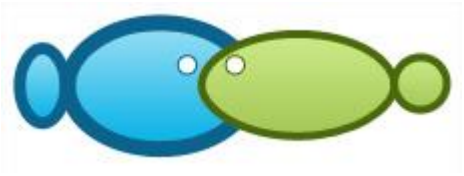

Protease enzymes of sponge-associated bacteria (Porifera: Demospongiae) in seagrass ecosystems at Nusa Lembongan Bali, Indonesia

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Abstract. Protease is an enzyme that is useful as a biocatalyst in the breakdown of proteins. Protease belongs to the main class of hydrolase enzymes which are widely used in the bioindustry. Results of this study lead to candidate bacteria that are useful in the bioindustry of shrimp culture. The study aimed to obtain screening and identification of symbiont sponge Demospongiae bacteria that can produce extracellular protease enzymes. The study conducted purposive sampling of sponges in the waters of the seagrass ecosystem in Nusa Lembongan, Bali, Indonesia, using an exploratory descriptive method. The research methods included: identification of sponges, screening of sponge symbionts, growth characteristics and protease activity of sponge symbionts, and identification of the selected bacteria using the molecular DNA method. The results of the morphological identification of sponges indicated *Chalinula pseudomolitba*. The study succeeded in obtaining 42 bacterial isolates, 7 isolates were active in protein hydrolysis, namely isolates 5, 6, 10, 19, 20, 37 and 39 with values of 13,135^{b}, 20,224^{e}, 11,033^{a}, 15,167^{c}, 17,412^{d}, 17,705^{bc} and 24,582^{f} mm. Isolate 39 had the highest protein hydrolysis zone, with an absolute growth of 14.159^{e}x10^{8} cells mL^{-1}, a number of generations 4.065^{e}, a generation time of 6.889^{a}, a growth rate of 0.100^{e}, a protease activity of 17.996 IU mL^{-1}, a protein content of 8.9 mg mL^{-1}, and a specific activity of 2.011^{g} IU mg^{-1}. The results of the identification of 16 S rRNA isolates 39 were identified as *Bacillus firmus* bacteria with the characteristics of bacillus-shaped, gram positive, indole (+), MR (+), VP (+), SC (+), moltyl and catalase positive.
Key Words: bacteria, symbionts, sponges, enzymes, proteases.

Introduction. Sponges are simple and sessile organisms that evolve, developing chemical defense mechanisms to protect themselves from competitors, predators and pathogenic microorganisms. Sponges are able to withstand interference from other organisms by relying on various bioactive compounds (Sabdono & Radjasa 2008). Sponges are marine biota that produces secondary metabolites with various groups of terpenoids, acetogenins, alkaloids, cyclic halides, cyclic peptides, nitrogen compounds. Bioactive compounds are produced by symbiotic microorganisms, not by the sponge itself (Sabdono & Radjasa 2008). Exploration of bacterial bioactive compounds from sponges from Indonesian waters has been carried out and has succeeded in isolating compounds with a broad-spectrum antimicrobial activity against pathogenic bacteria and pathogenic fungi (Sheikh 2010). Meanwhile, information about bacterial symbionts that have a protease enzyme activity is found in several bacterial symbionts, including symbionts of the stomach contents of sea cucumbers (Pringgenies et al 2020; Djunaedi et al 2021; Pringgenies et al 2021).

