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Original Article

Isolation of symbiont bacteria of *Stylissa massa* as potential candidates for producing antimicrobial compounds from the Waters of Rote Island, East Nusa Tenggara, Indonesia

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Abstract: Current work aimed to isolate and characterize symbiont bacteria of *Stylissa massa* inhabiting Rote Island of East Nusa Tenggara, Indonesia. The symbiont bacteria are potential candidates for producing antimicrobial bioactive compounds. A total of 22 bacteria were isolated from *S. massa* with different characteristics. These isolates were screened to understand their capability to inhibit the growth of *Escherichia coli* and *Staphylococcus aureus*. Further, the secondary metabolites of the SM10 isolate, with the best inhibition results. This isolate could inhibit pathogenic bacteria of *E. coli* (11.24 mm) and *S. aureus* (12.11 mm). The molecular identification using 16 S rRNA, revealed that the SM10 isolate is *Pseudomonas aeruginosa*. Based on the GC-MS results, the SM10 isolate had produced two alkaloid compounds, pyrrole alkaloid (hymenialdisine) and bromopyrrole alkaloid (agelongine and spongiacidin D), and 2 unknown compounds. This finding showed that *S. massa* has symbiont bacteria isolates that can be used in the biochemical industry.

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Introduction

East Nusa Tenggara (NTT) is an archipelagic province in Indonesia with approximately 200,000 km² of sea area. The wide open waters of NTT are rich in marine resources, including fisheries, seaweed, coral reefs, and sponges (Indonesia Statistics, 2018). Rote Island is one of the most advanced islands in the southern part of East Nusa Tenggara waters, the southern fence of the Republic of Indonesia, and its sponge symbiont bacteria diversity has not yet been explored. Rote Island waters have ecological conditions with specific characteristics that can be used as the main indicator to find new sponge symbiont bacteria that can produce antimicrobial compounds.

Stylissa massa is widely distributed in tropical Indonesian seas, including the Banda Sea, Sulawesi Sea, Mergui Islands, and Papua New Guinea (Van Soest, 2017). Several compounds that are categorized as potential medicinal ingredients have been isolated from sponges of this species, such as Stylissatin A (cyclic peptide) as an anti-inflammatory agent (Kita et al., 2013), Stylissatin B as an antitumor agent (Sun et al., 2015), Spongiacidin C as a USP7 inhibitor (Yamaguchi et al., 2013), and aldisine alkaloid as a MEK-1 inhibitor (Tasdemir et al., 2002). Currently, research concerning *S. massa* sponge symbiont bacteria is rare.

Stylissa sponges are abundant in Rote Island waters, and they are promising as an indicator of finding symbiotic bacteria. Bacteria are frequently found living symbiotically in various marine organisms, including sponges. Sponges are known as the producers of bioactive compounds, and some of them are also assumed to be produced by bacteria that live symbiotically with them. Such condition allows sponge symbiont bacteria to play a major role in producing bioactive compounds that have antimicrobial activity. Therefore, research on sponge

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symbiont bacteria through their isolation and characterization is crucial. Therefore, this study was conducted to identify the symbiotic bacteria of the *S. massa* sponge that produces antimicrobial compounds in the waters of Rote Island, East Nusa Tenggara.

Materials and Methods

Isolation of symbiont bacteria: The bacterial symbionts were isolated from the sponge as follows: 1 g of the sponge sample was ground finely using the pestle and mortar. The ground sample was diluted by adding 1 g of sponge into a test tube containing 9 ml sterile NaCl. Afterward, serial dilutions were performed from 10^{-1} to 10^{-4}. 0.1 ml of the dilution results were spread on a petri dish containing Na media and incubated at a temperature of 270°C for 24 hours.

Characterization of bacterial isolates: The morphological characteristics (macroscopic) of the bacterial isolates were investigated based on the colony's shape, color, elevation, size, and edge. Biochemically, they were analyzed using Citrate and TSIA tests.

