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Antibacterial Activities of *Micromonospora* sp. 2310 Isolated from Marine Sediment, Baru Island, West Kalimantan

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Abstract

The increase in cases of antibiotic resistance and the discoveries cessation of new classes of antibiotics encourages the exploration of various microorganism sources from unique environments to produce antimicrobial compounds. This study aimed to characterize actinobacteria isolated from marine sediment and evaluate the best medium and incubation time for its antibacterial activities. The sediment sample collected from Buru Island, West Kalimantan, was isolated actinobacteria and characterized based on morphological, biochemical, and molecular (16S rRNA gene) approaches. Antibacterial activities were tested using the well-diffusion methods. Twelve suspected colonies were successfully purified. Isolate 2310, which showed a different morphology colony, was close to *Micromonospora* based on morphological, biochemical, and 16S rRNA gene analysis and called *Micromonospora* sp. 2310. Extract isolate 2310 prepared from AM medium showed the best medium for antibacterial activities compared with the other media due to activity against 5 of 6 bacteria, namely *Staphylococcus aureus* ATCC 12600, *Bacillus subtilis* ATCC 6051, *Salmonella enterica* ATCC 14028, *Escherichia coli* ATCC 11775, *Pseudomonas aeruginosa* ATCC 9721 except *Mycobacterium smegmatis* ATCC 14468. Therefore, *Micromonospora* sp. 2310 could be considered a great potential antibacterial producer.

1. Introduction

Infectious diseases are caused by various infectious and pathogenic organisms such as multiple pathogenic bacteria, viruses, fungi, or worms. Antibiotics can treat infectious diseases caused by pathogenic bacteria and fungi [1]. Recently, most pathogenic bacteria have shown antibiotic resistance, such as methicillin-resistant *Staphylococcus aureus* [2] and nearly all antibiotic-resistant *Enterococcus faecium* [3]. The high rate of antibiotic resistance is a significant global health issue. The emergence of antibiotic-resistant increases morbidity and mortality predicted to reach 10 million cases in 2050 [4]. It causes high demand for novel antibiotics to overcome this problem.

Antibiotics in the market are generally isolated from secondary metabolites of actinobacteria [5]. Actinobacteria are Gram-positive bacteria with high G+C content and have mycelia and spores. Actinobacterial genera contributed to producing antibiotics in the market is dominated by *Streptomyces* [6]. For instance, streptomycin, erythromycin, chlortetracycline, and oxytetracycline [7]. Semisynthetic antibiotics also are inspired by secondary metabolites of actinobacteria having antimicrobial activities. For example, azithromycin is derived from erythromycin A. However, the discovery of novel antibiotic classes has been void since 1987 [8]. Therefore, novel antimicrobial compounds from various sources are needed to gain novel antibiotic classes.

Scientists have continually explored antimicrobial compounds from various actinobacterial sources other than *Streptomyces* genera to avoid re-isolation of known antimicrobial compounds. For example, to isolate rare actinobacteria such as *Micromonospora* sp. *Pseudonocardia*

sp. *Nocardia* sp. Rare actinobacteria are defined if actinobacterial abundance is lower than *Streptomyces* when isolated from sources [9]. *Micromonospora* sp. was considered a prolific and potential source of novel secondary metabolites such as antimicrobial compounds beside genus *Streptomyces* [10]. Some known antibiotics from *Micromonospora* sp. are gentamicin, rustamicin, neihumicin, neorustmicin C, dapiramicin, izumenolide, dotriacolide.

Actinobacteria are widespread on terrestrial and marine. Most scientists have explored terrestrial actinobacteria that probability reduces to get novel antimicrobial compounds [11]. Therefore, isolation of actinobacteria from the marine environment probably can increase to get novel antimicrobial compounds due to the unique environment and less explored microorganisms [12]. The unique environment probably can induce a cryptic secondary metabolite biosynthetic gene cluster from a bacterium or an actinobacterium to produce novel antimicrobial compounds. For example, streptocarbazoles obtained from mangrove *Streptomyces* sp. FMA [12]. Therefore, we isolated actinobacteria from marine sediment, Baru Island, West Kalimantan, Indonesia. This study aimed to characterize actinobacteria having antibacterial activities and obtain the best medium and incubation time for antibacterial production.

