Screening of the antifungal potential of nudibranch Chromodoris lineolata associated bacteria against Candida albicans

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Abstract. Skin disease is the fourth most common disease caused by infection of opportunistic pathogens on the skin. Skin diseases are generally treated by antibiotic products. However, overuse of antibiotics can increase the resistance of pathogens to various classes of antibiotics. Therefore, the study of new antimicrobial compounds against skin pathogens is urgently needed. This study aims to isolate nudibranch Chromodoris lineolata associated bacteria with antifungal activity against Candida albicans and detect the presence of type I polyketide synthase (PKS I), type II polyketide synthase (PKS II), and non-ribosomal peptide synthetase (NRPS) genes through a molecular approach. A total of 15 bacteria were successfully isolated from nudibranch at Panjang Island and Teluk Awur. Screening of the antifungal activity using an agar plug method showed that 1 isolate namely P.10.6 showed antifungal activity against Candida albicans. The detection of the biosynthetic gene cluster (BGC) showed the absence of BGC in the potential isolate. This finding was suspected due to the production of the antifungal compound from the other biosynthetic pathways. Based on molecular identification through BLAST homology, strain P.10.6 has been identified as Bacillus stratosphericus.

Keywords: antifungal; bacteria; nudibranch

1. Introduction
Skin disease is one of the most common diseases found in developing countries, especially in areas with dense populations [1,2]. It is estimated that around 20% - 25% of the human population around the world is infected by skin diseases caused by fungal infections [3]. The common treatment used to cure skin diseases is by applying antibiotic products [4]. Various types of antibiotics such as tetracyclines, macrolides, clindamycin, budesonide, fluconazole, and trimethoprim/sulfamethoxazole have been widely used to cure skin diseases [5,6]. However, excessive use of antibiotics with inappropriate procedures will cause the emergence of multi-drug resistant (MDR) skin pathogens [7]. Pathogens with the ability to survive against at least three classes of antibiotics are categorized as MDR pathogens [7-9]. The increasing number of MDR pathogens has resulted in up to 700,000 deaths worldwide [10]. The previous reports showed that various skin pathogens strains such as Propionibacterium acnes, Staphylococcus epidermidis, and Candida albicans had been categorized as MDR skin pathogens [11-13]. The emergence of MDR skin pathogens causes the treatment using antibiotics to cure skin diseases to become increasingly ineffective [14]. The increasing number of MDR infections with the difficulty of treatment using only a few antibiotic choices indicate the urgency for more research of new antimicrobial compounds.

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As an archipelagic country, Indonesia has abundant prospective marine resources as a potential source of new antimicrobial compounds. Marine invertebrates, especially sponges, are known as the most prospective sources of new bioactive compounds such as anti-bacterial and anti-fungal [15]. Several new compounds have been reported successfully isolated from Indonesian sponges Dysidea sp. namely biaketide and debromoantazirine [16]. In contrast to sponges, nudibranch is one of the invertebrates with potential as a source of antimicrobial compounds but has not been widely studied. Nudibranchs are gastropods from the Phylum Mollusca with the ability to accumulate compounds from the prey they consume. These compounds are accumulated by nudibranchs in their mantle as a defense mechanism against predators and have been studied for their bioactive properties [17-19]. The previous study has been reported that crude extract from nudibranch *Phyllidia varicose* has the potential as an antibacterial agent against *Shigella flexneri* and *Staphylococcus aureus* [18]. Unfortunately, only 0.4% of the new compounds from Indonesia's marine ecosystem were isolated from Phylum Mollusca [15]. However, isolation of bioactive compounds directly from nudibranchs would require large quantities of nudibranchs and may lead to decreasing nudibranchs diversity in the ecosystem. Therefore, another way that can be taken to overcome this obstacle is by using nudibranch-associated bacteria as a source of bioactive compounds. A previous study has reported that the nudibranch-associated bacteria from the genus Pseudoalteromonas showed potential antibacterial activity against methicillin-resistant *Staphylococcus aureus* [20,21]. Unfortunately, the antifungal potential of nudibranch-associated bacteria is still not widely reported. Therefore, this study aims at obtaining nudibranch-associated bacteria from Jepara, Central Java, Indonesia with antifungal potential against *Candida albicans* through an antagonistic and molecular approach.