Antagonistic test of bacterial isolates against pathogenic bacteria: Antagonistic test of the isolates was performed by testing against two pathogens of *Escherichia coli* and *Staphylococcus aureus* on Muller Hinton Agar (MHA) media based on the agar diffusion method. Bacteria were taken using an ose needle and added into a test tube. Furthermore, 10 microns of the suspension results were taken using a micropipette, dripped on paper discs, and incubated for 24 hours. The bacteria with the best results in terms of antimicrobial activity were used for the growth curve test, extraction of bioactive compounds, and molecular identification.

Growth curve of the potential bacterial isolate: The isolate's growth curve was measured by calculating the number of living bacterial populations based on the spread plate method. This method spread 0.1 ml of the bacteria on Plant Count Agar (PCA) media evenly. The isolates were grown on nutrient broth media and incubated in a shaker, and bacterial colonies were counted every 4 hours using a colony counter for 48 hours.

Extraction of bioactive compounds of the potential bacterial isolates: 100 ml of the Nutrient broth (NB) media was used to culture the bacterial isolates. Furthermore, these isolates were incubated for 3 x 24 hours. The grown isolates were then dissolved in 100 ml of petroleum ether and extracted using a funnel. The extraction results were stored in bottles.

Antibacterial activity of bioactive compounds extracted from potential symbiont bacterial isolates: The diffusion method was applied to conduct the antibacterial test (Brooks et al., 2007). This test was done first by making wells on the MHA media that had been inoculated previously using pathogenic bacteria. Furthermore, 0.1 ml of the selected symbiont bacterial extract was added to the well and incubated for 24 hours.

Molecular identification of potential bacterial isolates: DNA isolation (for SM10 isolate) was done using the PCR method. For this purpose, 1.5 ml of pure bacterial culture aged 24 hours on NB media was taken and centrifuged for 10 minutes. The supernatant was discarded and added with 100 L of distilled water under aseptic conditions. The cell suspension was frozen at -10°C until the solution crystallized and then thawed at 90°C for 10 minutes. This cycle was repeated 5-10 times so that the cell broke down efficiently. DNA amplification of the bacterial genome against the 16S rRNA gene was further carried out using a forward primer of 27f (5'-AGA GTT TGA TCA CTG GCT CAG-3') and reverse primer of 1492r (5'-TAC GGC TTA CCT TGT TAC GA-3') (Elavazhagan et al., 2009).

Gas chromatography-Mass spectrometry analysis: GC-MS analysis was done by a fraction of SM10 using Thermo Trace 1300GC coupled with Thermo TSQ 800 Triple Quadrupole MS equipped with Column BP 5MS 30 m X 0.25 mm, 0.25 mm. The oven was initially set at 60°C and further increased by a rate of 5°C/min, until reaching 230°C. The carrier gas was helium at the flow rate of 1 ml/min. The injection volume was 2.0 ml (split ratio of 5:1) while its temperature was maintained at

Table 1. Morphological character of *Stylissa massa* sponge symbiont bacteria.

Isolate Code	Colony Shape	Colony Edge	Elevation	Size	Color
SM1	Irregular	Entire	Flat	Medium	White
SM2	Irregular	Lobate	Flat	Medium	White
SM3	Irregular	Lobate	Flat	Medium	White
SM4	Irregular	undulate	Flat	Large	Yellow
SM5	Irregular	serate	Flat	Large	White
SM6	Irregular	serate	Flat	Large	White
SM7	Irregular	Entire	convex	Small	White
SM8	Irregular	entire	Raised	Medium	White
SM9	Irregular	undulate	Umbonate	Medium	White
SM10	Irregular	undulate	Flat	Small	Yellow
SM11	Irregular	Lobate	Flat	Small	White
SM12	Irregular	Entire	Flat	Medium	White
SM13	Irregular	undulate	Raised	Large	White
SM14	Rhizoid	Serate	Flat	Medium	White
SM15	Irregular	undulate	Flat	Small	White
SM16	Irregular	undulate	Raised	Small	White
SM17	Irregular	undulate	Raised	Medium	White
SM18	Irregular	Lobate	Flat	Large	White
SM19	Rhizoid	serate	Umbonate	Medium	White
SM20	Rhizoid	Filamentous	Flat	Small	White
SM21	Irregular	undulate	Flat	Medium	White
SM22	Irregular	Entire	Raised	Small	Yellow

250°C and the ion source temperature was 230°C (Ashraf et al., 2017).

