiotic microbes. 3 seaweed species (*Eucheuma spinosum*, *Gracilaria gracilis*, and *Gracilaria verrucosa*) were collected from July to December 2018, from the waters of Bunaken, Manado, North Sulawesi. The sample isolation was conducted at the Laboratory of Tropical Marine Biology, Integrated Laboratory of Diponegoro University, Semarang, Central Java. 2 kg of samples were placed into a Zobell 2216E marine broth, after which they were diluted in stages. Based on morphological characteristics, the microbial colonies were purified by culture using the swab method (Sanders 2012).

Screening of antimicrobial activity and Sensitivity test. The screening of antimicrobial activity of the microbial isolates was performed to determine the biological potency of seaweed symbiotic microbes against human pathogens like *Escherichia coli*, which represent gram-negative pathogens, and *Staphylococcus aureus*, which represent gram-positive pathogens. The antimicrobial activity was determined by the zone of inhibition formed around the symbiotic microbe (Isnansetyo & Kamei 2003). Antimicrobial quantitative test was performed by measuring the resulting zone of inhibition on a Kirby-Bauer agar disk diffusion assay. The pathogenic microbes tested in this study were *E. coli* and *S. aureus*. The assay was performed by introducing 100 µL of pathogenic microbe on agar media during its logarithmic phase. A paper disk (8 mm; Advantec Tokyo Roshi, Ltd, Japan) with 30 µL of symbiotic microbe strain is placed on the surface of each agar medium. The petri dish were then incubated for 48 hours at room temperature. The antimicrobial activity was determined by the formation of inhibition zones around the paper disk (Kemme & Heinzel-Wieland 2018). Isolates with advanced antimicrobial activity against the pathogens were further selected for molecular identification based on the amplification of PCR 16S rRNA genetic sequence.

Oxidative fermentative test. The oxidative fermentative (OF) test was performed to determine the oxidative of fermentative nature of the microbe on sugar. Microbial colonies were vertically inoculated using loops on 1 set (two tubes) of OF medium. 1 mL of paraffin was added to one of the tubes. Both tubes were incubated for 24 hours. Paraffin was added to prevent oxygen from entering the OF medium tube. Oxidative reaction was indicated by the yellow coloration on the tube without paraffin, whereas fermentative reaction was indicated by yellow coloration on the tube with paraffin or on both tubes. The resulting acid product with high concentration after the fermentation process will change the bromthymol blue indicator from green to yellow, by either the presence or lack of oxygen. OF media was made by increasing the amount of glucose and decreasing the amount of peptone. The increase of glucose concentration can increase the concentration of acid to a level that can be detected by bromthymol blue indicator. The reduction in peptone concentration reduces the resulting alkali product since it can neutralize pH, making no change in the color of the indicator (Jiang et al 2015).

Molecular identification of microbes. DNA genome from symbiotic microbe isolates were used as templates for PCR amplification. The amplification of 16S ribosomal RNA (rRNA) used universal primers 27F (5'AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'TACGGTTAACCTTGTTACGACTT-3'). Amplification was performed using a thermal cycler with denaturation at 95°C for 3 min, and then 30 cycles of annealing at 55°C for 1 min, with extension at 72°C for 1 min. The next stage was the final extension at 72°C for 7 min.

Data processing. The sequencing process was preformed by Genetika Science inc, Jakarta, Indonesia. Nucleotides from 16S rRNA gene region were processed and edited using BioEdit, after which they were submitted to the NCBI database. The phylogenetic tree was made using BLAST Toll designed by the NCBI Database Search Tool.

Results and Discussion

Isolation and antimicrobial activity screening of symbiotic microbes. Isolation of symbiotic microbes from the 3 seaweed species resulted in 88 microbial isolates. The isolation was performed based on the morphological characteristics of the colony. Out of the 88 isolates obtained, 16 microbial isolates showed capability in inhibiting the growth of *E. coli* and *S. aureus*.

The antimicrobial activity screening indicates that out of the 88 microbial isolates found in the 3 red seaweed species collected from Bunaken waters of Indonesia, 18% show antimicrobial activity against human pathogenic microbes. Kanagasabhapathy et al (2008) reported that 33% of the isolates from 9 red algae collected from Japanese waters, Pacific Ocean, showed antimicrobial activity. Symbiotic microbes are known to produce bioactive molecules to protect the seaweed host from pelagic environmental threats and to strengthen its own position in competition with other microbe species (Mazzoli et 2017).

