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Submission date: 23-Jun-2023 02:49PM (UTC+0800)
Submission ID: 2121289765
File name: 12884-Article_Text-1076145-1-10-20230302.pdf (418.79K)
Word count: 4139
Character count: 24405

BIODIVERSITAS
Volume 24, Number 2, February 2023
Pages: 1151-1156

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d240255

Antibacterial activity of sponge-associated bacteria from Torosiaje marine area, Gorontalo, Indonesia

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Manuscript received: 15 November 2022. Revision accepted: 14 February 2023.

Abstract. *Retnowati Y, Katili AS. 2023. Antibacterial activity of sponge-associated bacteria from Torosiaje marine area, Gorontalo, Indonesia. Biodiversitas 24: 1151-1156.* The marine sponge is a member of the Porifera class animal group that has the potential to produce secondary metabolites with various biological activities. These marine animals are often associated with various bacteria from the actinomycetes and non-actinomycetes groups. Currently, information about bacteria associated with sponges in Gorontalo coastal waters is still very limited. Bacteria associated with organisms from the marine environment have been explored as producers of new types of bioactive compounds. Exploring bacteria associated with sponges in the coastal waters of Gorontalo can potentially find new types of antibiotics. The aim of this study was to reveal the diversity of antibiotic-producing bacteria associated with sponges in the Torosiaje coastal area, Gorontalo. The research was carried out in the Torosiaje marine area as sponge sampling locations, including seawater physicochemical properties measurement. The sponges were identified based on morphological characteristics. The sponge-associated bacteria were isolated and screened for antibacterial potential against *Escherichia coli* and *Staphylococcus aureus*. The potential sponge-associated bacteria were identified based on a molecular approach. The result showed that a total of 10 sponge members of Demospongiae class. All ten sponge-associated bacteria were non-actinomycetes bacterial isolates that showed similar morphological characteristics. Only isolate ILM-1 associated with *Coelocarteria singaporensis* showed antibacterial potential in broad-spectrum mode against *Escherichia coli* and *Staphylococcus aureus*. The ILM-1 isolate was identified as *Vibrio diabolicus* closely related to *Vibrio diabolicus strain CW-9-11-1* with a similarity index of 99.70%.

Keywords: Actinomycetes, antibacterial activity, *Coelocarteria singaporensis*, sponge, *Vibrio diabolicus*

INTRODUCTION

Actinomycetes are a group of bacteria that produce bioactive compounds, especially antibiotics. Actinomycetes, especially Streptomyces, produce around 70-80% of bioactive compounds, and about 26% of them are produced by rare actinomycetes (Berdy 2005; Tiwari and Gupta 2013; Azman et al. 2015). Some rare actinomycetes that produce bioactive compounds are amycolatopsis, saccharopolyspora, actinoplanes, micromonospora, streptoverticillium, nocardia, streptosporangium, actinomadura, and nocardiopsis (Berdy 2005; Jose and Jebakumar 2005; Adegboye and Babalola 2013; Gebreyohannes et al. 2013). In addition, many reports revealed the rare group of actinomycetes produces new types of bioactive compounds with antibacterial (Retnowati 2018; Subramani and Sipkema 2019), antifungal (Subramani and Sipkema 2019; Sangkanu et al. 2021), antiparasitic (Pimentel-Elardo et al. 2010; Subramani and Sipkema 2019), and anticancer activities (Davies-Bolorunduro et al. 2019; Subramani and Sipkema 2019).

Currently, there are several cases of bacterial resistance to antibiotics, with various types of bacteria being resistant to more than one type of antibiotic (multidrug-resistant bacteria). For example, *staphylococcus aureus,* a multidrug-resistant bacteria against methicillin, cefepime, erythromycin, gentamycin, cotrimoxazole, netilmicin, amikacin, and imipenem (Sharma et al. 2011). While *Escherichia coli* was resistant to ciprofloxacin, cefotaxime, rifampicin, clindamycin, and cefoperazone (Sharma et al. 2011). In addition, several rediscoveries of similar bioactive compounds produced by actinomycetes from the terrestrial environment. Those rediscoveries have triggered efforts to shift target habitats to marine ecosystems to find new active compounds (Gong et al. 2018; Rosmine et al. 2016; Jiang et al. 2018).