2. Methodology

2.1. Materials

Media used in this study were ISP1 (International Streptomyces Project 1), ISP2 (International Streptomyces Project 2), ISP4 (International Streptomyces Project 4), LB (Luria-Bertani) agar, modified Bennet (MB) [13], amylostatin medium (AM) [14], basal mineral salt agar (BMSA), and artificial seawater (ASW). Chemical reagents used in this study were D-glucose, D-galactose, D-mannose, D-arabinose, D-xylose, D-fructose, maltose, sucrose, or inositol or dextrin, NaCl, nystatin, nalidixic acid, NaOH, and ethyl acetate. The kits used in this study were GenElute™ Bacterial Genomic DNA kits, Takara Taq™ DNA polymerase, EZNA purification kit, agarose, Gram staining kit (Himedia). The bacteria used in this study were *Staphylococcus aureus* ATCC 12600, *Bacillus subtilis* ATCC 6051, *Salmonella enterica* ATCC 14028, *Escherichia coli* ATCC 11775, *Pseudomonas aeruginosa* ATCC 9721 and *Mycobacterium smegmatis* ATCC 14468. All of this bacterium was a gift from Taifo Mahmud (School of Pharmacy, Oregon State University).

2.2. Sampling

The marine sediment samples were collected at a depth of 7 m with coordinate N 0°36'23.42" U: 108°45'22.41" T from Baru Island, Bengkayang District, West Kalimantan in July 2018. The marine sediment samples were put into a sterilized plastic bag, kept in an iced box, and brought to the laboratory. The sample was kept in a refrigerator (± 4℃) before being used. The seawater was collected and used to make media in isolation and production of isolates.

2.3. Isolation of Actinobacteria

A 1 g of the marine sediment sample was suspended with 9 mL of the sterilized seawater and mixed using a vortex for 15 min. Then, 1 mL of the suspension sample was mixed with warm ISP2 agar medium-enriched nalidixic acid (100 µg/mL), and nystatin (100 µg/mL) then poured on a plate and incubated at room temperature for four months. The suspected colonies that appeared on the medium surface, such as powdery or wrinkle or dull colonies, were purified by transferring into a new ISP2 agar medium to gain a pure isolate.

2.4. Characterization of Isolate

The isolate was characterized by morphological, physiological, biochemical, and molecular traits. The isolate was observed morphological and color colony, diffusible pigment, and melanoid pigmentation on ISP2 and ISP4 agar media. The physiological and biochemical isolate was examined based on diffusible pigment, melanoid pigmentation, salt tolerance, pH tolerance, hydrolysis of protein and starch, and utilization of carbohydrates [15]. The isolate was grown on different media depending on the test and incubated for 5–7 days at 30℃. Salt and pH tolerances were evaluated on ISP2 agar with varying salt concentrations and various pH, respectively. ISP2 agar medium enriched with corn starch (1%) or milk (1%) was used to evaluate the hydrolysis of protein and starch. The utilization of carbohydrate was evaluated using basal mineral salt agar (BMSA) medium added with different carbohydrates (D-glucose 1%, D-galactose 1%, D-mannose 1%, D-arabinose 1%, D-xylose 1%, D-fructose 1%, maltose 1%, sucrose 1%, or inositol 1% or dextrin 0.5%).

The molecular trait was evaluated by the 16S rDNA gene. The gDNA isolate was prepared by inoculating the isolate into an ISP2 broth medium and shaken at 200 rpm, room temperature. After 3 days, the isolated culture was isolated gDNA using GenElute™ Bacterial Genomic DNA kits. The gDNA isolate was used as a template to amplify a partial fragment of the 16S rRNA using a pair of universal primers 27F (5´-AGAGTTTGGATCMTGGCTCAG-3´) and 1492R (5´-CGGTTACCTTGTTACGACTT-3´) and Takara Taq™ DNA polymerase. The reaction and program of PCR were carried out based on the manufacturer's guidelines with 63℃ for the annealing temperature. The PCR products were cleaned up using the EZNA purification kit before sequencing on an ABI3730 capillary sequencing machine at the Oregon State University Center for Genome Research and Biocomputing.

16S rDNA sequence from isolate 2310 was used as representative query sequences to search the NCBI 16S rDNA database using Basic Local Alignment Search Tool for nucleotide (BLASTn) program, then some 16S rDNA sequences from 100% to 99.77% of identity were downloaded. All sequences were aligned using ClustalW from the MegaX program to gain pairwise percent identity [16]. The gap opening and extension penalty for pairwise and multiple alignments on the ClustalW option were 15 and 6.66, respectively. The sequences were re-aligned by Clustal W then calculated the evolutionary history using the Neighbor-Joining Methods [17] from

the MegaX program [18]. The evolutionary distances were calculated using the maximum composite likelihood method, which involved 39 nucleotide sequences [18]. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1537 positions in the final dataset.

2.5. Production and Extraction of Actinobacteria

The inoculum culture was prepared by inoculating the isolate spores into 50 mL of ISP2 broth medium (Erlenmeyer 250 mL with a ring) and grown at 30℃ for 7 days, shaking at 200 rpm. One mL inoculum culture was inoculated into each 100 mL of different medium and shaken at 200 rpm, 30℃ for a particular time. The medium and incubation in this study were ISP1 broth for 7 and 10 days, AM for seven days and modified Benneat suspended with artificial seawater (MB+ASW) for seven days. Then, each culture was centrifuged at 3,000 x g for 15 min to separate between the cells and the supernatant. Secondary metabolites were extracted from the supernatant using ethyl acetate as solvent three times, and the organic layer was collected and evaporated to obtain the crude extract.

