Molecular systematic and phylogenetic analysis of symbiotic bacteria of *Hydroclathrus* sp. producing antibiofilm enzyme alginate lyase

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Abstract. Alginolytic bacterial communities are known for producing antibiofilm enzymes that disrupt alginate, the main component of biofilm. Biofilm-associated infections are dangerous due to the resistance they cause against antibiotics and the human immune system. This work reported several marine alginolytic bacteria, potentially novel species, based on molecular systematics and phylogenetic analysis targeting 16S rRNA. They were isolated from distinct brown algae *Hydroclathrus* sp. inhabiting the sea surrounding Hoga Island, Wakatobi, Indonesia. The study aimed to reveal these bacterial isolates' molecular identity and kinship to understand more of their properties as symbionts of *Hydroclathrus* sp. Molecular identification and phylogenetic tree construction were performed based on sequences of 16S rRNA gene amplified using Polymerase Chain Reaction with 27F-1492R primers. A total of 31 isolates of the brown algae *Hydroclathrus's* symbiont bacteria could be obtained, indicating that the algae is an attractive symbiont host for marine bacteria. The number of isolates capable of producing alginate lyase and agarase was 15. Yet, after a confirmation test with minimal alginate media, only 12 out of 15 isolates were indeed alginate lyase producers. Molecular identification on the 8 isolates with the highest alginolytic indexes shows the closest relationship with 3 different genera: *Vibrio*, *Alteromonas*, and *Aestuariibacter*. Based on BLAST (Basic Local Alignment Search Tool) analysis, 5 have a lower than 97% similarity level to the top hits of their alignment results, revealing that they might be novel species. These findings showed the potential of marine brown algae *Hydroclathrus* sp. as a potential host of alginolytic bacteria.
Key Words: agarase, alginate lyase, marine bacteria, Wakatobi.

Introduction. Antibiofilm enzymes are types of enzymes that can be used to control and remove bacterial biofilms. These enzymes dissolve the polysaccharides, proteins, and nucleic acids that comprise bacteria's extracellular matrix. Antibiofilm enzymes include lipase, which prevents the growth of *Vibrio parahaemolyticus* biofilm, and cellulase, which breaks down the cellulose present in most biofilms (Gutiérrez 2019). It has also been demonstrated that combination enzymes such as lipase, cellulase, and proteinase K are efficient in preventing and eliminating *V. parahaemolyticus* biofilm (Li et al 2022). Other biofilm-controlling enzymes include β-glucanase, proteinase, and amylase, which can break down the EPS matrix and prevent the production of biofilms. Antibiofilm enzymes are considered more effective and environmentally benign than traditional methods, such as aggressive chemicals like sodium hydroxide or sodium hypochlorite, which can corrode machinery and materials (Blackman 2021).

The rich content of alginate in brown algae naturally makes it an attractive host for bacteria producing necessary antibiofilm enzymes; one of the notable ones is alginate lyase (Algl) (Barzkar et al 2022). This is because alginate is the naturally specific substrate for

the exolytic polysaccharide lyase enzymes such as Algl. Among brown algae with a high content of alginate is *Hydroclathrus* sp. It is an edible brown seaweed with a yellowish-brown, sponge-like appearance inhabiting shallow sea water surrounding islands (Santiañez et al 2018). A high alginate content of 46.0% was reported from *Hydroclathrus clathratus* in St. Martin's Island, Bangladesh (Rashedul & Zafar 2018). In Wakatobi Districts, especially in waters surrounding Hoga Island, *Hydroclathrus* sp. are pretty widespread in rainy seasons. However, in Indonesia, these brown seaweeds are generally considered less economical than other groups of brown algae, such as *Sargassum* or *Laminaria*. It is mainly because of the macroalgae do not belong to the primary producers of agar and carrageenan (Ethica et al 2021).