Actinomycetes are found in marine environments either in solitary or in symbiosis with marine macro-organism, one of which is sponges. Studies on actinomycetes associated with sponges in various locations have been widely reported (Sun et al. 2015; Shamikh et al. 2020; Beepath et al. 2016). Sun et al. (2015) successfully reported actinomycetes producing aromatic polyketides from the South China Sea sponges. Those identified as genera *Kocuria, Micromonospora, Nocardia, Nocardiopsis, Saccharopolyspora, Salinispora*, and *Streptomyces*. Shamikh et al. (2020) reported actinomycetes in sponges in Egypt with antibacterial, antifungal, and antiparasitic activity. However, the aquatic environment condition in which the sponge lives and the type of sponge determine the diversity and physiological activity of actinomycetes. Likewise, the environment and symbiont organisms strongly influence the bioactive compounds' characteristics. Gorontalo has a coastline in the north and south with great potential for marine waters. On the Northern Coast of

Gorontalo, Torosiaje marine area is maintained by the local wisdom of the Bajo Tribe. Various potentials of the marine area of Gorontalo need to be revealed and developed, one of which is the bacteria associated with sponges. These marine organisms are classified as porous animals group (Porifera). 5

In addition, given the large number of marine sponge species [more than 15,000 according to Hooper (2000)] represented in the oceans, current investigations into the actinomycetes associated with sponges are limited. Therefore, this study was conducted to reveal the different types of actinomycetes associated with sponges in the marine area of Torosiaje, Gorontalo, and their potential as producers of antibiotics.

MATERIALS AND METHODS

Study area

Sponge sampling was conducted in the Torosiaje water area, Pohuwato, Gorontalo, Indonesia. The observed sponges mostly had habitats and thrived on coral reef thickets with good cover (healthy coral conditions). The sampling location of the sponge was characterized by the measurement of ordinate points using GPS. The sampling location was divided into three sampling points with sampling point coordinates 1: 0°27'54.02"N; 121°26'0.35"E, sampling point 2: 0°28'1.85"N; 121°25'48.03"E; and sampling point 3: 0°27'56.00"N, 121°24'47.00"E (Figure 1). Environmental parameters measured included water depth, brightness, and salinity.

Procedures

Sampling and identification of sponge

The sampling of sponges on Torosiaje seas was based on a random sampling method. Furthermore, using the book Indonesian sponge exploration, the sponge was identified based on morphological characteristics.

Isolation and purification of sponge-associated bacteria

Sponge samples were surface sterilized using 70% alcohol for 1 minute, then rinsed with sterile distilled water. Sponge samples were cut aseptically using a scaper with a size of 1x1 cm and placed on the surface of Nutrient Agar (NA) and Starch Casein Agar (SCA) medium. The petri dishes were incubated at 37°C for 24-48 hours to isolate non-Actinomycetes bacteria and 7-14 days to isolate actinomycetes. The bacterial colonies that grew were then purified using the streak plate method on the NA and SCA medium surfaces. Furthermore, the separated colonies were transferred to NA and SCA slanted medium as pure cultures for further testing.