### 2. Material and methods

#### 2.1. Nudibranch sample collection

Sampling has been done by scuba diving at Teluk Awur (S 06° 37’ 16.9”; E 110° 38’ 07.2”) and Panjang Island (S 06° 34’ 33.7”; E 110° 37’ 54.3”), Jepara, Central Java, Indonesia (figure 1). Nudibranch samples were collected in ziplock and stored in the coolbox. Each sample was documented and identified according to Sabdono et al. (2021) based on morphological appearance [20,32].

#### 2.2. Isolation and purification

Bacterial isolation was carried out using the serial dilution method according to Kristiana et al. (2019) and Wijaya et al. (2020) with several modifications. Crushed sample was diluted to 10⁶, 10⁴, and 10² concentrations. Diluted samples were plated on ISP 4, ISP 4 + rose Bengal, and Humic Vitamin Agar (HVA) medium then incubated at 25°C. The bacterial colonies were separated into the MT medium using the streak method and incubated at 36°C. The composition of MT medium was as follows: meat extract 0,1%; yeast extract 0,1%; peptone 0,2%; soluble starch 0,5%; glucose 0,5%; CaCO₃ 0,1%; NaCl 0,2%; and agar 2% [20-22].

#### 2.3. Screening of antifungal activity

The antifungal activity screening was carried out using the agar plug method against *Candida albicans* according to Sibero et al. (2018) with several modifications. The agar plug of the cultured isolates were placed on the Mueller Hinton Agar (MHA) medium that has been inoculated with the pathogen and incubated for 3x24 hours. The formed clear zone around the agar plug indicated the presence of antifungal activity. Isolates with antifungal activity were used in further steps [23].

#### 2.4. Morphology characterization

The morphological characterization of nudibranch-associated bacteria with antifungal activity was performed macroscopically by observing the shape, margin, elevation, size, and color of bacterial colonies on MT agar medium [24].
2.5. Biochemistry test
Biochemical tests consist of gram staining, motility test, citrate test, indole test, H₂S test, and sugar fermentation. The gram staining test has been done according to Himedia K001 gram staining kit protocol [22]. The remaining biochemical tests were carried out by inculcating each of the isolates into a test tube containing Triple Sugar Iron Agar (TSIA), Sulfide Indole Motility (SIM), and Simmon Citrate Agar (SCA) media [25].

2.6. Salinity experiment
Salinity experiment was performed by inculcating isolates into an agar medium with a salinity of 0 ppm and 35 ppm using the streak method. The results were obtained by observing the presence of bacterial growth in the agar medium. The salinity test aims to determine whether the bacterial isolates are classified as marine obligate or marine facultative bacteria [22].

2.7. Molecular identification
DNA extraction has been done according to the Zymo Quick-DNA Miniprep Kit DNA extraction protocol. Amplification was performed using PCR mix consisting of 12.5 μl of GoTaq®Green Master Mix Promega, 1 μL of primer 27F (5’AGAGTTTGATCMTGGCTCAG-3’), 1 μL of primer 1492R (5’GGTTACCTTGTTACGACTT-3’), 1 μl of DNA template, and 9.5 μl of ddH2O. The PCR results were sent to PT. Genetics Science, Jakarta, Indonesia for the determination of the 16S rRNA sequence. Bacterial identification was performed by using DNA barcoding based on NCBI BLAST homology in the 16S ribosomal RNA region. Phylogenetic tree analysis was performed using MEGA X software with 1,000 bootstrap method [20,22,23,26].

2.8. Detection of biosynthetic gene cluster
Biosynthetic gene cluster (BGC) detection was carried out in order to detect the presence of polyketide synthase (PKS) and non-ribosomal peptide synthesis (NRPS) in each potential isolate. PKS I biosynthetic gene was detected using primer KSa-F (5’-TSGCS TGCTTGGAYGCSATC-3’) and KSα-R (5’-TGGAAAC CGCCGAAABCCGCT-3’) at 700 bp – 800 bp. Meanwhile, PKS II biosynthetic gene was detected using primer IIPF6 (50-TSG CST GCT TCG AYG CSA TC-30) and IIPR6 (50-TGG AAN CCG CCG AAB CCG CT-30) at 600 bp- 650bp. Meanwhile, NRPS biosynthetic gene was detected using primer A2gamForward (50-AAG GCN GCC GSB TAY STG CC-3’) and A3gamReverse (50-TTG GGB IKB CCG GTS GIN CCS GAG GTG-3’) at 300 bp. Amplification was performed using PCR mix with the exact composition as the previous step [27-30].