Results

The results of the isolated bacteria associated with *S. massa* showed the presence of 22 isolates. The isolated bacteria had different morphological characters, as shown in Table 1. Among the 22 isolates of symbiotic bacteria, 19 isolates had irregular colony shapes and 3 rhizoid colony shapes. The edges of colonies were lobate, entire, serrate, and undulate. The colony elevation was flat, raised, and umbonate. In addition, the color of the colonies was white and yellow and gram-negative staining. Five bacterial isolates, including isolates coded SM2, SM3, SM5, SM8 and SM10 were able to inhibit the pathogenic bacteria of both *E. coli* and *S. aureus*. SM10 had the best antimicrobial activity and was selected for further analysis.

Antagonist test of the isolates against *E. coli* and *S. aureus*: The antagonist results of bacterial isolates against pathogenic bacteria are shown in Table 2. Out of 22 bacterial isolates, 17 were able to inhibit *E. coli* growth, while 15 isolates inhibited *S. aureus* growth.

Curve of SM10 isolate growth: Based on the results of the SM10 growth curve (Fig. 1), the number of colonies increased in each phase. The adaptation phase (lag) had a relatively short time of growth increase in 0-4 hours. The logarithmic phase had a significant increase in growth at 8-20 hours, while the growth increase in the stationary phase occurred at 28-36 hours, and this phase was characterized by constant growth. The growth experienced a death phase after 42 hours, in line with the decrease in bacterial colonies.

Antibacterial test of the extract of SM10 isolate bioactive compounds: The results of SM10 isolate extract using petroleum ether are presented in Table 3. Based on the results of SM10 bacteria extract against *E. coli* and *S. aureus*, SM10 isolate could inhibit *E. coli* by an average of 11.24 mm, and *S. aureus* by an average of 12.11 mm (Fig. 2). Meanwhile, in the positive control test (+) using the antibiotic chloramphenicol, the inhibition against *E. coli* had a zone of 18.24 mm, and for *S. aureus*,

Table 2. Testing of the bacteria isolate activity against *E. coli* and *S. aureus* bacteria

Isolate Code	*E. coli*	*S. aureus*
SM1	++	+
SM2	+++	+
SM3	++	++
SM4	++	++
SM5	++	++
SM6	-	-
SM7	+	+
SM8	++	+
SM9	+	-
SM10	++	++
SM11	-	-
SM12	-	-
SM13	+	++
SM14	-	+
SM15	-	-
SM16	+	+
SM17	+	+
SM18	+	++
SM19	+	-
SM20	+	+
SM21	+	++
SM22	+	++

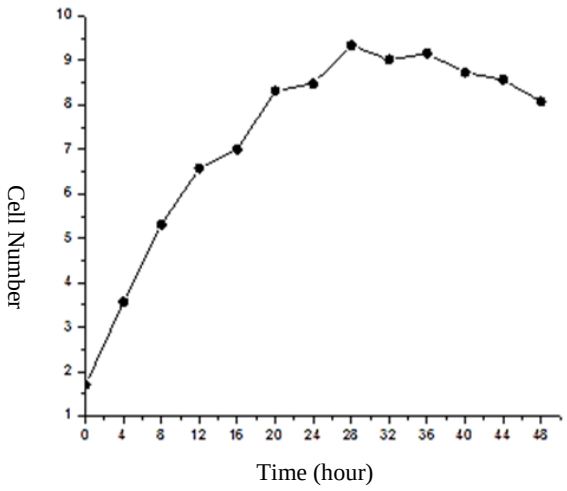

Figure 1. Diagram of Curve of SM10 isolate growth.