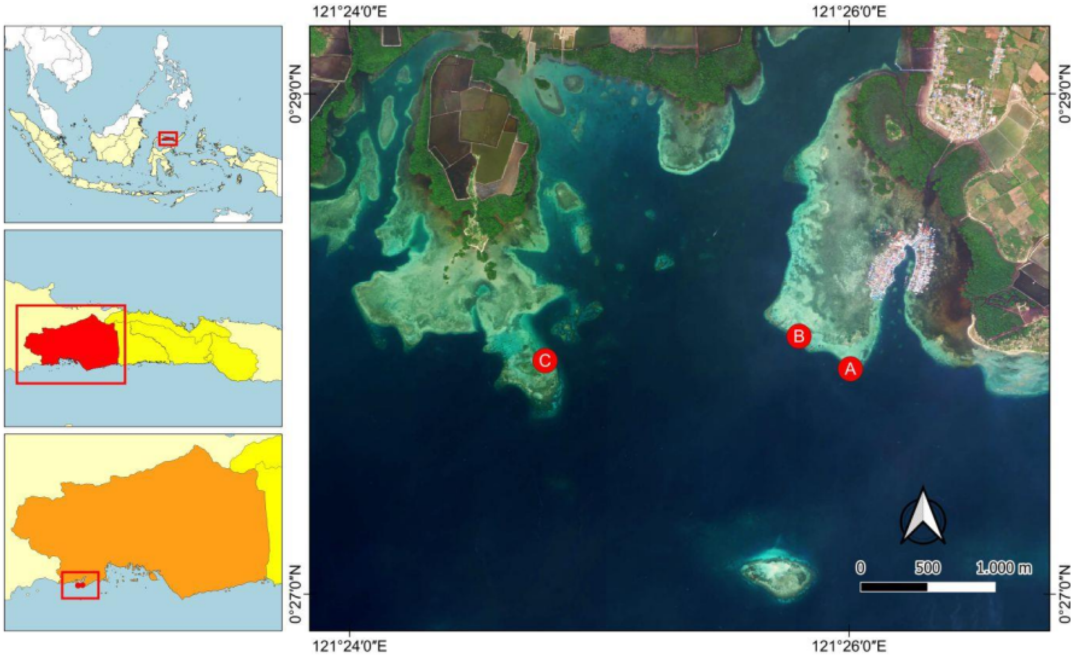

Figure 1. Sampling sponge location at marine area of Torosiaje, Gorontalo, Indonesia. A. Coordinates: 0°27'54.02"N; 121°26'0.35"E, B. Coordinates: 0°28'1.85"N; 121°25'48.03"E; C. Coordinates: 0°27'56.00"N, 121°24'47.00" E

Screening of isolates for antibacterial potential

Screening of antibacterial-potential isolates was based on the Kirby Bauer method and the agar block method against *Escherichia coli* and *Staphylococcus aureus*. The stock cultures of *Escherichia coli* and *Staphylococcus aureus* were re-culture in 50 mL of nutrient broth and incubated on a shaker incubator at 200 rpm at 37°C for 24 hours. A spectrophotometer equalized the mass cells at 0.7 of optical density. The bacterial cell of about 200 μL was spread onto a nutrient agar medium. Sterile paper discs 5 mm in diameter were immersed in the sponge-associated bacterial suspension for 1 minute, then aseptically placed on the surface of the NA medium. The petri dishes were incubated at 37°C for 24-48 hours, and antimicrobial activity was observed based on the formation of an inhibition zone. Therefore, the highest inhibition-zone formation of sponge-associated bacteria was selected for further investigation.

Identification of bacterial isolates

Identification of antibiotic-producing bacteria was based on morphological and molecular characteristics. Morphological characteristics included colony shape, colony color, cell shape, and response to gram staining, while molecular characteristics were based on the 16 S rRNA gene sequence. First, the chromosomal DNA of antibiotic-producing bacteria was isolated using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005). Next, the purity of genomic DNA was determined spectrophotometrically at 260 and 280 nm. In contrast, the concentration of genomic DNA was determined using nano drops. Then, the 16S rRNA gene was amplified using universal primers 27F and 1492R. The amplification product was purified using the DNA Purification Kit and continued with 16S rRNA gene sequencing based on the bidirectional method. Finally, the 16S rRNA gene sequence results were used as data to determine the identity of antibiotic-producing bacterial isolates based on the phylogenetic tree reconstruction.

Data analysis

The identity of sponge-associated bacteria was analyzed qualitative-descriptively based on the data interpretation of phylogenetic tree reconstruction.

RESULTS AND DISCUSSION

Sponge in the marine area of Torosiaje, Gorontalo

In the Torosiaje marine water area, Gorontalo contained a variety of sponges that showed specific morphological characteristics. The result showed that ten species belonged to one class, six orders, eight families, and ten genera (Table 1).

Sponge-bacteria associated at Torosiaje marine area

The results showed that ten bacterial isolates were associated with this sponge. Generally, the bacteria showed similar morphological characteristics that were milky white colony color and referred to a non-actinomycetes group (Figure 2).