Protease acts as the main component in reactions that can easily hydrolyse protein residues (de Souza et al 2015) so that the findings of protease bacteria are useful

as ingredients for low-toxicity enzymes, environmentally friendly cleaners and increased efficiency and stability, so that these bacteria can serve as a probiotic bacteria candidate for bioindustry, such as aquaculture activities. The application of protease bacteria is described as occurring in the decomposition process carried out by decomposers, namely probiotic bacteria, through the production of extracellular enzymes (Xiao et al 2020). Extracellular protease enzymes produced by bacteria hydrolyze feed waste proteins into simple peptides and amino acids. These simple peptides and amino acids are utilized by bacteria for their metabolic processes (Caballero et al 2020; Jasmin et al 2020). Thus, the study aimed to obtain bacterial isolates that have an extracellular protease-producing activity that can help provide nutrition and the nitrification process for sponges (Batista et al 2018; Hema et al 2019), and might become candidates in the making of probiotics for bioremediation of contamination with organic feed waste in shrimp farming. The study aimed to obtain a screening and identification of symbiont sponge Demospongiae bacteria that can produce extracellular protease enzymes.

Material and Method

Description of the study sites. The material in this research is sponge Demospongiae. Sponges were obtained from the seagrass ecosystem in Nusa Lembongan Bali, Indonesia, by purposive sampling. Next, the sponge was taken by cutting it using a knife and then stored in a cool box containing ice cubes for analysis in the laboratory.

Sponge identification. Sponges found were documented in the water and above the water surface for identification purposes in the sponge identification portal at http://spongeguide.org and http://www.marinespecies.org/porifera/porifera.php?p=specimens.

Isolation and purification of sponge association bacteria. Sponge samples were washed using sterile seawater, drained, and crushed using a mortar aseptically. After the sponge was crushed, 1 g was taken and put into a test tube containing 9 mL of sterile seawater. Then, the dilution was carried out in stages up to 10^{-5}. Bacterial colonies that grew were observed for the colony morphology (Jeanson et al 2015).

Proteolytic activity test with diffusion method. As much as 1 ose of isolates from purification on slanted media were inoculated into liquid Zobel 221E medium and incubated for 24 h at room temperature.

Culture in fermenter biocontroller. The culture was carried out in a 2 L scale fermenter with a working volume of 1 L. Conditions of the fermenter were: Zobell 2216 broth, 1% inoculum concentration with OD 0.01 at A600, pH 8, temperature of 35°C, salinity of 30 ppt, agitation speed of 150 rpm. Observations were made on the optical density (OD) of bacteria at A600, protease activity and protein content at 2, 4, 6, 12, 18, 24, 30, 36, 42 and 48 h of incubation.

Bacterial turbidity measurement. The OD measurement was carried out by centrifuging 10 mL of the sample at 3,000 rpm for 10 min. The supernatant was discarded and the natant obtained was dissolved in 10 mL of PBS solution and then vortexed. Furthermore, the OD value was observed using a spectrophotometer at a wavelength of 600 nm (Zainuddin et al 2017; Pringgenies et al 2020). The data from the OD measurement on the A600 was then converted with the standard Mc Farland equation into units of cells mL^{-1}. The cell mL^{-1} value is used to calculate the number of generations (g), generation time (Tg) and growth rate (μ) of bacteria.

Measurement of protease enzyme activity and protein levels. The substrate used was 1% casein dissolved in phosphate buffer (50 mM, pH 8). The protease absorbance data was converted to mM units using the standard tyrosine curve equation (Setyati et al 2016) and then to protein absorbance data (Setyati et al 2015).

Physiological test. Physiological tests were carried out to determine the physiological characteristics of bacteria. Physiological tests included: indole, Simmons citrate agar, urease, MR-VP, TSIA and glucose fermentation, H_2O_2, oxidase tests.

Bacteria identification. The bacterial isolates were cultured in MRS broth at 30ºC for 24 hours. DNA amplification was carried out using PCR with primers 9F (5'-AAGGAGGTGATCCAGCC-3') and 1541R (5'-AAGGAGGTGATCCAGCC-3'), which were added to the DNA solution, then purified using the Gel/DNA extraction kit. The 16S rRNA gene obtained was then sequenced using the Dye® terminator V 3.1 cycle sequencing kit. The DNA sequence equipment used is the ABI 300 genetic analyzer. Furthermore, the sequences obtained were compared with the database available in the NCBI Blast (Basic Local Alignment Search Tool) using the BLAST search engine (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Zhang et al 2020; Bahmani et al 2021).