Oxidative fermentative test. 16 isolates of symbiotic microbes from the 3 seaweed species were tested to determine their capability in breaking down sugar. Results indicate that out of 16 microbes with antimicrobial properties, 9 isolates are tested positive in the oxidative fermentative test. Positive results are found in isolates labeled ES 2.2, ES 3.2, ES 3.4, GM 4.4, GM 4.5, GV 4.2, GV 4.3, GV 4.5 and GV 5.1. All 9 microbial isolates are then used in the culture of microbial consortium. The change in coloration from blue to yellowish green/yellow on fermentative microbes (isolates GV 4.2, GV 4.3, GV 4.5 and GV 5.1) is caused by the formation of acid products in the OF media. During the fermentation process, pyruvic acid is converted into various other acid products, depending on the type of fermentation.

Sensitivity test. The 9 microbial isolates with fermentative properties were tested with the antimicrobial sensitivity test to determine the resulting zone of inhibition. The results of antimicrobial sensitivity test (Table 1) indicate that 4 symbiotic microbes isolated from *Gracilaria* sp., namely GV 4.2, GV 4.3, GV 4.5 and GV 5.1, are effective against *E. coli* and *S. aureus*. The remaining 5 microbial isolates only show effectiveness against *S. aureus*.

Table 1

Antimicrobial activity and sensitivity test results of isolates with fermentative properties from seaweeds against *Escherichia coli* and *Staphylococcus aureus*

No	*Isolate code*	*Inhibition zone diameter (mm)*			
		Escherichia coli		*Staphylococcus aureus*	
		24 hours	48 hours	24 hours	48 hours
1	ES 2.2	0.00±0.00	0.00±0.00	4.47±0.50	0.00±0.00
2	ES 3.2	0.00±0.00	0.00±0.00	4.33±0.58	0.00±0.00
3	ES 3.4	0.00±0.00	0.00±0.00	3.18±0.28	0.00±0.00
4	GM 4.4	0.00±0.00	0.00±0.00	5.67±0.58	0.00±0.00
5	GM 4.5	0.00±0.00	0.00±0.00	3.35±0.56	0.00±0.00
6	GV 4.2	6.03±0.06	4.50±0.50	4.23±0.68	4.07±0.81
7	GV 4.3	2.72±0.51	2.17±0.29	6.84±0.75	5.37±0.32
8	GV 4.5	3.42±0.52	3.40±0.36	2.58±0.53	2.20±0.72
9	GV 5.1	2.93±0.90	2.46±0.73	5.37±0.78	5.23±0.25

Note: ES - *Eucheuma spinosum*; GM - *Gracilaria gracilis*; GV - *Glacilaria verrucosa*.

Molecular identification of microbes. Molecular identification of the microbes in all 9 isolates with fermentation capabilities was performed. The DNA amplification of these isolates showed a single band of 1300 bp in accordance to the comparison using DNA markers, as presented in Figure 1.

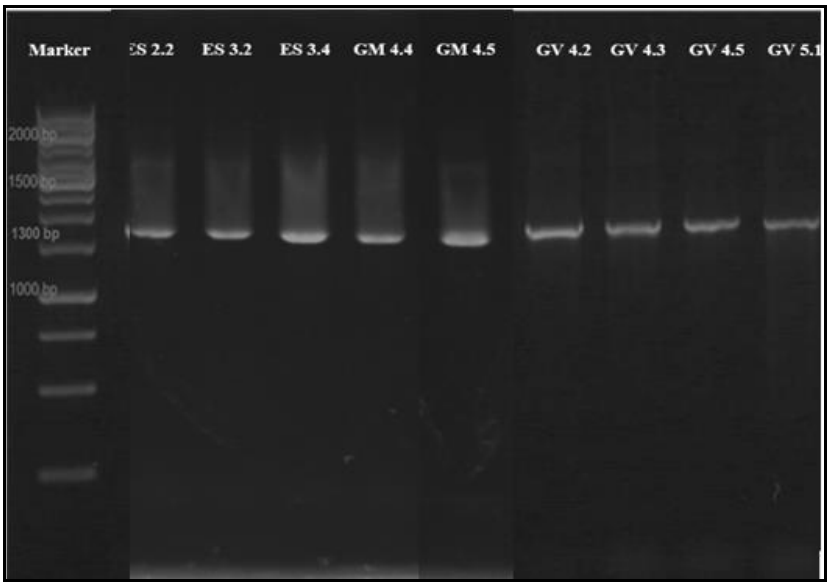

Figure 1. Results of 16S rDNA amplification.