Antibacterial activity of sponge-associated bacteria

The result showed that the antibacterial activity of sponge-associated bacteria varied based on the inhibitory-zone diameter and inhibition categories (Table 2). The bacteria associated with the sponge were *Stylissa massa, Holopsamma laminaefavos,* and *Clathria* sp., which did not inhibit the growth of *Escherichia coli* and *Staphylococcus aureus*. On the other hand, the ILM_1 bacterial isolate associated with *Coelocarteria singaporensis* showed the highest antibacterial activity (Table 2).

Figure 2. Morphology colony of sponge-associated bacteria from Torosiaje marine area, Gorontalo

Table 1. Taxonomy of Demospongiae Class sponges from the waters of Torosiaje, Pohuwato, Gorontalo, Indonesia

Class	Order	Family	Genus	Species
Demospongiae	Poecilosclerida	Isodictyidae	Coelocarteria	*Coelocarteria singaporensis*
		Microcionidae	Holopsamma	*Holopsamma laminaefavosa*
			Clathria	*Clathria* sp.
	Halichondrida	Dictyonellidae	Acanthella	*Acanthella pulcherrima*
	Verongida		Aplysina	*Aplysina aerophoba*
	Dictyoceratida	Thorectidae	Hyrtios	*Hyrtios erectus*
	Haplosclerida	Callyspongiidae	Callyspongia	*Callyspongia* sp.
		Petrosiidae	Petrosia	*Petrosia corticata*
		Niphatidae	Niphates	*Niphates* sp.
	Scopalinida	Scopalinidae	Styllissa	*Stylissa massa*

Table 2. Antibacterial activity of sponge-associated bacteria against *Escherichia coli* and *Staphylococcus aureus*

Isolates name	Sponge species	Diameter of inhibitory zone (mm)		Inhibition categories
		S. aureus	*E. coli*	
ILM_0	*Stylissa massa*	0	0	None
ILM_1	*Coelocarteria singaporensis*	8	8	Moderate
ILM_2	*Holopsamma laminaefavosa*	5	5	Moderate
ILM_3	*Acanthella pulcherrima*	5	5	Moderate
ILM_4	*Niphates* sp.	5	5	Moderate
ILM_5	*Aplysina aerophoba*	0	0	None
ILM_6	*Hyrtios erectus*	5	5	Moderate
ILM_7	*Callyspongia* sp.	5	5	Moderate
ILM_8	*Holopsamma laminaefavos*	0	0	None
ILM_9	*Petrosia corticata*	5	5	Moderate
ILM_10	*Clathria* sp.	0	0	None

Figure 3. Phylogenetic tree of ILM_1 bacteria associated with sponge type *Coelocarteria singaporensis* in the Torosiaje marine area, Gorontalo

Identification of ILM_1 bacterial isolate associated with Coelocarteria singaporensis

The 16S rRNA gene sequence of ILM1 isolates was aligned against ten strains on NCBI and subsequent reconstruction of phylogenetic trees based on neighbor-joining algorithms. The results showed that ILM_1 isolate, identified as *Vibrio diabolicus*, was closely related to *Vibrio diabolicus strain CW-9-11-1* on 99.70% index similarity (Figure 3).

Discussion

The sponge is one of the marine animals found in the Torosiaje marine area. A total of ten species of sponges belonged to ten genera, eight families, six orders, and one class of Demospongiae. Torsiaje marine environmental conditions showed the physicochemical condition of water in the salinity range of 67 to 83%, and pH tends to be acidic at values of 5-6, with coral reef clumps in the healthy category. Samawi et al. (2011) reported that the optimal growth temperature of marine sponges in their natural habitat ranges from 8 -18°C, with the optimal pH of marine sponges being about 6.5 because the pH value of cellular sponge fluid is 6.5. Haris et al. (2019) reported 22 sponge species in the flat reef flats of Barranglompo Island. The sponge is one organism that makes up coastal and marine ecosystems, especially in coral reef ecosystems and seagrass beds in tropical and subtropical waters (Samawi et al. 2011). According to Haedar et al. (2016), the diversity of sponge types in a habitat is generally determined by the condition of clear waters that do not have strong currents. Sponges can also be found in each depth condition with a sufficient brightness level for their growth.