Statistical analysis. Furthermore, the data obtained were tested by statistical analysis, namely One Way ANOVA.

Results and Discussion

Sponge identification. Sponge sampling was carried out in waters of a seagrass ecosystem with a substrate of coral rubble mud on the north coast of Nusa Lembongan. The collected sponges have a characteristic purple color in the water and purple outside the water, the surface did not secrete mucus, massive and sturdy. The oscules were large and spread all over the surface, so it can be concluded that the sponge sample is of the order Haplosclerida, family Chalinidae and genus Chalinula. The Chalinula genus is commonly found in the seagrass ecosystem in Nusa Lembongan Bali, Indonesia.

Bacterial proteolytic activity test with agar diffusion method. Results of the isolation found 42 isolates from 4 samples of the sponges, in which: sponge 1 had 12 isolates, sponge 2 had 9 isolates, sponge 3 had 13 isolates and sponge 4 had 8 isolates. Results of the proteolytic activity test showed that from 42 isolates, 7 isolates were active in protein hydrolysis (Table 1).

Table 1

Proteolytic activity of bacterial isolates by agar diffusion method

Sample	*Isolates*						
	5	*6*	*10*	*19*	*20*	*37*	*39*
Hydrolysis zone (mm)	13.135^{b}	20.224^{e}	11.033^{a}	15.167^{c}	17.412^{d}	17.705^{bc}	24.582^{f}

Different italics behind the value indicates that the value is significantly different ($p<0.05$).

Bacterial isolates had a proteolytic activity with a diameter of the hydrolysis zone between 11.033–24.582 mm. The isolate with the lowest zone activity was isolate 10. Meanwhile, the isolate with the highest zone activity was isolate 39. Results of One-Way ANOVA analysis showed that 7 isolates had a significantly different protein hydrolysis activity ($\alpha<0.05$). Sequentially isolates 10, 5, 19, 20, 6 and 39 had hydrolysis zone diameters that were significantly different, the larger were 11.033^{a}, 13.135^{b}, 15.167^{c}, 17.412^{d}, 20.224^{e} and 24.582^{f} mm, respectively.

Several studies have shown that heterotrophic bacteria are the main source of extracellular enzymes needed for the mineralization of organic matter including the production of amylase, protease, cellulase and lipase enzymes (Wilis et al 2015). Results of this study indicate that isolates with proteolytic activity have been successfully obtained. Then, these extracellular proteolytic enzymes hydrolyze protein compounds into oligopeptides, short-chain peptides and amino acids. This extracellular protease enzyme is essential for bacterial life because it provides the needs of nitrogen compounds that can be transported into cells.

Protease enzymes have two meanings; proteinase which catalyzes the hydrolysis of protein molecules into simpler fragments, and peptidase which hydrolyzes polypeptide fragments into amino acids. Proteolytic enzymes derived from microorganisms are proteases containing proteinases and peptidases (Kieliszek & Pobiega 2021). Factors that affect the size of the inhibitory zone of antimicrobial substances include the activity of the antimicrobial functional group of the substance itself, the resistance of bacteria to antimicrobial substances, the level of the active substance and the number of bacterial inoculum or the density of the test bacteria (Funovics et al 2005).

Growth of sponge-associated bacterial isolates. The results of the proteolytic activity test have succeeded in obtaining 7 active bacterial isolates. Furthermore, the 7 bacteria were cultured in a fermenter to determine the growth characteristics of the bacteria. The culture process was carried out for 2 days with observations at 2, 4, 6, 12, 18, 24, 30, 36, 42, and 48 hours. Based on the results of the study, it was shown that the 7 isolates had different growth chart patterns. Bacterial isolates 5 and 10 had the most sloping growth pattern than the other bacterial isolates. Furthermore, isolates 19 and 37 had a higher growth pattern and were close to each other. The bacterial growth curve is generally divided into four growth phases, namely the lag phase (adaptation), the log phase (exponential), the stationary phase and the death phase (Rocchetti et al 2020).