The search for new Algl is necessary to combat biofilm-involving infection. Concerns related to biofilm-associated infection are raised by the resistance it causes against antibiotics and the human immune system (Jamal et al 2018). The alginolytic bacterial community can produce an antibiofilm enzyme able to disrupt alginate, the main component of biofilm. Hence, alginate lyase (Algl) is widely reported as an anti-biofilm agent (Li et al 2019; Ethica et al 2021). The current study aimed to explore the symbiotic bacteria of *Hydroclathrus* sp., searching for new antibiofilm agents from marine environments.

Material and Method

Description of the study sites. *Hydroclathratus* sp. samples were collected in January 2021 from the waters (around 50-100 cm underwater) surrounding Hoga Island, Wakatobi Districts, South Eastern Sulawesi, at site coordinates of 5.28317° S and 123.45377° E. The white-sanded island (Figure 1) can be accessed by flight from Makassar Airport in South Sulawesi, apart from boat services from central Wakatobi Island.

Figure 1. Hoga Island waters located in Wakatobi Districts, South Eastern Sulawesi, Indonesia.

Isolation and selection of alginolytic bacteria. Isolation of symbiotic bacteria from macroalga samples was conducted on the spot using Zobell agar media (Kizhakkekalam et al 2019) before transporting them to the laboratory in Jakarta. To conduct alginolytic screening of symbiont bacteria, a minimal alginate medium agar was prepared as follows: The alginate powder was dissolved in sterile water gradually while stirring using a high-speed propeller-type stirrer. After the alginate material was dissolved as a homogeneous aqueous solution, Bacto agar was added to the alginate solution. The mixture was stirred until the agar was completely dissolved, the new solution was homogeneous, and the mixture was ready to pour into plates. As previously reported, alginolytic bacterial colonies were selected from the isolated *Hydroclathrus* sp. symbiotic bacteria using specially prepared minimal alginate agar media (Zilda et al 2019).

Molecular identification of alginolytic bacteria. All isolates of algal symbiont bacteria showing positive results for the alginate lyase production test were further identified molecularly by the PCR (Polymerase Chain Reaction) targeting the 16S rRNA gene (Zilda

et al 2019). This step was carried out to obtain biodiversity data on the alginolytic symbiont bacteria of the *Hydroclathrus* sp. For bacterial DNA extraction, pure isolated bacterial colonies previously refreshed on solid minimal alginate (MAL) medium were inoculated into 50 mL of liquid MAL medium and incubated at 30°C in a shaking incubator, at 150 rpm for 24 h. The bacterial cultures were then centrifuged at 10,000 x g for 10 min. The pelleted cells were then used for DNA extraction using the Genomic DNA Purification Kit (Fermentas, Lithuania) based on the kit's protocol (Kizhakkekalam et al 2019). Total DNA from 16 positive bacterial isolates for agarase was extracted using PCR (Qiagen). The PCR reaction consisted of 1 mL Forward primer (27F-5'- AGAGTTTGATCCTGGCTCAG-3'), 1 mL Reverse primer 1492R 5'-CGGTTACCTTGTTACGACTT-3'), 25 mL PCRmix (TIANGEN), 21 mL ultrapure water and 2 mL DNA (Aravena et al 2020; Hidayati et al 2021). PCR Amplification was performed using the GeneAmp® PCR System 2700 kit (Applied Biosystems, Carla, CA, USA) under pre-denatured conditions at 94°C for 5 mins and followed by 35 denaturation cycles at 94°C for 30 s, annealing at 54°C for 30 s and by a 72°C extension for 90 s (Mubarik et al 2022). The reaction was terminated by a post extension at 72°C for 10 min. The 16S rDNA Sanger sequencing was performed with the PCR products at Genetika Science, Singapore (Swacita et al 2022). The obtained 16S rRNA gene sequence was then searched for similarities to the bacterial 16S rDNA references in the NCBI, using BLASTn (Basic Local Basic Alignment Search Tool) (Agostino 2012). Results were then aligned using the ClustalW program (Thompson et al 2003) and separated by DNA electrophoresis (Mupid-EXu) using 1% agarose gel along with a 2000 bp DNA marker (TaKaRa), stained with ethidium bromide (Sigma Aldrich, USA) and visualized with a Geldoc UV transilluminator (Biometra-Herolab UVT-20M).