The 16S rDNA amplification has become the standard in phylogentic and marine biodiversity studies. The processing of the phylogenetic tree shows the clustering of the symbiotic microbe isolates with other organisms from the lowest to the highest levels (Figure 2). Isolates GV 4.2 and GV 4.5 cluster with a single organism, namely *Vibrio alginolyticus.* Isolate GV 4.3 clusters with *Vibrio parahaemolyticus* and isolate GV 5.1 clusters with *Vibrio hyugaensis*. The clustering of these organisms is based on the arrangement of nucleotide of each species, where genetically identical species are clustered together (Garcia-Ramon et al 2015).

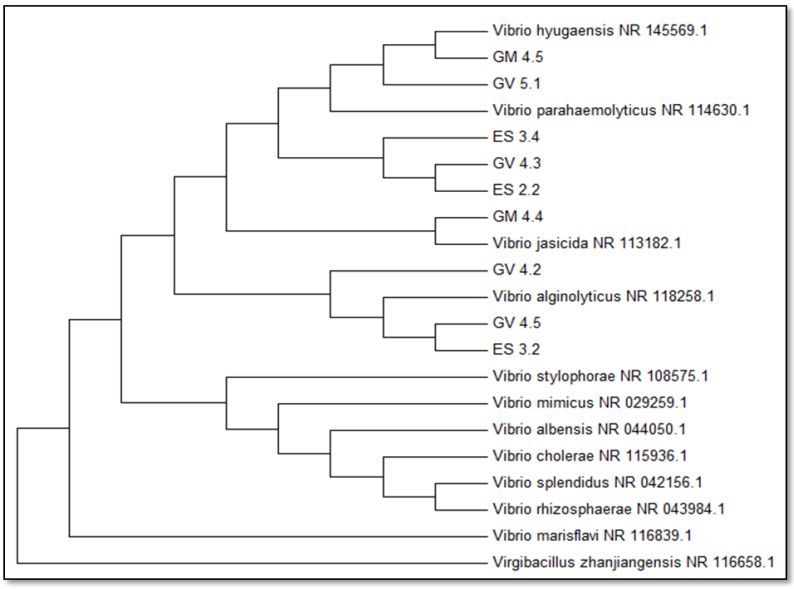

Figure 2. Phylogenetic tree of microbial isolates of red seaweed (*Eucheuma spinosum*; *Gracilaria gracilis; Glacilaria verrucosa*) symbiotic microbes, with *Virgibacillus zhanjiangensis* as the outer group.

The results of homology tracing of isolates ES 2.2, ES 3.2, ES 3.4, GM 4.4, GM 4.5, GV 4.2, GV 4.3, GV 4.5 and GV 5.1 with microbes from the Gene Bank database are presented in Table 2. The tracing resulted in more than 97% homology for all isolates. Isolates with more than 97% 16S rDNA are identified at species level (da Silva et al 2013). Sequence homology at 93%-97% can represent identity at the genus level. DNA amplification of single-band microbial isolates indicates that the primers used are specific primers to amplify 16S rDNA for microbes (Thijs et al 2017).

Table 2

DNA sequence tracing of microbial isolates from seaweeds with BLAST

No	*Code*	*Species*	*Homology*
1	ES 2.2	*Vibrio parahaemolyticus*	98%
2	ES 3.2	*Vibrio alginolyticus*	99%
3	ES 3.4	*Vibrio parahaemolyticus*	98%
4	GM 4.4	*Vibrio jascida*	99%
5	GM 4.5	*Vibrio hyugaensis*	98%
6	GV 4.2	*Vibrio alginolyticus*	99%
7	GV 4.3	*Vibrio parahaemolyticus*	98%
8	GV 4.5	*Vibrio alginolyticus*	99%
9	GV 5.1	*Vibrio hyugaensis*	98%

Note: ES - *Eucheuma spinosum*; GM - *Gracilaria gracilis*; GV - *Glacilaria verrucosa*.

Molecular identification indicate that all identified symbiotic microbes are from the genus *Vibrio*. BLAST analysis of isolates ES 3.2, ES 3.4, and GV 4.3 resulted in 98% homology with *Vibrio parahaemolyticus*. Isolates ES 3.2, GV 4.2 and GV 4.5 present homology with *Vibrio alginolyticus* (99%), isolates GM 4.5 and GV 5.1 with *Vibrio hyugaensis* (98%) and isolate GM 4.4 with *Vibrio jascida* (99%).