The lag phase (adaptation) is a period of microbial adjustment. Cells must first acclimate to new growth conditions. The log phase is a phase where bacteria experience very fast growth. In addition, the need for energy for bacteria in this phase is higher than in other phases and the cell becomes very sensitive to its environment (Saberi et al 2020). Therefore, in this phase, bacteria produce a lot of metabolites needed to meet their nutritional needs. The growth then becomes slow, this is because the nutrients in the media have been greatly reduced.

Based on the results, 7 isolates had significant absolute differences in growth. Isolates 5 and 10 had the lowest absolute growth compared to other isolates, namely 2.519^{a} and 2.724^{a}. Furthermore, isolates 19 and 37 had higher absolute growth but were not significantly different from each other, namely 7.060^{b} and 5.904^{b}. The two groups of isolates had lower absolute growth than isolates 20, 16, and 39. The three isolates were significantly different from each other, namely 9.886^{c}, 12.301^{d} and 14.159^{e}x10^{8} cells mL^{-1} (Table 2).

Table 2

Absolute growth value, growth rate, number and generation time of bacterial isolates

Isolate sample	*Regression in exponential phase*			*Pm (x 10^{8} cells mL^{-1})*	*μ (hours $^{-1}$)*	*g (generation)*	*Tg (hours)*
	Equation	*R^2*	*R*				
Isolate 5	y=0.0046x+0.0908	0.9430	0.971	2.519^{a}	0.052^{b}	2.124^{b}	13.19^{d}
Isolate 6	y=0.0236x+0.0166	0.9930	0.996	12.301^{d}	0.093^{d}	3.765^{d}	7.437^{b}
Isolate 10	y=0.0047x+0.1064	0.9134	0.956	2.724^{a}	0.047^{a}	1.922^{a}	14.581^{e}
Isolate 19	y=0.0127x+0.1310	0.8355	0.914	7.060^{b}	0.076^{c}	3.075^{c}	9.119^{c}
Isolate 20	y=0.0196x+0.0660	0.9555	0.977	9.886^{c}	0.089^{d}	3.602^{d}	7.791^{b}
Isolate 37	y=0.0106x+0.1165	0.8721	0.934	5.904^{b}	0.071^{c}	2.894^{c}	9.717^{c}
Isolate 39	y= 0.0264x+0.0941	0.9651	0.982	14.159^{e}	0.100^{e}	4.065^{e}	6.889^{a}

Different italics behind the values in the same column indicate that the values are significantly different ($p<0.05$).

Based on the results of the study, 7 isolates had some generations and growth rates that were significantly different. The number of generations value and the growth rate ranged from 1.922[a] to 4.065[e] and 0.047[a] to 0.100[e], respectively. The lowest number of generations value and the lowest growth rate was isolate 10 and the highest was isolate 39. The isolates with the highest number of generations and growth rates were isolates 6 and 20: isolate 6 with values of 3.765[d] and 0.093[d], isolate 20 with values of 3.602[d] and 0.089[d]. Based on the results of the study, it was shown that the 7 isolates had significantly different generation times. The growth of a microorganism is strongly influenced by environmental factors such as physico-chemical factors (temperature, pH, aeration, agitation) and the composition of the growing media (Bonnet et al 2019).

Sponge association bacterial isolate protease activity. Bacterial isolate samples were cultured in a fermenter and observed for extracellular protease enzyme activity. The aim was to determine the optimal time and the resulting protease production. The results show that 7 isolates had significantly different patterns of extracellular protease enzyme activity. Isolates 5 and 10 had the lowest pattern of protease activity compared to other isolates and coincided with each other. Proteases are enzymes that catalyse the breakdown of peptide bonds in peptides, polypeptides and proteins through hydrolysis reactions into simpler molecules such as short-chain peptides and amino acids (Wilis et al 2015). In the genus Bacillus, the synthesis of the greatest number of extracellular enzymes normally occurs just before sporulation, i.e., at the end of the exponential or early stationary phase (Subagiyo et al 2015).