2.9. Metabolite extraction
Isolates with antifungal activity were cultivated into 10 ml of MT broth medium for 3 days as a seed culture. Seed culture was transferred into 100 ml of MT broth medium and cultivated for 7 days as production culture. Both microbial cultures were carried out at 200 r.p.m and at 25°C. Extraction of metabolites has been done by using the maceration method with ethyl acetate at 200 r.p.m. for 1 hour. The ratio of solvent: media used was 1:1. The ethyl acetate phase was separated using a rotary evaporator at 40°C. The crude extract was then weighed and stored in the freezer for further step [22,23].

2.10. Antifungal assay of crude extracts
The crude extract was diluted with dimethyl sulfoxide (DMSO) to 1 mg/ml and then 10μl of crude extract was injected into sterile paper disks (Ø 6 mm OxoidTM). Paper disks of each extract were tested against Candida albicans. Pathogen was refreshed on potato dextrose agar (PDA) medium for 24 hours and swabbed evenly into the new PDA medium. Paper disks were placed into MHA and PDA medium containing swabbed pathogen and incubated for 3x24 hours. The antifungal activity of crude extracts were observed by the presence of a clear zone around the paper disks [20,23,31].
3. Results and discussion

In this study, a total of 2 nudibranchs were successfully collected from two different locations, namely Teluk Awur and Panjang Island (Figure 1). Teluk Awur sample was coded as TA.3 while Panjang Island sample was coded as P.10. Both samples showed identical morphological appearance with blackish-brown body color, cream-colored gill, and a yellowish-cream striped body pattern (Figure 2). Based on the previous study by Sabdono et al. (2021), who studied the diversity of nudibranchs in the Jepara sea, nudibranchs with these morphological characteristics were classified as *Chromodoris lineolata* [32].

The diversity and abundance of nudibranchs in the sampling sites are known to be affected by various factors such as temperature, salinity, and substrate in the environment. The sampling sites in Teluk Awur and Panjang Island have an optimal temperature (28.9°C and 30.5°C) and salinity (31 ppm and 29 ppm) as a habitat for nudibranch from Chromodorididae Family [33]. Another factor affecting the abundance of *Chromodoris lineolata* in the sampling site is the distribution of this nudibranch food. A previous study has reported the presence of this invertebrate food, which is a Dysidea sponge in the Jepara marine environment [32,34]. The abundance of this invertebrate food became another supporting factor for *Chromodoris lineolata* abundance in the sampling location. After the identification step, samples went through the isolation and purification step to obtain associated bacteria. A total of 15 isolates was successfully isolated from nudibranch P.10 (9 isolates) and TA.3 (6 isolates). All of the pure cultures were purified from ISP 4 medium. There is no bacterial growth found in the HVA and ISP 4 + rose bengal medium. The absence of bacterial growth on HVA and ISP 4 + rose bengal medium was thought to be due to the unsuitable nutrients provided by the medium for the growth of nudibranch-associated bacteria [35-36]. Therefore, it is highly recommended to isolate nudibranch-associated bacteria using ISP 4 medium in future studies.

All purified isolates went through the screening of antifungal activity against *Candida albicans*. Among 15 isolates, isolate P.10.6 formed a clear zone around the agar plug indicated the presence of antifungal activity against *Candida albicans* [23]. Details of antifungal screening results are presented in Table 1. According to Hodges et al. (2012), secondary metabolites are produced by bacteria as a form of self-defense mechanism [28]. These metabolites usually possess the potential as a bioactive compound such as antifungals that can be used as a source of antibiotics. The diameter of the clear zone gradually decreases in every observation, indicating the antifungal compound produced by each isolate is only able to inhibit the growth of pathogens.

