Screening of Culturable Seaweed associated Bacteria with Polysaccharidases Activity Isolated from the Ambon Waters, Indonesia

Gintung Patantis[1*], Dewi Seswita Zilda[2], Jiang Li[3], Xiaoqian Gu[4], Yuanyuan Gui[5], and Stalis Norma Ethica[6]

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[1] Research Center for Marine and Land Bioindustry, National Research and Innovation Agency, Ds. Teluk Kodek, Kec. Pemenang, Kab. North Lombok, West Nusa Tenggara, 83352, Indonesia

[2] Research Center for Deep Sea, National Research and Innovation Agency. Jl. Pasir Putih Raya, Pademangan, North Jakarta City, Jakarta 14430, Indonesia

[3] Marine Biological Resources and Environment Center, First Institute of Oceanography, Qingdao 266061, China

[4] Chinese Academy of Science, Qingdao, China

[5] College of Environmental Engineering, Qingdao University, China

[6] Magister Program of Clinical Laboratory Science, Universitas Muhammadiyah Semarang, Jl. Kedungmundu Raya, Semarang, 50273, Indonesia

[*]Corresponding Author:
gint001@brin.go.id

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Abstract

Seaweed is a rich source of phycocolloids, which include agar, alginate, and carrageenan. Low molecular weight polysaccharides, namely oligomers or oligosaccharides, can be produced from seaweed polysaccharides through enzymatic degradation. Most of these enzymes are produced by microorganisms closely associated with seaweed. This study aimed to isolate and select polysaccharidases-producing (SPases) bacteria associated with wild seaweed from the sea around Ambon Island, an area famous for its high marine biodiversity index. A total of 11 types of marine algae samples could be collected, and as many as 92 bacterial isolates could be cultured from all these algae samples. Screening used a clear zone method on a solid medium containing substrates agar, alginate, or carrageenan and followed by Lugol's iodine staining solution showed that a total of 74 of the 92 bacterial isolates obtained were SPases-producing with the composition: agarase-producing (28 isolates), and alginate lyase-producing (26 isolates), and carrageenase-producing (20 isolates). The 16S rRNA identification results showed that the 74 bacterial isolates were representative of 13 species and belong to 2 classes, namely Gammaproteobacteria and Bacillus. The bacterial isolates in the Gammaproteobacteria class obtained consisted of three genera: *Pseudoalteromonas* (32 isolates), *Cobetia* (18 isolates), and *Microbulbifer* (15 isolates). Bacterial isolates in the Bacillus class obtained only contain a genus consisting of 8 isolates. In conclusion, the sea around Ambon Island is a potential source of polysaccharidases-producing algal symbiont bacteria.

Keywords: agar, alginate, carrageenan, seaweed polysaccharides, bacterial polysaccharidases

Introduction

Seaweeds are natural sources of polysaccharides and are essential resources that humans have used for decades. Based on their photosynthetic pigments, seaweeds are classified as red, brown, and green. Red seaweeds contain carrageenan and agar as the main extracellular polysaccharides, whereas brown seaweeds mainly contain alginate. These polysaccharides consist of small sugar residues linked by glycosidic bonds. Significant research into the industrial applications of these polysaccharides has been conducted because of their economic potential as biomass for energy production, proteins, feed for animals, food for humans, fine chemicals, pharmaceuticals, and cosmetics (Ahmed et al., 2014; Otero et al., 2023).

Low molecular weight polysaccharides derived from seaweed (seaweed oligosaccharides) can be generated naturally or produced by chemical or enzymatic hydrolysis (Ahmad et al., 2019; Giordano et al., 2006; Hong et al., 2017; Xu et al., 2018; Zheng et al., 2023). Seaweed oligosaccharides have been used in various industries for nutraceuticals, wastewater management, pharmaceuticals, biomaterials, cosmetics, prebiotics, and feed (Chi et al., 2015; Liu et al., 2015; Yun et al., 2013; Zhang et al., 2023). These oligosaccharides are generated using seaweed-degrading enzymes such as carrageenases, agarases, and alginate lyases to degrade carrageenan, agar, and alginate, respectively. Enzymatic depolymerization could generate higher yields and produce more specific oligosaccharides than chemical and physical methods.