Construction of phylogenetic tree. Mid-point neighbor-joining (NJ) analysis was performed using the MEGA X software (Saitou 1991; Kumar et al 2016; Stevens et al 2019). The method is based on the neighbor-joining (NJ) algorithm, a distance-based method that constructs trees by iteratively joining pairs of taxa, that minimize the total branch length at each clustering stage. This method is generally more reliable than other methods, which assume a constant rate of evolution. Next, the summary of 16S rRNA gene-based identification results of selected isolates of alginolytic bacteria symbiont of *Hydroclathrus* sp. was evaluated. The lengths of branches in the tree, which should be proportional to the amount of genetic divergence between the bacteria, were analyzed to understand their evolutionary relationship (Purwaningrum et al 2021).

Results. This work reported several alginolytic bacteria, potentially novel species based on molecular systematics and phylogenetic analysis targeting 16S rRNA. The marine bacteria were isolated from rarely studied brown algae *Hydroclathrus* sp. inhabiting the sea surrounding Hoga Island, Wakatobi, Indonesia. Four samples of *Hydroclathrus* were coded WKTB 01 to 08, one of which is displayed in Figure 2. Symbiotic bacteria of each algal sample were index-coded by HI03, HI04, HI08, and HI09, where HI refers to Hoga Island.

Figure 2. A fresh sample of *Hydroclathrus* sp. isolated from 50-100 cm underwater from the surroundings of Hoga Island, Wakatobi, Indonesia.

Alginolytic bacterial isolation from Hydroclathrus sp. A total of 31 isolates of the brown algae *Hydroclathrus* symbiont bacteria were obtained, indicating that algae are an attractive symbiont host for many marine bacteria. The number of isolates capable of producing alginase (alginate lyase) and agarase was 15. Yet, after a confirmation test on solid MAL medium, it was found that only seven of the 15 isolates (all originated from sample WKTB 03) were indeed alginate lyase producers, i.e., the isolates HI03-1a and HI03-1b, HI03-3a and HI03-3b, HI03-5a- and HI03-5b, and HI03-8b (Figure 3).

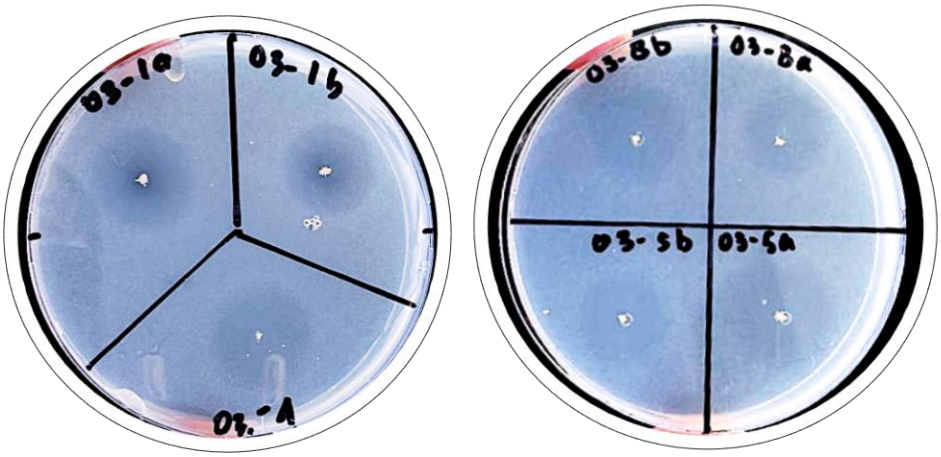

Figure 3. The alginolytic clear zone surrounding the colonies of HI03 and HI04 marine symbiotic bacterial isolates from *Hydroclathrus* sp. (harvested in the Wakatobi surroundings) on minimal agar alginate (MAL) media.