Based on morphological characterization, isolate P.10.6 has a cream-colored circular colony with flat elevation and entire margin. This isolate produced extracellular cream pigment on the MT medium at 25°C. The extracellular pigment produced by microbes is known to have a biological activity such as antibacterial [37]. A biochemical test was carried out to understand the physiological properties of the potential isolate. The results of the biochemical test were obtained based on the gram staining, cell shape, motility, indole, citrate, H2S, and sugar fermentation test (Table 2). The results of gram staining showed that isolate P.10.6 was categorized as gram-positive bacteria. Gram-positive and negative bacteria are distinguished based on their composition of the peptidoglycan and lipid layer of the cell wall [38,39].

Observation of cell shape using a light microscope showed that isolate P.10.6 has rod-shaped cells. Meanwhile, the motility test result showed that isolate P.10.6 formed a spreading growth and were suspected as motile-bacteria. Non-motile bacteria move by a rolling mechanism and are commonly found as bacteria associated with an organism [40]. No isolates formed black deposits on the bottom of SIM medium, indicating that all isolates could not produce H2S. The production of hydrogen sulfide is indicated by the presence of black deposits or ferric sulfide which is the result of the reaction of H2S with ferrous ammonium sulfate [25]. Isolate P.10.6 did not show any indole production since there was no color change in the Kovacs reagent added. These findings showed that this isolate could not produce the enzyme tryptophanase. The citrate test of isolate P.10.6 showed no color change in the SCA medium, indicated the absence of citrate utilization ability. Citrate utilization is the ability to convert citrate to pyruvate as a source to produce energy. This ability is commonly found in bacteria from the Enterobacteriaceae group [41]. The carbohydrates fermentation test of isolate P.10.6 showed the presence of lactose and/or sucrose fermentation. The positive result was indicated by the change of the
medium color from red to yellow in the slanted part of the TSIA medium due to the fermentation of carbohydrates to acid products. Glucose is fermented in the bottom of the tube anaerobically. Meanwhile, lactose and/or sucrose are fermented on the slant side of the medium aerobically [25].

The salinity experiment aims to determine either the isolate classified as marine obligate or marine facultative bacteria. Isolate P.10.6 was able to growth in both 0 ppm and 35 ppm medium (Table 3). These findings showed that isolate P.10.6 belong to the marine facultative group. The presence of facultative marine bacteria in the marine environment can be caused by various factors, such as natural drainage systems, natural phenomena, rain, and human activities. Bacteria found in marine ecosystems have unique mechanisms that distinguish them from terrestrial bacteria. These mechanisms include regulating of cell morphology and anatomy, modulation of cation transport entities, and metabolite production [42-45].

Figure 1. Sampling site in Teluk Awur and Panjang Island, Jepara, Central Java, Indonesia.

Figure 2. *Chromodoris lineolata* from Teluk Awur (A) and Panjang Island (B).
The 16S rRNA gene of the potential isolates was amplified and the species of each isolate was determined using the BLAST homology (Figure 3). Based on molecular identification, isolate P.10.6 was identified as *Bacillus stratosphericus* with 98.31% similarity with *Bacillus stratosphericus* NR_118441.1. These species are known to belong to the Terrabacteria group. *Bacillus stratosphericus* has been reported found in both terrestrial and marine environments [46-47]. The previous study has shown that *B. stratosphericus* has the potential as a source of a bioactive compound with antibacterial activity.

**Table 1.** Antifungal activity of nudibranch-associated bacteria.

| Isolate | Candida albicans |
|---------|-----------------|
|         | 1 | 2 |
| TA.3.1  | - | - |
| TA.3.2  | - | - |
| TA.3.3  | - | - |
| TA.3.5  | - | - |
| TA.3.7  | - | - |
| TA.3.8  | - | - |
| P.10.1  | - | - |
| P.10.3  | - | - |
| P.10.5  | - | - |
| P.10.6  | + | + |
| P.10.7  | - | - |
| P.10.8  | - | - |
| P.10.9  | - | - |
| P.10.10 | - | - |
| P.10.11 | - | - |