Sponges in the Torosiaje marine ecosystem are found to be associated with bacteria. The results showed that all sponge species were associated with non-actinomycetes bacteria. Riyanti et al. (2020) reported sponge-associated bacteria found in Enepahembang East Tahuna, Sangihe Islands, North Sulawesi, Indonesia. Sabhapathy et al. (2005) reported that sponge *Pseudoceratina purpurea* associated with marine water bacteria could form close and complex associations with diverse microbial consortia. In sponge-microorganism associations, sponges can provide a food source for other organisms in the marine environment (Wulff 2001). Xi et al. (2012) reported that various microbial groups found in symbiosis with sea sponges include Proteobacteria, Chloroflexi, Crenarchaeota, and Actinomycetes. More than 30 genera of sponges are known to be associated with actinomycetes.

Bacteria associated with sponges in the Torosiaje marine area were successfully isolated from 11 kinds of sponges. Sponges-associated bacteria exhibited a uniform colony morphological characteristic. The low diversity of sponges-associated bacteria was suspected to be influenced by the isolation technique approach based on classical isolation. According to Sun et al. (2010), classical isolation has limitations where they are biased toward most unculturable microbes that can reach 99% of any environment. Moitinho-Silva et al. (2017) reported that molecular techniques revealed relationships of more than 60 bacterial and archaeal phylum associated with 268 different sponge species, where each sponge sample holds at least a total of 13 different phyla. Studies using PCR techniques in combination with denaturing gradient gel electrophoresis (PCR-DGGE), cloning, and sequencing of fragments of the 16S rRNA gene prove that different sponge species are dominated by various bacterial phylum. That phylum includes Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Gemmatimonadetes, Nitrospirae, and Proteobacteria (Yang et al. 2011; Simister et al. 2012; Kuo et al. 2019; Moreno-Pino et al. 2020).

Non-actinomycetes bacteria associated with sponges in the Torosiaje sea area showed antibacterial activity against multidrug-resistant bacteria *E. coli* and *S. aureus* on moderate inhibition. Morales et al. (2003) reported that the antimicrobial activity was grouped into four categories based on the diameter of the inhibitory zone: weak on less than 5mm, moderate on 5 to 10mm, strong on 10 to 20 mm, very strong on more than 20 mm of inhibitory zone diameter.

Riyanti et al. (2020) reported that sponge-associated bacteria showed antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA). Microorganisms associated with sponges can be composed of about 40% of the host's biomass and exhibit various functions, such as photosynthesis (Wilkinson 1983), nitrogen fixation (Wilkinson and Fay 1979), sulfate reduction (Hoffmann et al. 2005), and secondary active compounds production of metabolites (Graça et al. 2015). Bacteria associated with sponges can produce antimicrobial substances to compete and maintain stable populations (Esteves et al. 2017), whereas sessile sponges rely on secondary metabolites as a defense mechanism against potential threats (Engel and Pawlik 2000).

Isolate ILM_1 bacteria associated with sponge *Coelocarteria singaporensis* in the water of Torosiaje marine area was identified as *Vibrio diabolicus* closely related to *Vibrio diabolicus strain CW-9-11-1* on 99.70% of index similarity. Turner et al. (2018) reported that *Vibrio diabolicus* is a member of the genus Vibrio which is Gamma proteobacteria originating from marine environments around the world. David et al. (2014) reported that *Vibrio diabolicus* associated with *Alvinella pompejana* in the deep hydrothermal sea could produce HE800 exopolysaccharide. Conde-Martínez et al. (2019) also revealed that crude extract of *Vibrio diabolicus* showed antibacterial activity against methicillin-resistant *Staphylococcus aureus* and cytotoxic activity against human lung cell line.

ACKNOWLEDGEMENTS

The authors are grateful for the non-tax revenue of Universitas Negeri Gorontalo, Indonesia year 2022 and to the Chancellor of Universitas Negeri Gorontalo, who has allocated PNBP research funds in 2022. We are also thankful to the laboratory of the biology department Universitas Negeri Gorontalo for the facility in this research.

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