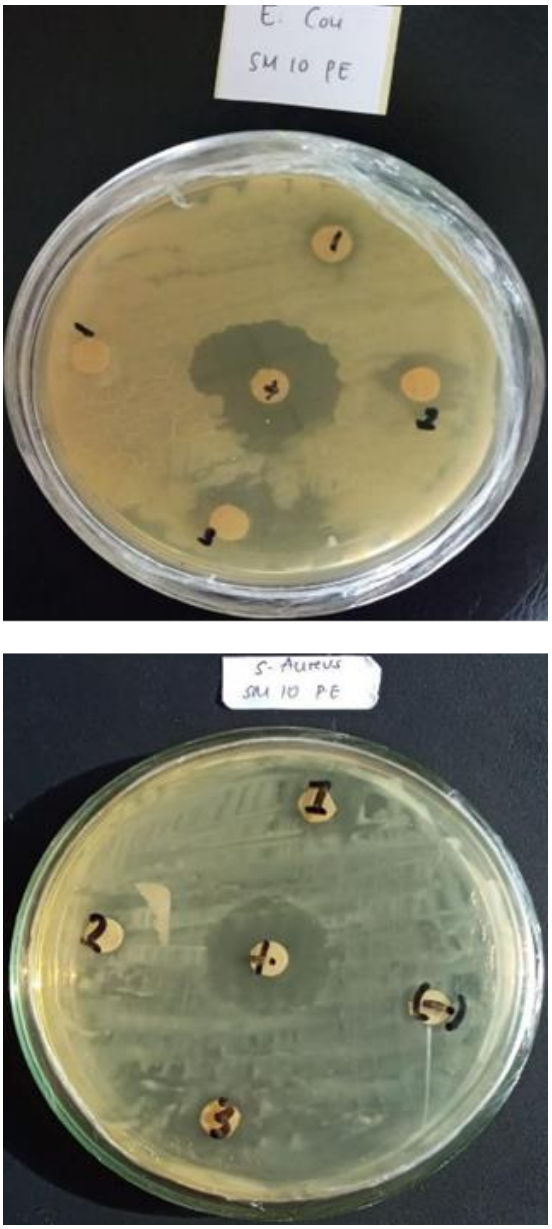

Figure 2. Antagonistic test of the SM10 isolate against the pathogenic bacteria; (A) *E. coli*, (B) *Aureus*.

18.86 mm. Furthermore, in the negative control test (-) using petroleum ether, the isolates could not inhibit *E. coli* and *S. aureus*.

Identification of isolates based on the 16S rRNA gene: The results of molecular identification based on 16S rRNA gene revealed that isolate SM10 was *Pseudomonas aeruginosa* bacteria with 100% similarity to a bacterium with the accession number of CP012001 (Fig. 3).

Identification of compounds produced from GC-MS: The identification of compounds extracted from SM10 was made by comparing the data spectrum with other literatures. The compound at the retention time of 43.525 (Fig. 4) had a fragmentation pattern of m/z 281 $[M\text{-Isopropyl}]^+$, and m/z 325 $[M+H]^+$, indicating that the mass was 324 sma. The compound with a mass of 324 sma was assumed to be 2-Hymenialdisine (Fig. 5) that has been isolated from *S. massa* sponge from Guam Island (Rohde et al., 2012), and *Stylissa carteri* sponge (Hamed et al., 2018), *Axinella verrucosa* sponge, and *Acantella*

Table 3. The antibacterial activities of SM10 isolate bioactive compounds.