The 16S rDNA sequences of 7 positive isolates/ alginate lyase producers were aligned with those of other bacterial strains retrieved from the DDBJ/EMBL/GenBank databases using the BLASTn tool (Agostino 2012). The BLASTn analysis results are shown in Table 1. As seen in Table 1, the seven isolates belong to 3 genera, namely: *Vibrio*, *Alteromonas*, and *Aestuariibacter*.

A phylogenetic tree construction based on 16S rRNA gene sequences using neighbor-joining (NJ), as Saitou (1991) first reported, was performed for seven alginolytic isolates. The tree generated by MEGA 7 based on a previously reported protocol by Kumar et al (2016) is shown in Figure 5.

Table 1

Result of Basic Local Alignment Search Tool (BLAST) analysis on 16S rRNA sequences of 7 isolates of alginolytic bacteria associated with *Hydroclathrus* sp. from Hoga Island

No.	*Isolate code*	*Isolate name*	*Top species hit*	*Highest homology level (%)*
1	A	03-3a	*Vibrio* sp. JC009	98.00
2	B	03-3b	*Vibrio* sp. JC009	96.65
3	C	03-4	*Alteromonas gracilis* SCSIO_43745	98.03
4	D	03-5a	*Alteromonas gracilis* SCSIO_43745	97.12
5	E	03-5b	*Alteromonas gracilis* SCSIO_43745	96.49
6	F	03-8a	*Alteromonas* sp. strain 139 (*forward*)	80.55
7	G	03-8b	*Aestuariibacter* sp.	78.79

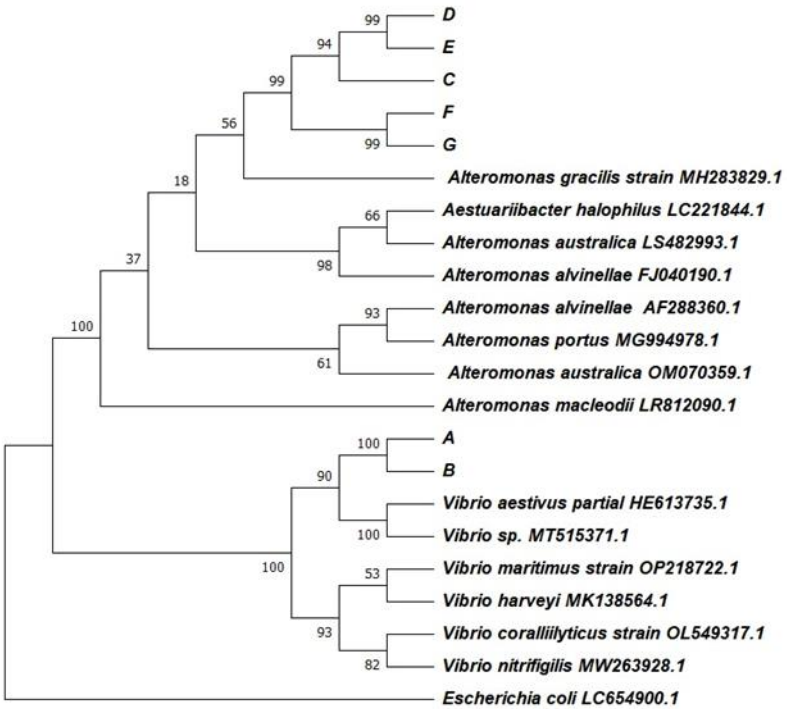

Figure 4. A neighbor-joining tree showing the phylogenetic relationship of the obtained symbiotic bacterial isolates with alginolytic activity (coded A-G) based on 16S rRNA gene partial sequences. *Escherichia coli* (GenBAccession no. LC654900.1) was used as an outgroup, the substitution (The scale bar represents 0.005 nucleotide substitutions per site.)