Based on the results, it is shown that 7 isolates had an absolute increase in extracellular protease enzyme activity that was significantly different. The absolute increase in extracellular protease enzyme activity of bacterial isolates from low to high was found in isolate 10, isolate 5, isolate 37, and isolate 19, with values of 5.932[a], 6.291[b], 7.585[c] and 8.572[d] IU mL^{-1}, respectively. The next group comprises isolate 20, isolate 6 and isolate 39, with values of 10.175[e], 12.170[f] and 15.091[g] IU mL^{-1} (Figure 1).

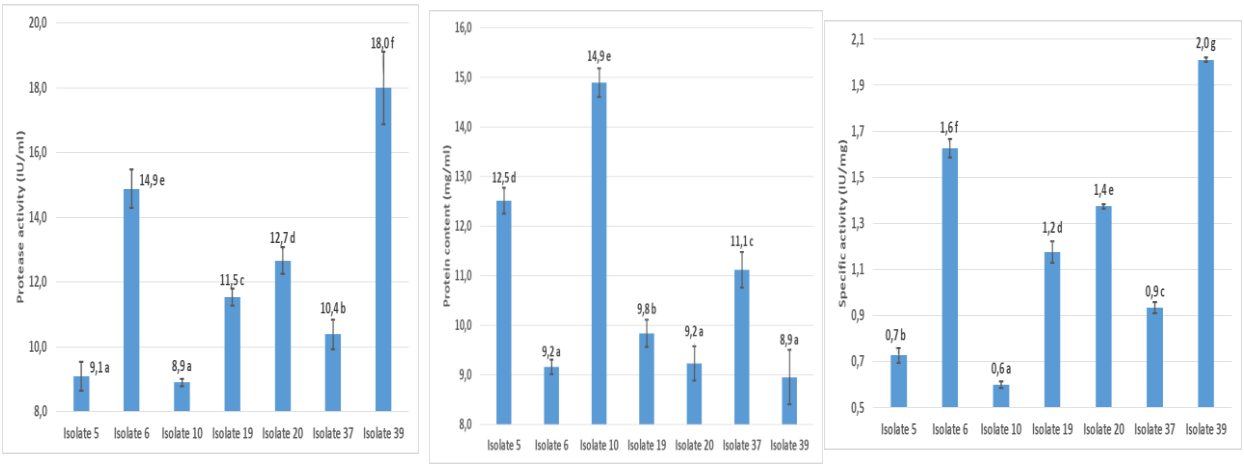

Figure 1. The protease activity of sponge-associated bacteria isolates at an exponential peak (a) and protein content of sponge-associated bacteria isolates at the lowest point of exponential decline (b), specific activity of sponge-associated bacterial isolates at an exponential peak (c).

Based on the results of the study, it was shown that 7 isolates had significantly different rates of increase in extracellular protease enzyme activity (Table 3). Isolate 10 had the lowest rate of increase in protease activity compared to the other isolates, which was 0.039[a]. Isolate 5, isolate 19 and isolate 37 had a higher rate of increase in protease activity, which were 0.042[b], 0.048[c], and 0.046[bc]. Furthermore, isolates 6 and 20 had a higher rate of increase in protease activity and were not significantly different from each other, which were 0.060[d] and 0.058[d]. The isolate that had the highest rate of increase in protease activity from the other isolates was for isolate 39, with a value of 0.065[e] (Table 3).