E. coli				*S. aureus*			
Inhibition zone diameter (mm)				Inhibition zone diameter (mm)			
Repetition 1	Repetition 2	Repetition 3	Average	Repetition 1	Repetition 2	Repetition 3	Average
9.22	12.58	11.92	11.24	15.50	8.44	12.40	12.11

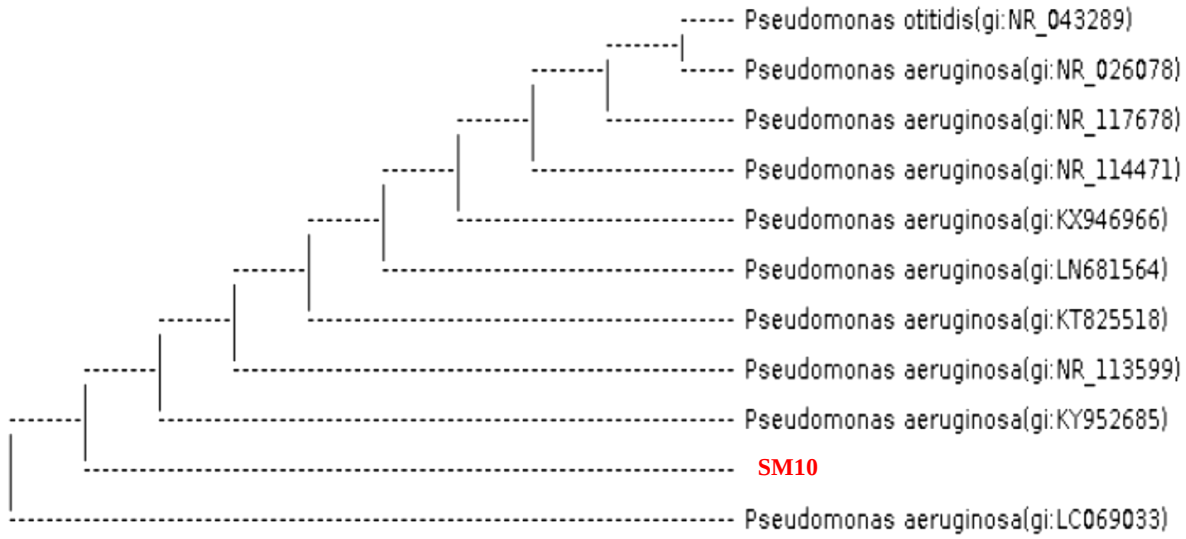

Figure 3. Phylogenic tree of SM10 isolates.

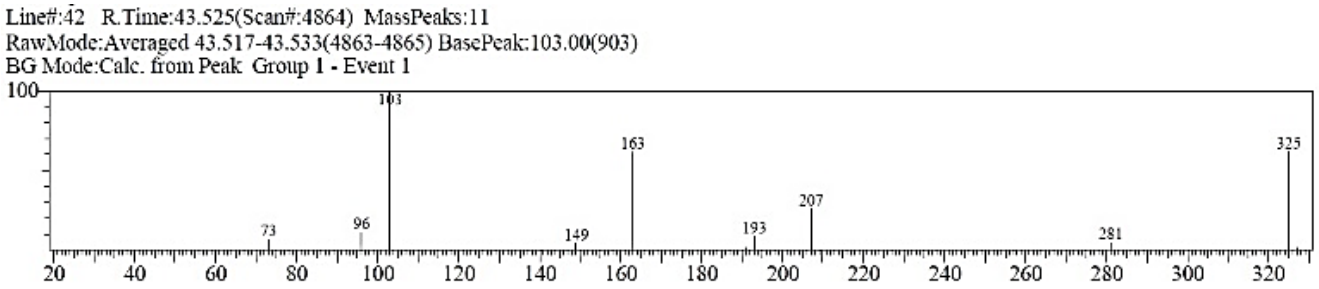

Figure 4. Mass spectrum results at retention time of 43.525.

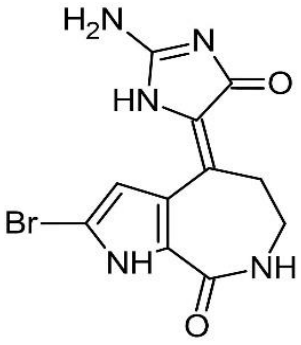

Figure 5. Hymenialdisine.

aurantiaca (Sharma et al., 2004). This compound has the inhibitory activity of interleukin-2 (IC_{50} = 2.4 µM) (Sharma et al., 2004).