Polysaccharidases that degrade seaweed polysaccharides (SPases) have been found in various organisms, including herbivores and marine mollusks. However, microorganisms associated with algae are among the most valuable sources of SPases because of their easy cultivation, rapid growth, and small space needed for production (Madgwick et al., 1973; Martin et al., 2014; Rahman et al., 2012; Suda et al., 1999). Of all culturable seaweed-associated bacteria, only 30% were reportedly able to degrade seaweed polysaccharides (Goecke et al., 2013). SPases have been reported in microbiota associated with the macroalgae *Delisea pulchra* and *Ulva australis* (Burke et al., 2011; Fernandes et al., 2012). A cultured approach is still nedeed to explore the diversity of seaweed-associated bacteria and determine their ability to produce SPases.

Several bacteria that produced SPases have been isolated and their enzymes have been characterized. They include *Wenyingzhuangia fucanilytica* (Pei et al., 2019), *Isoptericola halotolerans* CGMCC 5336 (Chen et al., 2018), *Sphingomona*s sp. (He et al., 2018), *Vibrio furnissii* (Zhu et al., 2018) and *Microbulbifer sp*. ALW1 (Zhu et al., 2016), which produce alginate lyases; *Flavobacterium* sp. YS-80-122 (Li et al., 2017), *Wenyingzhuangia fucanilytica* (Shen et al., 2017, 2018) *Wenyingzhuangia aestuarii* OF219 (Shen et al., 2018), and *Pseudoalteromonas carrageenovora* (Xiao et al., 2018), which produce carrageenases; and *Microbulbifer* sp. (Zhu et al., 2019), *Gayadomonas joobiniege* G7 (Jung et al., 2017). *Cellulophaga omnivescoria* W5C (Ramos et al., 2018), *Pseudoalteromonas* sp. H9 (Chi et al., 2015) and *Aquimarina agarilytica* ZC1 (Lin et al., 2017), which produce agarases. However, only a few studies have reported SPases obtained from bacteria associated with Indonesian seaweeds.

In this study, seaweeds were collected, and SPase producing-bacteria were isolated from Ambon Island surrounding sea. The aquatic environment is located in eastern Indonesia known to have a high marine biodiversity index (Tuapetel et al., 2019). This study aimed to isolate and screen polysaccharide producing bacteria by growing them on media containing specific substrates, subsequently identifying the species through molecular analysis of 16S rRNA.

Materials and Methods

Collection of Seaweed Samples

Seaweed samples were collected by scuba diving at 2-10 m depths in the sea around Ambon Island, Indonesia, in September 2018 at the following four coordinates: (i) 3°29.9730'S - 128°43.0930'E; (ii) 3°29.7750'S - 128°42.7330'E; (iii) 3°38.7470'S - 128°48.7090'E; and (iv) 3°38.7710'S - 128°47.97 60'E. The seaweed codes were ANL for coordinates i and ii; AMLN for coordinates iii and iv. The in-situ temperature, pH, and dissolved oxygen were 27.4-27.9°C, 8.57-8.91, and 0.20%-2.10% respectively. The samples were placed directly from the site into sterile bags, one for each species. The plastic bags containing the fresh seaweeds were stored in cool boxes with packed ice. Subsequently, the seaweeds were washed with sterilized seawater previously prepared by filtering seawater collected from the sampling site. The washed seaweeds were weighed aseptically prior to storing in sterile conical tubes. The seaweed samples were transported to the Biotechnology Laboratory, Research and Development Center for Marine and Fisheries Product Processing and Biotechnology (Jakarta Pusat, Indonesia) in cool boxes with packed ice and stored at 4°C until used.

Isolation and Screening of Microorganism that Produce SPases

Isolation of bacteria from the seaweed samples was carried out a week after the collection date. Ten grams of seaweed were mixed with 20 mL sterile seawater and shaken by vortex for 10 min to remove bacteria symbiont from the surface of the seaweed. One mL of solution was diluted in a series of 10^{-2}-10^{-4}, then spread on a solid medium containing 0.1% of peptone (Oxoid, UK) and 0.05% of yeast extract (Oxoid, UK) in seawater with 5% of carrageenan (TCI, Japan) or 0.1% of sodium alginate (Solarbio, China) for screening for carrageenase and alginate lyase activity respectively (Sawant et al., 2015). For agarase screening, 2% of bacteriological agar (Oxoid, UK) was added to the medium. Agar plates were incubated at 30°C for 1-3 days. The colonies growing on each agar plate were purified and checked for clear zones by streaking the pure culture on a solid medium. After two days, Lugol's iodine solution (10 g potassium iodide and 5 g iodine in 1000 mL dH_2O) was poured into the colonies (Kasana *et a*l., 2008). The clear zone will be formed around the colony, indicating the capability of isolates to produce polysaccharidase. The isolate codes followed these sequences: screened medium (Car for carrageenan, Alg for Alginate and Aga for agar) - the source of seaweed - number of isolates.