Table 3

Absolute increase value and rate of increase in extracellular protease activity

Isolate sample	*Regression in exponential phase*			*Absolute protease activity (IU mL^{-1})*	*Rate of protease activity (IU mL^{-1} hours^{-1})*
	Equation	R^2	R		
Isolate 5	y=0.2096x+2.9249	0.9734	0.987	6.291^{b}	0.042^{b}
Isolate 6	y=0.4217x+2.2157	0.9806	0.990	12.170^{f}	0.060^{d}
Isolate 10	y=0.2045x+3.6250	0.8651	0.930	5.932^{a}	0.039^{a}
Isolate 19	y=0.2905x+2.4127	0.9844	0.992	8.572^{d}	0.048^{c}
Isolate 20	y=0.3574x+2.2070	0.9914	0.996	10.175^{e}	0.058^{d}
Isolate 37	y=0.2645x+3.1331	0.9496	0.974	7.585^{c}	0.046^{bc}
Isolate 39	y=0.5171x+1.4971	0.9496	0.974	15.091^{g}	0.065^{e}

Different italics behind the values in the same column indicate that the values are significantly different ($p<0.05$).

The protein content of sponge association bacteria isolates. In addition to observing the activity of extracellular protease enzymes, in the fermenter culture, protein levels were also observed. The results of the study showed that the 7 isolates had a significantly different pattern of decreasing protein levels. Furthermore, isolates 5, 19 and 37 had a higher pattern of decreasing protein content. The isolate that had the highest pattern of decreasing protein content was isolate 10.

Based on the protein reduction data for each isolate, there was a decrease in the protein content from the beginning to the end of the research. The decrease in the protein content of the media was probably due to the presence of protease enzymes produced by bacteria. Enzyme activity depends on the proper structure and conformation of the protein molecule. Changes in the structure and conformation of protein molecules, for example by changes in temperature, pH and metal ions, cause the biochemical activity of proteases to be reduced or denatured. This causes the biochemical activity of the protease to be reduced or denatured. Each enzyme has a certain temperature and pH that causes its activity to reach an optimum state (Ojha et al 2020). The presence of protease activity after the optimum temperature is probably due to the protein is coagulating or conjugated with other molecules, so that the enzyme activity is reduced.

Based on the results of the study, 7 isolates had a significant decrease in absolute protein content: isolate 5, isolate 10 and isolate 20 had a low absolute decrease in protein content, with values of -6.906^{a}, -5.460^{a} and -6.573^{ab} mg mL^{-1}. The isolate with the highest absolute decrease in protein content compared to the other isolates was isolate 6 in -11.487 d mg mL^{-1} (Table 4). Furthermore, it was shown that the 7 isolates had significantly different rates of decrease of protein levels. Isolate with the lowest rate of decrease in protein content was isolate 10, with a value of was -0.011^{a}. In addition, isolates 5, 19, 20, and 37 had a higher rate of decrease in protein content, namely -0.015^{ab}, -0.020^{b}, -0.019^{b} and -0.020^{b}, respectively. Isolate 39 has a higher rate of decrease in protein content, with a value of -0.025^{c}. The isolate with the highest rate of decrease in protein content compared to the other isolates was isolate 6, with a value of -0.029^{d}.

Table 4

Absolute decreasing value and rate of decrease in protein levels

Isolate sample	*Regression in exponential phase*			*Decrease in absolute protein content (mg mL^{-1})*	*Decrease in rate of protein content (mg mL^{-1} hours^{-1})*
	Equation	*R^2*	*R*		
Isolate 5	y=-0.2708x+20.006	0.9716	0.986	6.906^{a}	0.015^{ab}
Isolate 6	y=-0.3660x+19.014	0.8738	0.935	11.487^{d}	0.029^{d}
Isolate 10	y=-0.1779x+20.123	0.9701	0.985	5.460^{a}	0.011^{a}
Isolate 19	y=-0.2808x+17.997	0.9889	0.994	7.768^{b}	0.020^{b}
Isolate 20	y=-0.2279x+16.256	0.9933	0.997	6.573^{ab}	0.019^{b}
Isolate 37	y=-0.3242x+19.984	0.9574	0.978	8.375^{b}	0.020^{b}
Isolate 39	y=-0.3441x+18.459	0.9721	0.986	9.451^{c}	0.025^{c}

Different italics behind the values in the same column indicate that the values are significantly different ($p<0.05$).