The compound at the retention time of 43.683 (Fig. 6) had a fragmentation pattern of m/z 249 [M-Methyl]$^+$, m/z 207 [M-Isobutyl]$^+$, m/z 193 [M-tertpentyl]$^+$, and m/z 265 [M+H]$^+$, and the compound had mass of 264 sma. However, the compound with a mass of 264 sma has not been reported from *Stylissa* sponge, so it is a new compound.

The compound with the retention time of 43.725 (Fig. 7) had a fragmentation pattern of m/z 327 [M-Metyl]$^+$, m/z 283 [M-Isobutyl]$^+$, and m/z 341 [M]$^+$. The compound of the mass of 341 sma has been isolated from *S. carteri* sponge from Read Sea –

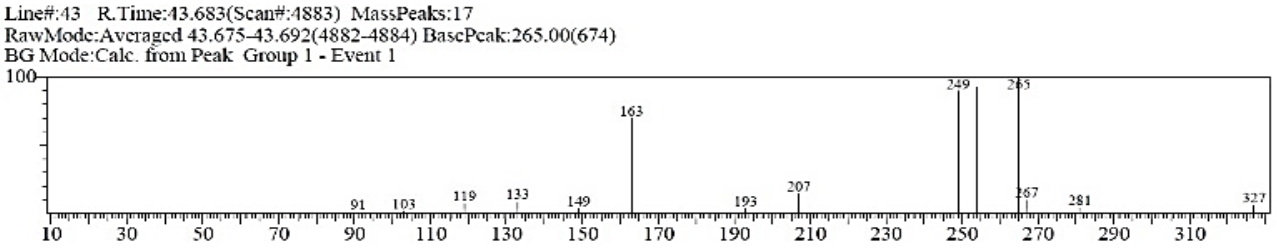

Figure 6. Mass spectrum at retention time of 43.683.

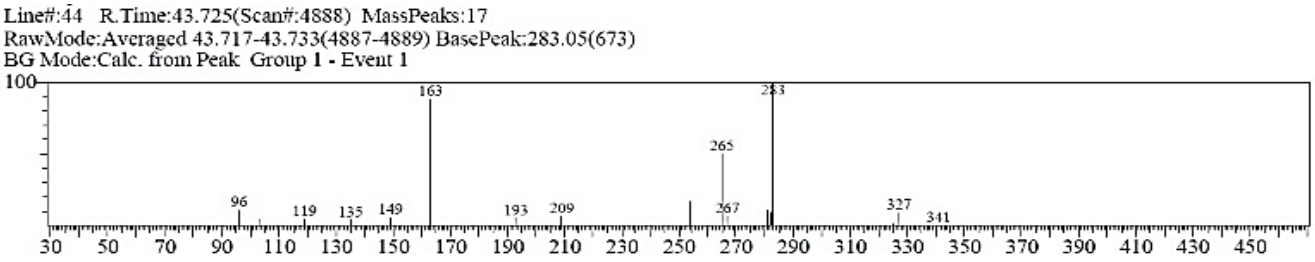

Figure 7. Spectrum of mass at retention time of 43.725.

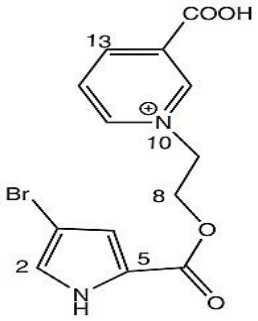

Figure 8. Agelongine.

Mesir (Hamed et al., 2018). This compound was Agelongine (Fig. 8), categorized as pyrrole alkaloid group.