(+) : presence of clear zone;  
(-) : absence of clear zone

**Table 2.** Biochemical test results of isolate P.10.6.

| Test       | Result |     |
|------------|--------|-----|
| Gram       | Positive | Rod |
| Cell shape |         |     |
| Motility   | +       |     |
| Indole     | -       |     |
| H2S        | -       |     |
| Citrate    | -       |     |
| Glucose    | -       |     |
| Lactose    | +       |     |
| Sucrose    | +       |     |

(+) : Positive reaction  
(-) : Negative reaction

**Table 3.** Salinity experiment results of isolate P.10.6.

| Isolate | Concentration |
|---------|---------------|
|         | 0 ppm | 35 ppm |
| P.10.6  | +     | +      |

(+) : Presence of bacterial growth  
(-) : Absence of bacterial growth
activity against P. syringae [46]. Interestingly, there has been no report regarding the potential of B. stratosphericus antifungal activity against Candida albicans.

Biosynthetic gene cluster detection showed the absence of NRPS, PKS I, PKS II biosynthetic genes in Bacillus stratosphericus (P.10.6). The absence of biosynthetic genes cluster in potential isolate was suspected caused by the bioactive compounds of this isolate produced from the other biosynthetic pathways, such as PKS III, terpene, bacteriocin, beta-lactam, and other clusters [48]. BGC in bacteria plays an important role in producing secondary metabolites as defense mechanisms against various environmental conditions [21]. Apart from being a self-defense mechanism, these metabolites have been well known to have various benefits in the industrial sector, such as antibiotics, anti-tumor agents, and cholesterol-lowering agents [49-51]. The antifungal assay was carried out on crude extracts of isolate P.10.6 against Candida albicans. The result showed that the crude extract did not show the presence of a clear zone in the test media. The antifungal assay using the disc diffusion method showed completely different results with the screening of antifungal activity results using the agar plug method. The negative result was suspected to be caused by various factors, such as differences in culture medium, the influence of solvents, and the multi-drug resistance properties of the test pathogens [35,36,52-54].