DNA Extraction and 16S rRNA Identification

The genomic DNA of bacteria was extracted using a heat-shock method by boiling 250 µL pure 24-h bacterial cultures for 15 min, then transferring them immediately on ice for 10 min. The treatments were done twice. The PCR mixture contained 1 µL forward

primer (27F, 52 -AGAGTTTGATCCTGGCTCAG-32), 1 µL reverse primer (1492R, 52-GGTTAC CTTG TTACGACTT-32), 25 µL of 1× PCRmix (TIANGEN, China), and 2 µL of DNA templates. The PCR cycles were denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 90 s. The reaction was ended at 72°C for 10 min (TaKaRa, Japan). The PCR products were electrophoresed on 1% agarose gel (Mupid-EXu, Japan) along with a 2000-bp DNA ladder (TaKaRa, Japan), then stained with ethidium bromide (Sigma Aldrich, USA) and visualized using a Geldoc UV transilluminator (Biometra-Herolab UVT-20M, Germany). Then, all PCR products were sequenced (Personalbio, Qingdao, China) by the Sanger sequencing method (Winand et al., 2019).

Sequence Analysis

The obtained sequences were used as queries in a homology search against the NCBI nucleotide sequence database (nr) using BLASTN (Basic Local Alignment Search Tool) (Altschul et al., 1990), then aligned using ClustalW (Higgins et al., 1996). A phylogenetic tree was constructed by neighbor-joining mid-point analysis (Kim, 1993) using MEGA7 with 1000 replicates of the bootstrap test (Kumar et al., 2016). The Codon Code Aligner (Codon Code Corporation, Massachusetts, USA) was used to analyze the similarity of 16S rRNA sequences (1400-bp fragments). The sequence with similarity >98% was considered to represent one operational taxonomic unit (OTU).

Sequence Submission

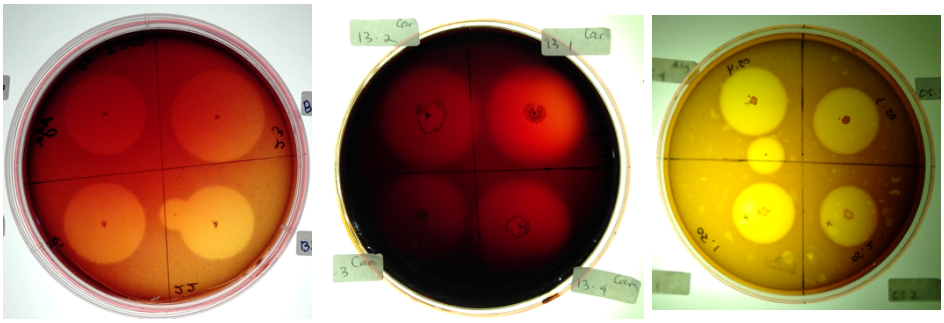
The DNA sequences of the 74 SPase-producing bacteria have been deposited in the DNA Data Bank of Japan (DDBJ) with accession numbers of MK453420-MK453493.

Results and Discussion

Isolation and Screening the Seaweed Samples

We collected a total of 92 bacterial isolates from 11 differnt seaweeds inhabiting the sea around Ambon Island, Indonesia. A total of 74 from 92 bacterial isolates formed a clear zone on a solid medium containing substrates of seaweed polysaccharides (carrageenan, sodium alginate, and agar) after Lugol's iodine staining. The clear zone indicated that these bacteria are SPase producers (Figure 1). The clear zone was formed because the degraded polysaccharide by the enzyme did not form a complex with the dye. In contrast, in the dark zone, the polysaccharide and dye solution interacted (Hodgson & Chater, 1981; Kasana et al., 2008). In terms of agarase and carrageenase-producing bacteria, only one substrate, agar or carrageenan, was added to the solid medium, so the clear zone formed on the solid medium was undoubtedly caused by the degradation of the one added substrate. For screening carrageenase-producing bacteria, the solid medium should contain up to 5% carrageenan to compact the medium. However, the clear zone formed on a solid medium must be confirmed for alginate-degrading bacteria by measuring enzyme activity in a liquid medium using two substrates, sodium alginate and agar, added on a solid medium. This is because the degradation of agar, sodium alginate, or both could have formed the clear zone.