The compound at the retention time of 44.067 (Fig. 9) had fragmentation patterns of m/z 283 [M-Isopropyl]$^+$, m/z 267 [M-Isobutyl]$^+$, m/z 254 [M-Tertpentyl]$^+$, and m/z 325 [M]$^+$. The compound at the mass of 325 sma has been reported from *S. carteri* from the Red Sea – Egypt (Hamed et al., 2018), and *Hymeniacidon* sp. sponge from Ishigaki Island – Japan (Inaba et al., 1998). This compound was categorized as bromopyrrole group as Spongiacidin D (Fig. 10). The compounds at the retention time of 45.175 (Fig. 11) had fragmentation patterns of m/z 156 [M-Cl]$^+$, m/z 135 [M-Isobutyl]$^+$, m/z 191 [M]$^+$, and m/z 193 [M+2]$^+$, and the compound had mass of 191 sma. The spectrum of this mass indicated that the compound contained Cl atom, seen at the peak of [M]$^+$ and [M+2]$^+$ with the ratio of 3:1. With the amount of odd mass (191 sma), this compound also indicated to have Nitrogen atom. However, a compound with 191 sma has never been reported from *Stylissa* sponge, and it is assumed as a new compound.

Discussion

Based on the results, 22 bacterial isolates were identified that 17 isolates inhibited the growth of *E. coli*, and 15 inhibited the growth of *S. aureus*. Five bacterial isolates, including isolates coded SM2, SM3, SM5, SM8 and SM10 were able to inhibit the pathogenic bacteria of both *E. coli* and *S. aureus*. Nineteen isolates had irregular colony shapes and three rhizoid colony shapes. The morphological characters of the edge in the colony were lobate, entire, serrate, and undulate. The colony elevation was flat, raised, and umbonate. There were two bacterial colonies colors, white and yellow. This result is in line with the findings of Kandio's (2021) that reported white and orange colony bacteria isolated from *Stylissa* sp. sponge. One isolate produced yellow pigment and inhibited both *E. coli* and *S. aureus*. Bacteria that produce pigment generally have different physiological and biochemical properties than others. In addition, such ability was affected by environmental factors, and in

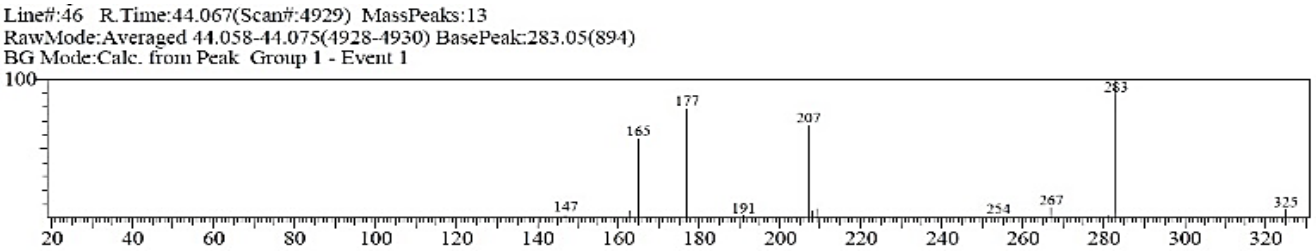

Figure 9. Spectrum of mass at the retention time of 44.067.

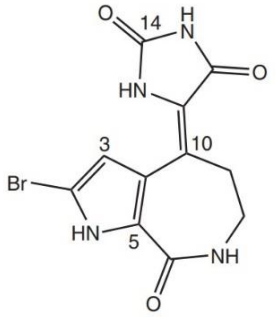

Figure 10. Spongiacidin D.

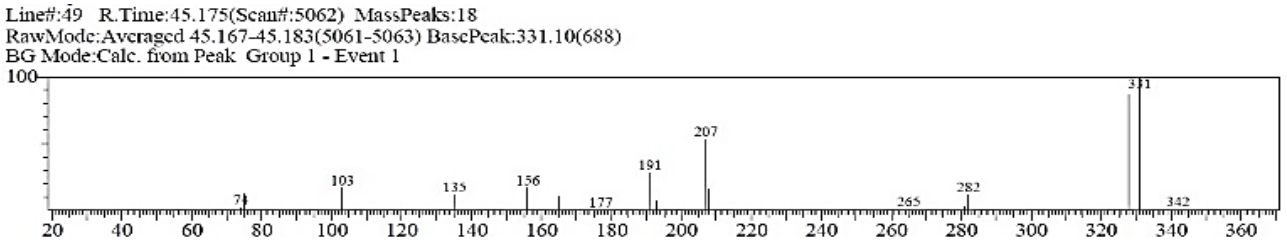

Figure 11. Spectrum of mass at the retention time of 45.175.

this case, the bacteria could produce various pigments with unique characteristics that can be correlated to the symbiosis between bacteria and their ecosystem (Celedón and Díaz, 2021).