4. Conclusions
This study successfully isolated 15 bacteria from nudibranch Chromodoris lineolata from Panjang Island and Teluk Awur, Jepara, Indonesia. Among the associated bacteria, 1 isolate namely P.10.5 exhibits antifungal activity against Candida albicans. The absence of biosynthetic genes cluster was suspected due to the production of bioactive compound from the other biosynthetic pathways. The results of morphological and molecular identification in the 16S rRNA region using the BLAST homology showed that this isolate was identified as Bacillus stratosphericus.
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5. References
[1] Karimkhani C, Dellavalle R P, Coffeng L E, Flohr C, Hay R J, Langan S M, Nsoesie E O, Ferrari A J, Erskine H E, Silverberg J I, Vos T and Naghavi M 2017 Global skin disease morbidity and mortality an update from the global burden of disease study 2013 JAMA Dermatology. 153 406–412.
[2] Hay R J, Johns N E, Williams H C, Bolliger I W, Dellavalle R P, Margolis D J, Marks R, Naldi L, Weinstock M A, Wulf S K, Michaud C, Jl. Murray C and Naghavi M 2014 The global burden of skin disease in 2010: An analysis of the prevalence and impact of skin conditions Journal of Investigative Dermatology. 134 1527–1534.
[3] Kühbacher A, Burger-Kentischer A and Rupp S 2017 Interaction of Candida Species with the Skin Microorganisms. 5 32.
[4] Yang J, Yang H, Xu A and He L 2020 A Review of Advancement on Influencing Factors of Acne: An Emphasis on Environment Characteristics Frontiers in Public Health. 1–16.
[5] Bienefeld A, Nagler A R and Orlow S J 2017 Oral Antibacterial Therapy for Acne Vulgaris: An Evidence-Based Review American Journal of Clinical Dermatology. 18 469–490.
[6] Li X, Yu C, Huang X and Sun S 2016 Synergistic effects and mechanisms of budesonide in combination with flucanazole against resistant candida albicans PLoS ONE. 11 1–20.
[7] Chang H.-H, Cohen T, Grad Y H, Hanage W P, O’Brien T F and Lipsitch M 2015 Origin and Proliferation of Multiple-Drug Resistance in Bacterial Pathogens Microbiology and Molecular Biology Reviews. 79 101–116.
[8] Jacopin E, Lehtinen S, Débarre F and Blanquart F 2020 Factors favouring the evolution of multidrug resistance in bacteria: Factors favouring the evolution of multidrug resistance in bacteria Journal of the Royal Society Interface. 17 168.
[9] Basak S, Singh P and Rajurkar M 2016 Multidrug Resistant and Extensively Drug Resistant Bacteria: A Study Journal of Pathogens. 1–5.
[10] Wang T Z, Kodiyamplakkal R P L and Calfee D P 2019 Antimicrobial resistance in nephrology In Nature Reviews Nephrology. 15 8.
[11] Ksiezopolska E and Gabaldón T 2018 Evolutionary emergence of drug resistance in candida opportunistic pathogens Genes. 9 9.
[12] Lee J Y H, Monk I R, Gonçalves da Silva A, Seemann T, Chua K Y L, Kearns A, Hill R, Woodford N, Bartels M D, Strommenger B, Laurent F, Dodémont M, Patel R, Larsen A R, Korman T M, Stinear T P and Howden B P 2018 Global spread of three multidrug-resistant lineages of Staphylococcus epidermidis Nature Microbiology. 3 1175–1185.
[13] Aoki S, Nakase K, Nakaminami H, Wajima T, Hayashi N and Noguchi N 2020 Transferable Multidrug-Resistance Plasmid Carrying a Novel Macrolide-Clindamycin Resistance Gene, erm(50), in Cutibacterium acnes February. 1–6.
[14] Jacopin E, Lehtinen S, Débarre F and Blanquart F 2020 Factors favouring the evolution of multidrug resistance in bacteria: Factors favouring the evolution of multidrug resistance in bacteria Journal of the Royal Society Interface. 17 168.
[15] Hanif N, Murni A, Tanaka C and Tanaka J 2019 Marine natural products from Indonesian waters. Marine Drugs. 17 6.
[16] Trianto A, De Voodg N J and Tanaka C 2014 Two new compounds from an Indonesian sponge Dysidea sp. Journal of Asian Natural Products Research. 16 163–168.
[17] Cheney K L, White A, Mudianta I W, Winters A E, Quezada M, Capon R J, Mollo E and Garson M J 2016 Choose your weaponry: Selective storage of a single toxic compound, latrunculin a, by closely related nudibranch molluscs PLoS ONE. 11 1–16.
[18] Reddy K V, Mohanraju R, Murthy K N, Ramesh C, Karthick P and Islands N 2015 Antimicrobial properties of nudibranchs tissues extracts from South Andaman, India Journal of Coastal Life Medicine. 3 582–584.

[19] Winters A E, White A M, Dewi A S, Mudianta I W, Wilson N G, Forster L C, Garson M J and Cheney K L 2018 Distribution of Defensive Metabolites in Nudibranch Molluscs Journal of Chemical Ecology. 44 384–396.

[20] Kristiana R, Sibero M T, Farisa M Y, Ayuningrum D, Dirgantara D, Hanafi M, Radjasa O K, Sabdono A and Trianto A 2019 Antibacterial potential of nudibranch-associated bacteria from Saparua and Nusa Laut Islands, Indonesia Biodiversitas. 20 1811–1819.

[21] Böhringer N, Fisch K M, Schillo D, Bara R, Hertz C, Grein F, Eisenbarth J H, Kaligis F, Schneider T, Wägele H, König G M and Schäferle T F 2017 Antimicrobial potential of bacteria associated with marine sea slugs from North Sulawesi, Indonesia Frontiers in Microbiology. 8 1–8.

[22] Wijaya A P, Bondar K G, Frederick E H, Igarashi Y and Sibero M T 2020 Identification of marine bacteria HPP. 4A and HPP. T13 and its anticancer activity against P388 murine leukaemia cell IOP Conference Series: Earth and Environmental Science. 584 012005.

[23] Sibero M T, Radjasa O K, Sabdono A, Trianto A, Triningsih D W and Hutagaol I D 2018 Antibacterial activity of indonesian sponge associated fungi against clinical pathogenic multidrug resistant bacteria Journal of Applied Pharmaceutical Science. 8 088–094.