Bioactive compounds produced by symbiotic microbes depend of several factors, namely temperature, aeration, media acidity, incubation period and media composition. These factors must be taken into account and adjusted to reproduce the natural habitat of the symbiotic microbe (Penesyan et al 2009). For example, *Laminaria* microbial isolates can produce antibiotic compounds if the culture media is modified by adding extract of *Laminaria,* which is the natural host (Girão et al 2019). Microbes have several antimicrobial mechanisms, including inhibiting the formation of cell walls, damaging plasma membranes, inhibiting protein synthesis, the synthesis of nucleic acids, and the synthesis of essential metabolites (Prabhu & Poulose 2012; Holmes et al 2016).

Phylogenetic analysis results of this study indicate that all isolates which have antimicrobial and fermentative properties are identified as the genus *Vibrio*, which falls into the phylum Proteobacteria. It is reported that 63% of all epibiotic marine microbes are categorized into the phylum Proteobacteria (Wahl et al 2012; Saraiva & Dimopoulos 2019). Antimicrobial compounds successfully isolated are reported to be derived from various genera of bacteria such as *Pseudomonas*, *Pseudoalteromonas*, *Stenotrophomonas*, *Vibrio*, *Alteromonas*, *Shewanella*, *Streptomyces*, and *Bacillus* (Goecke et al 2010; Uzair et al 2018).

Conclusions. This study obtained 88 microbes symbiotic with seaweed species *E. spinosum*, *G. gracilis*, *G. verrucosa*, out of which 16 present antimicrobial activity. Out of the 16 isolates, 9 are determined to be fermentative. Antimicrobial sensitivity test in this study found 4 microbial isolates from *E. spinosum* with active antimicrobial activity against pathogenic *E. coli* and *S. aureus,* while the 5 remaining isolates from *G. verrucosa* and *E. gracilis* only showed active antimicrobial activity against pathogenic *S. aureus*. Molecular identification of fermentative isolates indicate that all the isolates are of the genus *Vibrio*.

References

Behnken S., Hertweck C., 2012 Anaerobic bacteria as producers of antibiotics. Applied Microbiology and Biotechnology 96(1):61-67.

da Silva M. A. C., Cavalett A., Spinner A., Rosa D. C., Jasper R. B., Quecine M. C., Bonatelli M. L., Pizzirani-Kleiner A., Corção G., Oliveira de Souza Lima A., 2013 Phylogenetic identification of marine bacteria isolated from deep-sea sediments of the eastern South Atlantic Ocean. Springerplus 2:127, 10 p.

Garcia-Ramon D. C., Luque-Navas M. J., Alfonso Molina C., del Val C., Osuna A., Vilchez S., 2015 Identification, sequencing and comparative analysis of pBp15.s plasmid from the newly described entomopathogen *Bacillus pumilus* 15.1. Plasmid 82:17-27.

Girão M., Ribeiro I., Ribeiro T., Azevedo I. C., Pereira F., Urbatzka R., Leão P. N., Carvalho M. F., 2019 Actinobacteria isolated from *Laminaria ochroleuca*: a source of new bioactive compounds. Frontiers in Microbiololgy 10:683, 13 p.

Goecke F., Labes A., Wiese J., Imhoff J. F., 2010 Review chemical interactions between marine macroalgae and bacteria. Marine Ecololgy Progress Series 409:267-300.

Holmes A. H, Moore L. S. P., Sundsfjord A., Steinbakk M., Regmi S., Karkey A., Guerin P. J., Piddock L. J. V., 2016 Understanding the mechanisms and drivers of antimicrobial resistance. Lancet 387(10014):176-187.

Isnansetyo A., Kamei Y., 2003 MC21-A, a bactericidal antibiotic produced by a new marine against methicillin-resistant *Staphylococcus aureus*. Antimicrobial Agents and Chemotheraphy 170(2):481-490.

Jiang Y., Mikova G., Kleerebezem R., van der Wielen L. A. M., Cuellar M. C., 2015 Feasibility study of an alkaline-based chemical treatment for the purification of polyhydroxybutyrate produced by a mixed enriched culture. AMB Express 5:5, 13 p.

Kanagasabhapathy M., Sasaki H., Nagata S., 2008 Phylogenetic identification of epibiotic bacteria possessing antimicrobial activities isolated from red algal species of Japan. World Journal of Microbiology & Biotechnology 24(10):2315-2321.