The SM10 isolate had optimal growth after 28 hours of incubation. The incubation time can be used as an indicator of producing secondary compounds by bacteria and the final logarithmic phase until entering the initial stationary phase, has the optimal condition to produce useful secondary compounds. Antimicrobial compounds can be applied as drug candidates to prevent the growth of pathogenic bacteria. Based on our findings, crude extract of SM10 isolate inhibits the growth of *E. coli* by 11.24 mm and *S. aureus* by 12.11 mm. Krishnan et al. (2013) reported S8 isolate, symbiont with *Stylissa* sp., can inhibit the growth of *Klebsiella pneumoniae*. Pardosi et al. (2022) reported that SM4 isolate from *S. massa* could inhibit *S. aureus* and *E. coli*.

Molecular identification of SM10 isolate identified it as *P. aeruginosa*. Several studies have reported *Pseudomonas* spp. symbiosis with sea sponges. *Pseudomonas stutzeri* lives in symbiosis with the sponges of *Hyrtios erectus*, *Clathria*, and *Callyspongia* sp. (Marzuki et al., 2021), and *Pseudomonas putida* was symbiosis with the *Mycale microsigmatosa* sponge (Marinho et al., 2009). *Pseudomonas putida* isolated from the *Mycale microsigmatosa* sponge showed strong antimicrobial activity, which was active against multidrug-resistant bacteria (*S. aureus, S. epidermidis,* and *E. coli*). In our study, *P. aeruginosa* could produce antimicrobial compounds. Interpretation of spectra using GC-MS found two groups of alkaloid compounds, including pyrrole alkaloids (hymenialdisine) and bromopyrrole alkaloids (agelongine and spongiacidin D), and two other unknown compounds.

Sponges are one of the most prolific marine organisms for producing new bioactive compounds (Thomas et al., 2010; Joseph and Sujatha, 2011). The pigmentation produced by the sponge symbiont bacteria indicates the characteristics of their habitat (sponges) that produce various bioactive compounds. Sponges of the genus *Stylissa* are unique because of having bioactive compounds, including dimeric alkaloids such as hymenialdisine (Fouad et al., 2012; Ebada et al., 2015; Presson et al., 2021; Win et al., 2021), agelongine (Hamed et al., 2018), and pongiacidin (Yamaguchi et al., 2013; Hamed et al., 2018; Wang et al., 2022). The agelongine and pongiacidin D compounds are members of the bromopyrrole alkaloid family (Scala et al., 2010; Aktas et al., 2011). Bromopyrrole alkaloids have several pharmacological activities that chemists tied to their synthesis in the last two decades because of having antiproliferative, antimicrobial, and immunosuppressive activities (Ebada et al., 2015). Furthermore, the hymenialdisine compound is a member of the pyrrole alkaloid family (Hamed et al., 2018). SM10 isolate is not one type of bioactive compound to prevent the growth of *E. coli* and *S. aureus*, but it also produces other bioactive compounds.

In conclusion, SM10 isolate, a symbiont of the *Stylissa* sponge from the waters of Rote Island, East Nusa Tenggara showed antimicrobial activity. The SM10 isolate was identified as *P. aeruginosa* and the application of its crude extract can inhibit the growth of *E. coli* by 11.24 mm and *S. aureus* by 12.11 mm. The crude extract contains two alkaloid compounds: pyrrole alkaloids (hymenialdisine) and bromopyrrole alkaloids (agelongine and spongiacidin D). In addition, there were two unknown compounds in the crude extract of SM10.

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