[24] Margarida A, Olívia M and Lourenço A 2015 MorphoCol: An ontology-based knowledgebase for the characterisation of clinically significant bacterial colony morphologies Journal Of Biomedical Informatics. 55 55–63.

[25] Talaiekhozani A, Alaee S, Mohanadoss P and Knowledge I 2015 Guidelines for quick application of biochemical tests to identify unknown bacteria Accounts of Biotechnology Research. 64–82.

[26] Ayuningrum D, Liu Y, Riyanti Sibero M T, Kristiana R, Asagabaland M A, Wuisan Z G, Trianto A, Radjasa O K, Sabdono A and Schäferle T F 2019 Tunicate-associated bacteria show a great potential for the discovery of antimicrobial compounds PLoS ONE. 14 1–14.

[27] Sibero M T, Herdikiawan D, Radjasa O K, Sabdono A, Trianto A and Triningsih D W 2018 Antibacterial activity of sponge associated fungi against vibriosis agents in shrimp and its toxicity to Litopenaeus vannamei AACL Bioflux. 11 10–18.

[28] Hodges T W, Slattery M and Olson J B 2012 Unique Actinomycetes from Marine Caves and Coral Reef Sediments Provide Novel PKS and NRPS Biosynthetic Gene Clusters Marine Biotechnology. 14 270–280.

[29] Sibero M T, Igarashi Y, Radjasa O K, Sabdono A, Trianto A, Zilda D S and Wijaya Y J 2019 Sponge-associated fungi from a mangrove habitat in Indonesia: species composition, antimicrobial activity, enzyme screening and bioactive profiling International Aquatic Research. 11 173–186.

[30] Wijaya A P, Sibero M T, Zilda D S, Windiyana A N, Wijayanto A, Frederick E H, Murwani R, Wijayanti D P, Sabdono A, Pringgenies D and Radjasa O K 2021 Preliminary Screening of Carbohydrase-Producing Bacteria from Preliminary Screening of Carbohydrase-Producing Bacteria from Chaetomorpha sp. in Sepanjang Beach IOP Conf. Ser.: Earth Environ. Sci. 750 012027.

[31] Frederick E H, Sibero M T, Wijaya A P, Syafitri E, Puspitarini A, Siswanto Murwani R, Wijayanti D P, Sabdono A, Pringgenies D and Radjasa O K 2021 Preliminary Evaluation of Anti Fish Pathogenic Bacteria and Metabolite Profile of Andaliman Fruit (Zanthoxylum acanthopodium DC.) Ethanol Extract Preliminary Evaluation of Anti Fish Pathogenic Bact IOP Conference Series: Earth and Environmental Science. 750 012026.

[32] Sabdono A, Radjasa O K, Trianto A, Sibero M T, Martynov A and Kristiana R 2021 An Ecological Assessment of Nudibranch Diversity Among Habitats Receiving Different Degrees of Sedimentation in Jepara Coastal Waters, Indonesia International Journal of Conservation
Ompi M, Lumoindong F, Undap N, Papu A and Wägele H 2019 Monitoring marine heterobranchia in lembeh strait, north sulawesi (Indonesia), in a changing environment AACL Bioflux. 12 664–677.

Radjasra O K 2007 Identification of Sponge-Associated Bacteria with Antibacterial Property against Staphylococcus aureus based on Molecular Approach A Scientific Journal. 24 98-104.

Al-Ansari M, Kalaiyarasai M, Almalki M A and Vijayaraghavan P 2020 Optimization of medium components for the production of antimicrobial and anticancer secondary metabolites from Streptomyces sp. AS11 isolated from the marine environment Journal of King Saud University – Science. 32 1993–1998.

Fang B Z, Salam N, Han M X, Jiao J Y, Cheng J, Wei D Q, Xiao M and Li W J 2017 Insights on the effects of heat pretreatment, pH, and calcium salts on isolation of rare Actinobacteria from Karstic Caves Frontiers in Microbiology. 8 1–9.

Sibero M T, Sahara R, Syafiqoh N and Tarman K 2017 Antibacterial activity of red pigment isolated from coastal endophytic fungi against multi-drug resistant bacteria BIOTROPIA-The Southeast Asian Journal of Tropical Biology. 24 161-172.