Different types of SPase bacteria were isolated from different seaweeds. Seaweeds ANL 24 and AMLN 02 only produced one type SPase which was agarase and carrageenase, respectively. Seaweeds AMLN 05, 06 and 08 produced alginate lyase bacteria. Whereas three



A B C

Figure 1. Clear zones formed by polysaccharidase-producing bacteria on seawater agar medium containing seaweed polysaccharides (A) Agar, (B) Carrageenan, and (C) Sodium alginate after incubation for 48 h at 30°C. The colonies were stained with 10% Lugol's iodine solution.

types of SPase bacteria were isolated from seaweeds ANL 00, AMLN 12, AMLN 14 and AMLN 15. These seaweeds also had the highest number of SPsase-associated bacteria, with 10, 7, 24, and 16 isolates, respectively (Table 1).

Most of SPase-producing bacteria were reported to be associated with seaweed (Martin et al., 2014). Only a small number were reported from the seawater columns by metagenomic (Ferrer et al., 2016) and culturable approaches (Neumann et al., 2015). In addition, the type of some SPases-producing bacteria is related to the polysaccharide type of seaweed. Verrucomicrobia, known as fucoidan degrading bacteria, inhibit specific brown algae, particularly those within the *Fucus* genus (Singh & Reddy, 2014). Like other marine microorganism, polysaccharide-degrading bacteria play vital roles in carbon recycling from seaweed polysaccharides. In the marine ecosystem, polysaccharide-degrading bacteria are associated with seaweeds and overcome oligotrophic conditions by degrading seaweed polysaccharides into biologically versatile forms (Imran et al., 2017).

The specificity associations between algae and bacteria have been described. Some bacterial groups (Aquificae, Chlorobi, Dyctioglomi, Lentisphaerae, and Tenericutes) were reported in macroalga Rhodophyta and Gemmatimonadetes. At the same time, Proteobacteria was found in all macroalgal genera (Martin et al., 2014). Regarding the type of seaweed, the *Laurencia* genus belonging to the Rhodophyta class had the highest bacterial diversity (Florez et al., 2017). Some studies found that the specificity of bacteria with the macroalgae was influenced by various factors: differences in cell walls, polysaccharidases, and bioactive compounds of the macroalgae. Polysaccharide cell wall variations distinguish environmental interactions by governing biological and biomechanical processes within an individual and among different organisms such as bacteria. Microorganism has ability to produce different types of polysaccharide-modifier enzymes. These enzymes degrade the polysaccharides as organic carbon sources for them. Bioactive substances such as antimicrobial, antifouling, vitamins and growth factors function as defense mechanisms or attraction for the microorganism (Martin et al., 2014).

Identification of SPase-Producing Bacteria

The DNA was successfully isolated from the 24-h cell cultures of all the SPase-producing bacteria. The amplified 16S rRNA sequences obtained by PCR for each isolate formed a single band at around 1,400 bp. The 74 culturable isolates represented 26 strains and 13 species, and their codes, reference names, similarity, and accession numbers are listed in Table 2. While a neighbor-joining tree based on the 16S rRNA sequences of the SPase-producing bacteria is presented in Figure 2.

Based on Table 2, most of the isolates belonged to phylum Proteobacteria with class Gammaproteobacteria and represented three genera *Pseudoalteromonas, Cobetia* and *Microbulbifer.*

Table 1. Numbers of polysaccharidase-producing bacteria isolated from seaweed collected from the sea around Ambon Island, Indonesia

Seaweeds	Number of isolates			Total
	Carrageenase	Alginate lyase	Agarase	
ANL 00	4	3	3	10
ANL 08	2	NA	1	3
ANL 24	NA	NA	1	1
AMLN 02	1	NA	NA	1
AMLN 05	NA	1	NA	1
AMLN 06	NA	1	NA	1
AMLN 08	NA	3	NA	3
AMLN 12	2	3	2	7
AMLN 14	5	9	10	24
AMLN 15	4	1	11	16
AMLN 18	2	5	NA	7
Total	20	26	28	74