Discussion. Molecular identification on seven selected alginolytic isolates showed they have the closest relationship with three different *Vibrio*, *Alteromonas*, and *Aestuariibacter genera*. Interestingly, the performed BLASTn had lower than 97% similarity levels to the top hits, suggesting that they can probably be identified as a novel species. In the definition of species identification, Janda & Abbott (2007) stated that similar species have ≥99%, while different species showed <99% sequence similarities on 16S rRNA gene sequence. As seen in Figure 4, phylogenetic analysis results of 7 selected isolates show that they belong to only two genera, Alteromonas and Vibrio. The tree showed that all seven isolates were situated in only two clades. A lower score of BLASTn hits (only 78.9%) of isolate code G (HI03-8b) explained why the species does not fall into the group *Aestuariibacter*. However, this family is positioned in the same branch as *Alteromonas*, together with the species *Alteromonas australica*. The tree analysis underlines the novelty possibility of isolating HI03-8b as previously predicted by a low BLASTn hit score. To confirm the novelty of species of isolate HI03-8b as well as three other isolates (HI03-3b, HI03-5a, and HI03-5b), a Whole Genome Sequencing (WGS) analysis should be performed to reveal further the uniqueness of bacterial DNA composition (Huang et al 2018; Ethica et al 2023). Bacterial symbionts may play a crucial role in the growth, morphogenesis, and overall health of brown algae such as *Hydroclathrus* sp. found in the South Eastern Sulawesi Sea (de Mesquita et al 2019; Ethica et al 2021). These symbionts can be divided into two main categories: epiphytic and endophytic. The epiphytic bacteria attach to the surface of the algae and can interact with the algal cells through various mechanisms. They may provide nutrients to the algae, such as nitrogen, phosphorus, and other essential elements (Singh & Reddy 2014). Additionally, epiphytic bacteria can enhance the growth of the algae by producing extracellular polymeric substances (EPS), which can help to hold marine aggregates and keep bacterial networks intact, eventually facilitating bacterial biofilm formation. This can lead to improved survival of the algae and enhanced growth rates (Wichard 2023). Endophytic bacteria live inside the algae cells and can influence the algae's growth and development. They may provide essential nutrients or role in the algae's

metabolism, contributing to its overall health and growth (Singh & Reddy 2014). Additionally, endophytic bacteria may help the algae to cope with environmental stresses, such as temperature fluctuations, salinity changes, and exposure to pollutants (Ghaderiardakani et al 2020).

In previous studies, *Hydroclathrus* sp. was barely mentioned as a potential host of alginolytic bacteria. However, bacteria attached to the brown algae wall *Hydroclathrus* sp. were used in a study that reported broad-spectrum bioactivity against pathogenic bacteria and cytotoxic activity against cancer cell lines (Kaaria 2018). This study identified a variety of bacterial isolates, including *Actinobacteria*, *Bacillus*, and Proteobacteria, that clustered with different bacterial species, such as *Bacillus* sp.: *Bacillus pumilus*, *Bacillus aerius*, and *Bacillus safensis*. Interestingly, bacteria reported from the anticancer screening were other genera from those obtained using alginolytic screening in our study. The results of the current study also showed that brown algae *Hydroclathrus* sp. is a potential marine bacterial host that could support the production of valuable enzymes, including alginate lyase. Such enzymes are beneficial for human health, particularly in biofilm-related infection treatment. Today, the enzyme plays a significant role in treating cystic fibrosis caused by *Pseudomonas aeruginosa* (Blanco-Cabra et al 2020). The findings suggest that *Hydroclathrus* sp. is the only novel range of bacterial symbionts with *in vitro* antibiofilm properties. More extensive studies may support the alginate hypotheses, which can be expected from novel bacterial species associated with *Hydroclathrus* sp.

Conclusions. *Hydroclathrus* sp. from waters around Hoga Island, Wakatobi, is an attractive symbiont host for many marine bacteria. Seven of the 15 symbiotic bacterial isolates associated with *Hydroclathrus* sp. were found to be alginate lyase producers, thus having potential as an anti-biofilm agent. Molecular identification of these seven isolates based on 16S rRNA gene sequences suggested they might be novel species.

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Conflict of interest. The authors declare no conflict of interest.

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