Kemme M., Heinzel-Wieland H., 2018 Quantitative assessment of antimicrobial activity of plga films loaded with 4-hexylresorcinol. Journal of Functional Biomaterials 9(1):4, 11 p.

Mazzoli R., Riedel K., Pessione E., 2017 Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Frontiers in Microbiology 13(10):701-712.

Monciardini P., Iorio M., Maffioli S., Sosio M., Donadio S., 2014 Discovering new bioactive molecules from microbial sources. Microbial Biotechnology 7(3):209-220.

Penesyan A., Marshall-Jones Z., Holmstrom C., Kjelleberg S., Egan S. 2009 Antimicrobial activity observed among cultured marine epiphytic bacteria reflects their potential as a source of new drugs. FEMS Microbiology Ecology 69(1):113-124.

Prabhu S., Poulose E. K., 2012 Silver nanoparticles: mechanism of antimicrobial action, synthesis, medical applications, and toxicity effects. International Nano Letters 2:32, 10 p.

Rocha-Martin J., Harrington C., Dobson A. D. W., O'Gara F., 2014 Emerging strategies and integrated systems microbiology technologies for biodiscovery of marine bioactive compounds. Marine Drugs 12(6):3516-3559.

Sanders E. R., 2012 Aseptic laboratory techniques: plating methods. Journal of Visualized Experiments 63:3604, 18 p.

Saraiva R. G., Dimopoulos G., 2019 Bacterial natural products in the fight against mosquito-transmitted tropical diseases. Natural Product Reports 37(3):338-354.

Thijs S., De Beeck M. O., Beckers B., Truyens S., Stevens, V., Van Hamme J. D., 2017 Comparative evaluation of four bacteria-specific primer pairs for 16S rRNA gene surveys. Frontiers in Microbiology 8, 15 p.

Uzair B., Menaa F., Khan B. A., Mohammad F. V., Ahmad V. U., Djeribi R., Menaa B., 2018 Isolation, purification, structural elucidation and antimicrobial activities of kocumarin, a novel antibiotic isolated from actinobacterium *Kocuria marina* CMG S2 associated with the brown seaweed *Pelvetia canaliculata.* Microbiological Research 206:186-197.

Wahl M., Goecke F., Labes A., Dobretsov S., Weinberger F., 2012 The second skin: ecological role of epibiotic biofilms on marine organisms. Frontiers in Microbiology 3:292, 1–21.
***Ministry of Energy and Mineral Resources, 2018 Bioenergy in Indonesia: rules and implementations. International Workshop: Future Perspectives of Bioenergy Development, Tokyo, Japan, 6-7 September.

Received: 10 March 2020. Accepted: 12 May 2020. Published online: 27 August 2020.
Authors:
Delianis Pringgenies, Department of Marine Sciences, Faculty of Fisheries and Marine Sciences, Diponegoro University, Tembalang Campus, 50275 Semarang, Indonesia, e-mail: delianispringgenies@lecturer.undip.ac.id
Endang Is Retnowati, Department of Chemical Engineering, Faculty of Engineering and Informatics, University of PGRI, 50125 Semarang, Indonesia, e-mail: endangisretnowati@upgris.ac.id
Dafit Ariyanto, Department of Aquaculture, Faculty of Agriculture, University of Asahan, 21216 Kisaran, Sumatera Utara, Indonesia, e-mail: dafitriyanto676@gmail.com
Kartika Dewi, Department of Marine Sciences, Faculty of Fisheries and Marine Sciences, Diponegoro University, Tembalang Campus, 50275 Semarang, Indonesia, e-mail: kakartikadewi@gmail.com
Muhamad Afwan Shadri Viharyo, Department of Marine Sciences, Faculty of Fisheries and Marine Sciences, Diponegoro University, Tembalang Campus, 50275 Semarang, Indonesia, e-mail: fwanshadriv2401@gmail.com
Ragil Susilowati, Department of Marine Sciences, Faculty of Fisheries and Marine Sciences, Diponegoro University, Tembalang Campus, 50275 Semarang, Indonesia, e-mail: susilowati_ragil@yahoo.com

How to cite this article:
Pringgenies D., Retnowati I. E., Ariyanto D., Dewi K., Viharyo M. A. S., Susilowati R., 2020 Symbiotic microbes from various seaweeds with antimicrobial and fermentative properties. AACL Bioflux 13(4):2211-2217.