NA : Not Available

Pseudoalteromonas was the most common genus with 32 isolates, followed by *Cobetia* (18 isolates) and *Microbulbifer* (15 isolates). Phylum Firmicutes class Bacilli had only one genus *Bacillus* (8 isolates). This study was similar to the study of seaweed-associated bacteria isolat1ed that six major phyla of seaweed-associated bacteria had been cultured, namely Bacteroidetes (42%), Proteobacteria (35%), Firmicutes (10%), Actinobacteria (8%), Verrucomicrobia (5%), and Planctomycetes (1%). That study showed that 30% of isolates degraded seaweed components or nutrients available in living seaweed. Another study identified 36 different bacterial genera associated with the brown seaweed *Ascophyllum nodosum*; the most abundant genus was *Marinomonas* (13.1%), followed by *Cellulophaga* (10.1%) and *Pseudoalteromonas* (9.1%). Among 324 isolates, 74 (34%) had polysaccharidase activity (Martin et al., 2014). *Cobetia* and *Pseudoalteromonas* were the most abundant genera found among the Ambon seaweeds. Furthermore, this result was similar to a study by Lin et al. that found that *Pseudoalteromonas* was the dominant genus among

Table 2. Details of representative bacteria from the 26 strains of polysaccharidase-producing bacteria isolated from seaweed collected from the sea around Ambon Island, Indonesia

Code of Represented Isolates	References strain (GenBank)	Similarity	Gen Bank Accession Number	Total of similar isolates	Phylum; Class; Genera
Alg-AMLN-18-1	*Cobetia* sp. 37	99%	MK453430	1	Proteobacteria; Gammaproteobacteria; *Cobetia*
Car-AMLN-14-6	*Cobetia sp* . strain JCG-23	99%	MK453457	1	
Alg-AMLN-05	*Cobetia* sp. strain P4	100%	MK453445	3	
Aga-AMLN-15-5	*Cobetia* marina strain JCM 21022	99%	MK453452	13	
Aga-AMLN-14-8	*Microbulbifer* sp. HB09007	99%	MK453473	7	Proteobacteria; Gammaproteobacteria; *Microbulbifer*
Alg-AMLN-14-4	*Microbulbifer* sp. HB09008	99%	MK453461	4	
Aga-AMLN-14-4	*Microbulbifer* sp. strain THAF38	99%	MK453461	1	
Car-ANL-00-4	*Microbulbifer variabilis* strain SCSIO_43706	99%	MK453422	3	
Aga-AMLN-15-2	*Pseudoalteromonas* sp. BSi20622	99%	MK453449	1	Proteobacteria; Gammaproteobacteria; *Pseudoalteromonas*
Alg-AMLN-15-1	*Pseudoalteromonas* sp. BSi20396	99%	MK453436	13	
Aga-ANL-00-3	*Pseudoalteromonas* sp. strain 8-13	99%	MK453493	3	
Car-AMLN-12-Mix1	*Pseudoalteromonas* sp. strain 22-16	99%	MK453482	4	
Alg-AMLN-12-Mix2	*Pseudoalteromonas donghaensis* strain HJ51	99%	MK453478	7	
Car-AMLN-14-Mix1	*Pseudoalteromonas issachenkonii* strain KMM 3549	99%	MK453479	1	
Aga-AMLN-14-Mix2	*Pseudoalteromonas issachenkonii* strain 5-4-4	100%	MK453472	1	
Alg-ANL-00-2	*Pseudoalteromonas lipolytica* strain K-W45	99%	MK453490	1	
Alg-ANL-00-Mix2	Uncultured *Pseudoalteromonas* sp. clone Cl17	99%	MK453490	1	
Alg-AMLN-14-9	*Bacillus* sp. strain 201705CJKOP-80	100%	MK453431	1	Firmicutes; Bacilli; *Bacillus*
Alg-AMLN-14-1	*Bacillus* sp. CNJ796 PL04	99%	MK453433	1	
Alg-AMLN-14-7	*Bacillus* sp. strain CK-35	100%	MK453434	1	
Aga-AMLN-15-6	*Bacillus* sp. BEK11	99%	MK453451	1	
Car-AMLN-18-1	*Bacillus altitudinis* strain -Y118	100%	MK453426	1	
Car-AMLN-14-3	*Bacillus pumilus* strain JMB004	100%	MK453444	1	
Alg-AMLN-14-2	*Bacillus pumilus* strain CFC-5	100%	MK453427	1	
Car-AMLN-18-2	*Bacillus oceani* strain SCSIO 04524	99%	MK453425	1	
Car-AMLN-14-1	*Bacterium* strain GU1721	100%	MK453455	1	