In the screening phase of the research, a qualitative hydrolysis activity test was carried out. Results of the reshuffle of protein polymers are only shown by the presence of a clear zone which indicates that the protein has been remodeled into peptide and amino acid compounds that are dissolved in the medium. The hydrolysis activity is a qualitative illustration of the ability of proteolytic bacterial isolates to break down proteins by comparing the size of the clear zone around the colony with the size of the colony diameter (Sun et al 2019).

The specific activity of protease isolates of sponge-associated bacteria. Based on the value of protease activity and protein content in the fermenter culture, further determination of the specific activity value of extracellular protease enzymes was carried out. Results of the study show that 7 isolates had significantly different patterns of specific activity of extracellular protease enzymes. The lowest specific activity pattern was found in isolates 5 and 10. The highest specific activity pattern and overlapping each other was found in isolates 6 and 20. The highest specific activity pattern compared to the other isolates was found in isolate 39, which had a peak of specific activity at 30 hours, with a value of 2.011 IU mg^{-1}.

Based on the results of the study, 7 isolates had values for the absolute increase in specific activities that were significantly different. The isolate with the lowest absolute increase in specific activity was isolate 10, with a value of 0.452^{a} IU mg^{-1}. Furthermore, isolates 5, 37, 19, 20 and 6 had higher absolute values of specific activity, respectively, with values of 0.581^{b}, 0.790^{c}, 1.006^{d}, 1.215^{e}, and 1.494^{f} IU mg^{-1}. The isolate with the highest absolute increase in specific activity was isolate 39 with a value of 1.852^{g} IU mg^{-1} (Table 5).

Table 5

Absolute increase value and rate of increase in specific activities

Isolate sample	*Regression in exponential phase*			*Absolute specific activity (IU mg^{-1})*	*Rate of specific activity (IU mg^{-1} $hours^{-1}$)*
	Equation	*R^2*	*R*		
Isolate 5	y=0.0206x+0.1080	0.9842	0.992	0.581^{b}	0.057^{b}
Isolate 6	y=0.0518x-0.0103	0.9749	0.987	1.494^{f}	0.089^{e}
Isolate 10	y=0.0156x+0.1653	0.9358	0.967	0.452^{a}	0.050^{a}
Isolate 19	y=0.0337x+0.0544	0.9674	0.984	1.006^{d}	0.069^{c}
Isolate 20	y=0.0415x+0.0445	0.9804	0.990	1.215^{e}	0.077^{d}
Isolate 37	y=0.0295x+0.0922	0.9791	0.989	0.790^{c}	0.066^{c}
Isolate 39	y=0.0644x-0.1037	0.9279	0.963	1.852^{g}	0.090^{e}

Different italics behind the values in the same column indicate that the values are significantly different ($p<0.05$).

Based on the results of the study, it showed that 7 isolates had significantly different rates of increase in the specific activity. The isolate with the lowest rate of increase in specific activity was isolate 10, with a value of 0.050^{a}. Furthermore, isolates 5, 37, 19, and 20 had a higher rate of increase in specific activity, with values of 0.057^{b}, 0.066^{c}, 0.069^{c}, and 0.077^{d}, respectively. Isolates with the highest specific activity increase rate were isolate 6 and isolate 39, with values which did not differ significantly from each other: 0.089^{e} and 0.090^{e}, respectively.