Sandle T 2004 Gram’s Stain: History and Explanation of the Fundamental Technique of Determinative Bacteriology IST Science and Technology. 54 3-4.

Hiremath P S and Bannigidad P 2011 Automated Gram-staining characterization of bacterial cells using colour and cell wall properties International Journal of Biomedical Engineering and Technology 7 257-265.

Dunn C L and Pandya D D 2013 The General Morphology and Biology of Bacteria The Chemistry and Bacteriology of Public Health. 109–121.

Almas M, Tahir U, Zameer M, Shafiq M I, Farrukh S Y, Zahra N, Mazhar M, Gill S A, Hadi F, Muhammad T, Ali Q and Malik A 2021 Detection of INVA Gene and Cytotoxin of Salmonella enteridis in Food Samples Using Molecular Methods. Journal of Pharmaceutical Research International. 33 20–28.

Hohmann S 2002 Osmotic Stress Signaling and Osmoadaptation in Yeasts Microbiology and Molecular Biology Reviews. 66 300–372.

Ariño J, Ramos J and Sychrová H 2010 Alkali Metal Cation Transport and Homeostasis in Yeasts Microbiology and Molecular Biology Reviews. 74 95–120.

Nagano Y, Nagahama T, Hatada Y, Nunoura T, Takami H, Miyazaki J, Takai K and Horikoshi K 2010 Fungal diversity in deep-sea sediments - the presence of novel fungal groups. Fungal Ecology. 3 316–325.

Sharma A and Sharma S C 2017 Physiological Basis for the Tolerance of Yeast Zygosaccharomyces bisporus to Salt Stress HAYATI Journal of Biosciences. 24 176–181.

Durairaj K, Velmurugan P, Park J H, Chang W S, Park Y J, Senthilkumar P, Choi K M, Lee J H, and Oh B T 2017 Potential for plant biocontrol activity of isolated Pseudomonas aeruginosa and Bacillus stratosphericus strains against bacterial pathogens acting through both induced plant resistance and direct antagonism FEMS Microbiology Letters. 364 1–8.

Hentati D, Chebbi A, Hadrich F, Frihka I, Rabanal F, Sayadi S, Manresa A and Chamkha M 2019 Production, characterization and biotechnological potential of lipopeptide biosurfactants from a novel marine Bacillus stratosphericus strain FLU5 Ecotoxicology and Environmental Safety. 167 441–449.

Belknap K C, Park C J, Barth B M and Andam C P 2020 Genome mining of biosynthetic and chemotherapeutic gene clusters in Streptomyces bacteria Scientific Reports. 10 1–9.

Chen R, Wong H and Burns B 2019 New Approaches to Detect Biosynthetic Gene Clusters in the Environment Medicines. 6 32.

Martinet L, Naômé A, Deflandre B, Maciejewska M, Tellatin D, Tenconi E, Smargiasso N, De Pauw E, Van Wezel G P and Rigali S 2019 A single biosynthetic gene cluster is responsible for the production of bagremycin antibiotics and ferroverdin iron chelators Mbio. 10 1–15.
[51] Reynolds K A, Luhavaya H, Li J, Dahesh S., Nizet V, Yamanaka K and Moore B S 2018 Isolation and structure elucidation of lipopeptide antibiotic taromycin B from the activated taromycin biosynthetic gene cluster *Journal of Antibiotics*. 71 33–338.

[52] Kabir M A, Hussain M A and Ahmad Z 2012 *Candida albicans*: A Model Organism for Studying Fungal Pathogens *ISRN Microbiology*. 2012 1–15.

[53] Ren, Q, Liao G, Wu Z, Lv J and Chen W 2020 Prevalence and characterization of *Staphylococcus aureus* isolates from subclinical bovine mastitis in southern Xinjiang, China *Journal of Dairy Science*. 103 3368–3380.

[54] Srikanth M, Kalyani C S, Mohan N, Sridhar K and Padmaja I J 2015 Bacteriology of Acne *Journal of Evolution of Medical and Dental Sciences*. 4 3267–3274.