bacteria associated with the giant kelp *Macrocystis pyrifera*. However, *Cobetia* were not found with either the cultured or metagenomic approaches in that study (Lin et al., 2018). *Pseudoalteromonas* is also found as a dominant bacterium in infecting disease of *Saccharina japonica* (Wang et al., 2008).

The microalgae-associated bacteria not only produced SPases but also have essenstial functions for the algae. Some studies found that these bacteria contributed to macroalgae's defense, growth, and nutrient uptake. The bacteria associated improved the defense of macroalgae by produce antimicrobial and

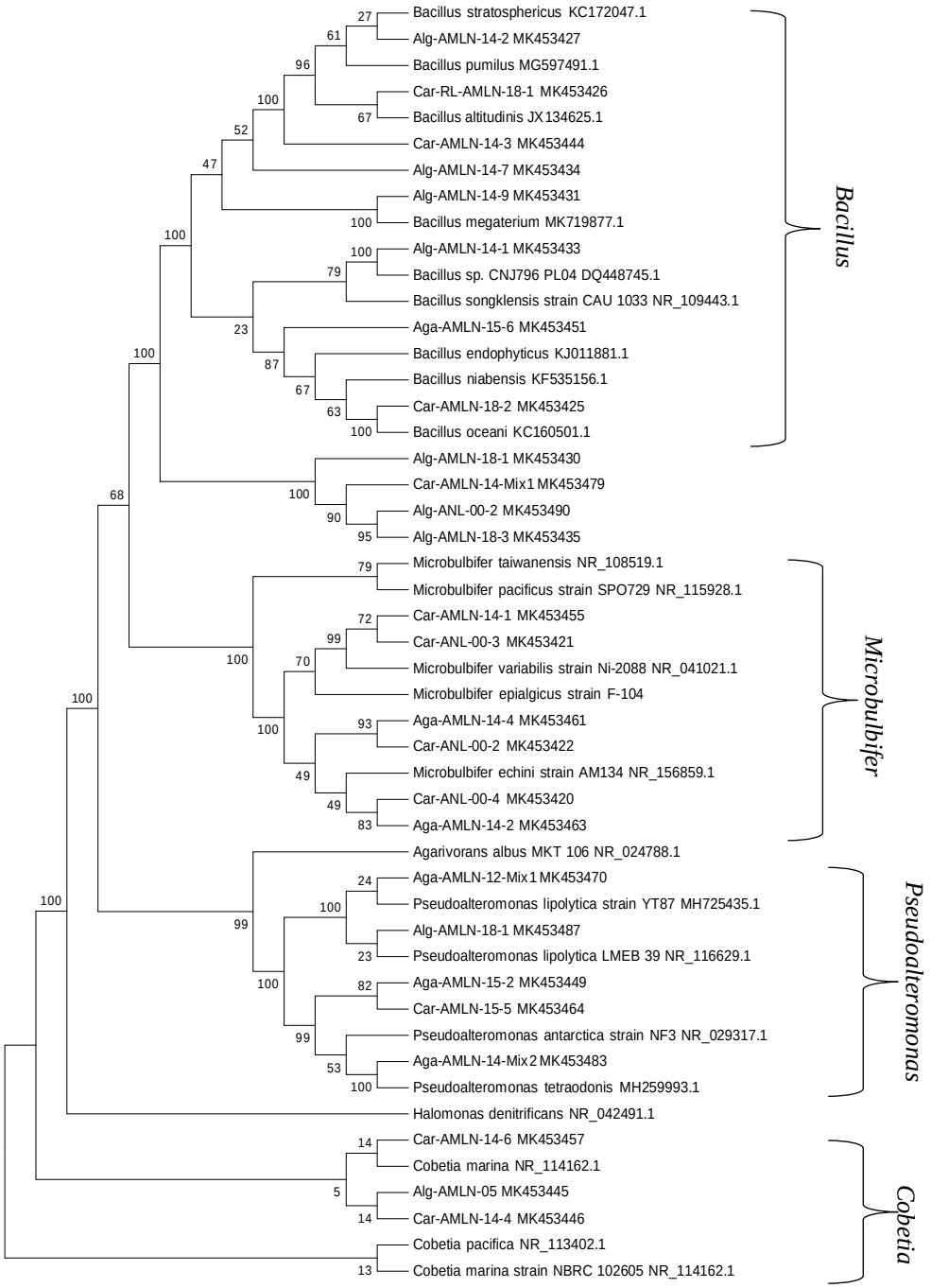


Figure 2. Neighbor-joining phylogenetic tree constructed using 16S rRNA sequences of the represented isolates of 74 polysaccharidase-producing bacteria associated with seaweed and related strains from GenBank. MEGA7 was used to construct the tree and the % bootstrap values are shown at the nodes.

antifouling compounds. The number of macroalgae isolated strains with antimicrobial activity was higher than seawater or terrestrial strains (Penesyan et al., 2009). Some microbes produced vitamins and growth elements in plentiful amounts for macroalgae growth. In addition, bacteria isolated from macroalgae produced various hydrolytic enzymes that enhanced the nutrient uptake of macroalgae. A bacteria isolate from red algae has agarase activity and amylase, phosphatase, esterase, urease and lipase activities (Kim & Hong, 2012)

The Composition of SPase-Producing Bacteria

The compositions of SPase-producing bacteria capable of degrading the three substrates are shown in Figure 3. Among the 13 species representing the 74 isolates, four were in class Bacilli, eight were in class Gammaproteobacteria, and one was unclassified. Some of these species could degrade all three types of seaweed polysaccharides, whereas others degraded only one or two types of seaweed polysaccharides. Three species only degraded carrageenan; one degraded alginate; one degraded