The relationship between growth and proteases of sponge-associated bacterial isolates. Based on the results of the study, there was a positive correlation between bacterial growth and the specific activity of the protease enzyme. The longer the incubation time in the culture process, the higher the bacterial growth, and the specific production of extracellular protease enzymes. The growth decrease in the death phase also has the effect of reducing the specific activity of the protease enzyme. Based on the results, there was a negative correlation between protease activity and protein content in the culture medium. The longer the incubation time in the culture process, the higher the

protease activity, accompanied by a decrease in the protein content of the culture medium. Decreased protease activity also prevent protein levels to be stationary.

B. subtilis strain 38 all bacterial cells appeared in the form of vegetative cells during the first 12 hours, then formed spores, after 16 to 24 hours of incubation period. These results indicated that the highest protease production was achieved during the exponential phase and remained constant when the spores were formed (stationary phase). Protease production occurs at the end of the exponential phase or the beginning of the stationary phase (Sun et al 2019a).

Extracellular protease synthesis usually occurs in the stationary phase. This is related to the catabolite repression mechanism. During the exponential growth phase, cells will experience inhibition of catabolite repression, thereby reducing intracellular cAMP concentrations which can activate the transcription of protease-encoding mRNA. Entering the stationary phase, the catabolite repression begins to decrease thereby activating the enzyme biosynthesis (Sun et al 2019b).

Molecular identification of association bacteria. Bacterial isolate 39 was subjected to DNA extraction and PCR amplification of 16 S rRNA for molecular identification purposes (Table 6). Then, on the results of the PCR there were performed electrophoresis and visualization of DNA bands with Doc gel. Results showed that isolate 39 had single and intact bands with a value of 2,300 bp.

Table 6

BLAST homology of 39 isolates of sponge-associated bacteria

Name	*Percent identity*	*Accession length*	*Accession*
Bacillus sp. (in: Bacteria) strain B4P095b	99.99%	1460	MN989043.1
Bacillus firmus strain WS2-R2A-64	99.99%	1474	MK026774.1
Paenibacillus sp. PP8	99.86%	1430	KF554095.1
Cytobacillus firmus strain AMB2	99.79%	1530	MZ669892.1
Bacillus firmus strain JCM 2512	99.66%	1474	LC379133.1
Bacterium strain SML_M2	99.65%	1440	MG937744.1
Bacterium msa3	99.38%	1461	KT152820.1
Bacillus sp. 2-8	99.24%	1517	KJ955376.1

The 16S-rRNA gene is of choice because it is present in all prokaryotes and has a very variable portion of conservative sequence and other sequences (Ribeiro et al 2012). 16S-rRNA gene has been used as a universal, representative and practical molecular systematic parameter for constructing phylogenetic relationships at the species level (Zahidah et al 2013). Based on the results, it is shown that BLAST isolate 39 had a homology value against Bacillus bacteria; against Bacterium strains SML_M2 and msa3 it was of 99.65% and 99.38%, respectively; against *B. firmus* strains WS2-R2A-64 and JCM 2512 it was of 99.97% and 99.79%, respectively; the highest was against the *Bacillus* sp. (in: Bacteria) strain B4P095b, reaching 99.99%.

Conclusions. The study concluded that 39 isolates of sponge symbionts of the genus Chalinula and bacteria had extracellular protease enzymes. The identification showed that 8 isolates had the potential to have a closeness above 99% with Bacillus bacteria, namely *Bacillus* sp. (in: Bacteria) strain B4P095b (99.99%), *B. firmus* strain WS2-R2A-64 (99.99%), *Paenibacillus* sp. PP8 (99.86%), *Cytobacillus firmus* strain AMB2 (99.79%), *B. firmus* strain JCM 2512 (99.66%), Bacterium strain SML_M2 (99.65%), Bacterium msa3 (99.38%) and *Bacillus* sp. 2-8 (99.24%). The bacteria found have the potential to be candidates for the formation of probiotics that are useful as bioremediation of the shrimp culture pollution with organic feed waste.

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