carrageenan and alginate; two degraded alginate and agar; two degraded agar and carrageenan; and four degraded all three polysaccharides. It was found that all species capable of degrading agar were also capable of degrading carrageen and/or alginate. (Figure 4). *Microbulbifer* sp., *Cobetia marina*, and *Pseudoalteromonas* sp. were found in high numbers and could degrade all three polysaccharides. *Pseudoalteromonas donghaensis*, which was also found in high numbers, degraded alginate but had low activity against agar. Three species, *Bacillus altitudinis*, *Bacillus oceani*, and an unclassified bacterium specifically degraded carrageenan, whereas *Pseudoalteromonas lipolytica* degraded alginate.

Pseudoalteromonas, *Microbulbifer* and *Cobetia* species are important in biotechnology because of their metabolic versatility. Some *Pseudoalteromonas* Spp. were reported to produce agarase (Borchert et al., 2017; Mauro et al., 2013; Oh et al., 2011; Hinojosa et al., 2018; Schroeder et al., 2003; Vera et al., 1998), alginate lyase (Li et al., 2011; Zhang et al., 2020) and carragenase (Xiao et al., 2018; Zhao et al., 2021). Similarly, various *Microbulbifer* were found produced agarose (Li et al., 2018; Ma et al., 2019), alginate lyase (Yang et al., 2020; Zhu et al., 2016) and carrageenase (Jonnadula et al., 2018). In addition, the genome sequences of *Microbulbifer* Spp. were reported to contain various genes coding for polysaccharidase, including agarase, alginate lyase and carragenase (Imran et al., 2017; Jung et al., 2018). *Cobetia* were reported to have alginolytic activity (Gong et al., 2017; Yagi et al., 2016). These species also were found to produce bioflocculants (Ugbenyen et al., 2012), phosphatases (Golotin et al., 2015), sulfated O-polysaccharides that have anticancer activity (Kokoulin et al., 2016) and hydrocarbon degradation activity (Ibacache-Quiroga et al., 2013). However, only a few studies are available about the agarase and carragenase activity of *Cobetia* genus.

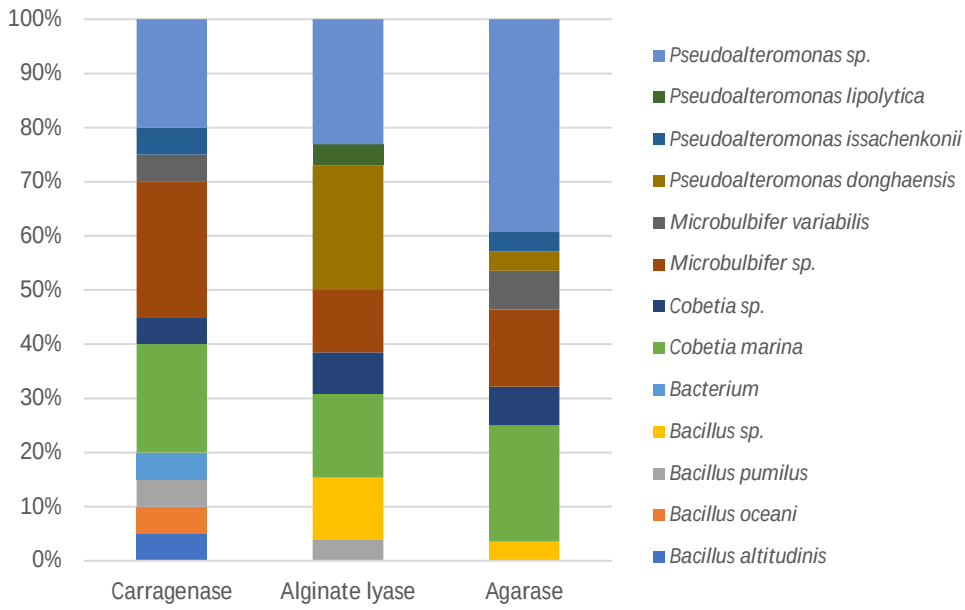


Figure 3. The composition of 74 polysaccharidase-producing bacteria which grouped in to 13 species based on their capability to degrade carrageenan, alginate, and agar.

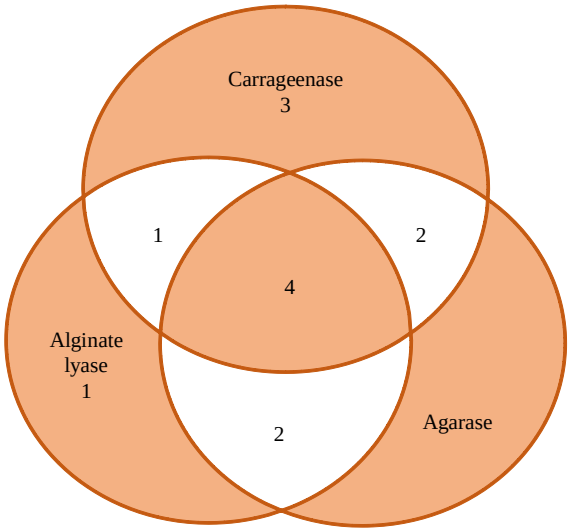


Figure 4. A Venn diagram shows the distribution of the 13 species in degrading the seaweed polysaccharides i. e. carrageenan, alginate, and agar.

Furthermore, some of the species that had been isolated from Ambon seaweeds had limited information of SPase producing ability, such as *Pseudoalteromonas lipolytica*, *Microbulbifer variabilis*, and *Bacillus oceani*, which provided new insights into the diversity of SPase-producing bacteria.

Conclusion

In this study, 74 SPase-producing bacteria were isolated from 11 types of marine algae samples collected from Ambon Sea waters. The 74 isolates consist of 28 isolates agarase-producing, alginate 26 isolates alginate lyase-producing and 20 isolates carrageenase-producing. The identification results showed that 13 species represent 74 isolates and belong to 2 classes, namely Gammaproteobacteria and Bacillus. The Gammaproteobacteria class contained three genera: Pseudoalteromonas (32 isolates), Cobetia (18 isolates), and Microbulbifer (15 isolates). The Bacillus class only contained a genus consisting of 8 isolates. *Microbulbifer* sp., *Cobetia marina*, and *Pseudoalteromonas* sp. were found in high numbers and were able to degrade all three types of polysaccharides. Some species have limited information on the ability of SPase-bacteria which provided new insights into the diversity of SPase-producing bacteria. Further work will be conducted to determine the characteristics of the polysaccharidases produced by some of these seaweed-associated bacteria to find suitable applications for these enzymes.

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Supplementary Materials

Supplementary materials is not available for this article.

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