2.6. Screening of Antibacterial Activities

2.6.1. Types of Bacteria

This study used Staphylococcus aureus ATCC 12600, Bacillus subtilis ATCC 6051, Salmonella enterica ATCC 14028, Escherichia coli ATCC 11775 Pseudomonas aeruginosa ATCC 9721, and Mycobacterium smegmatis ATCC 14468.

2.6.2. Bacterial Isolation

All bacteria except *M. smegmatis* were inoculated into LB broth medium and grown overnight at 200 rpm, 37℃, to produce bacterial culture. *M. smegmatis* culture was inoculated into an ISP2 broth medium and grown at 200 rpm, 30℃ for 3 days.

2.6.3. Antibacterial Bioassay

Antibacterial bioassays were carried out using the agar well diffusion technique. A 300 µL of bacterial culture and 15 mL of warm LB agar medium were carefully mixed, plated out in a petri dish with a diameter of 9 cm, and let cool down. Then, the medium was punched using a sterilized puncher to make wells. The extract (200 µg/µL) was then dispensed into the well. Once the extract dried, the plate was incubated at 37℃ (30℃ for *M. smegmatis*). Positive antibacterial activity was signed with forming a clear zone around the well, then its diameter was measured using a vernier caliper.

3. Results and Discussion

3.1. Isolation and Characterization of Isolate 2310

The marine sediment sample from Baru Island, Bengkayang District, West Kalimantan was collected at a depth of 7 m with coordinate N 0°36'23.42" U: 108°45'22.41" T. The sample was inoculated on an ISP2 agar medium supplemented with nalidixic acid and nystatin. Nalidixic acid can inhibit the growth of both Gram-negative bacteria and minor Gram-positive except for actinobacteria. Nystatin was used to inhibit the growth of fungi (particularly *Candida* sp.). Twelve suspected colonies of actinobacteria arose on the surface of the medium that morphological colonies were dull, powdery, and wrinkle. One of them was isolate 2310 focused on this study due to the different morphology of the other colonies (Figure 1).

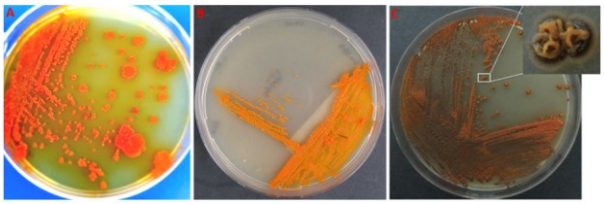

Figure 1. Morphological colonies of Micromonospora sp. A. Isolate 2310 on ISP2 agar medium; B. Micromonospora gifhornensis on GYM Streptomyces medium [19] (https://bacdive.dsmz.de/strain/8029 accessed on 18/06/2021); *C. Verrucosispora* sp. MS100047 on VER01 medium [20]

Table 1. Morphological and biochemical characteristics of isolate SM1A

Tests	Results	Tests	Results
Morphology		**Utilization of carbohydrate**	
		1% (w/v) D-glucose	-
Gram staining	Positive	1% (w/v) D-galactose	-
		1% (w/v) D-mannose	-
Colony		1% (w/v) D-arabinose	++
		1% (w/v) D-xylose	-
ISP2	orange	1% (w/v) D-fructose	-
ISP4	orange	1% (w/v) Maltose	-
		0.5% (w/v) Dextrin	+
NaCl tolerance		1% (w/v) Inositol	-
		1% (w/v) Sucrose	-
0% NaCl	+		
2.5% (w/v) NaCl	+	**pH Tolerance**	
5% (w/v) NaCl	+	pH 4.0	-
7.5% (w/v) NaCl	+	pH 5.0	+
10% (w/v) NaCl	+	pH 8.0	+
12.5% (w/v) NaCl	+	pH 9.0	+
15% (w/v) NaCl	-	pH 10.0	+
		pH 11.0	-
ISP2 Tween 20%	-	pH 12.0	-
ISP2 starch 2%	+	pH 13.8	-
Diffusible pigment		**Melanoid pigment**	
ISP2	d, yellow	ISP2	-
ISP4	nd	ISP4	-

Note: Sign for pigment tests: d, distinctive; nd, not distinctive. Sign for melanoid pigment: -, no melanoid pigment; Sign for salt and pH tolerance: +, growth; -, no growth; Sign for the carbohydrate utilization: ++, the isolated growth on tested carbon in the basal medium is equal to or greater than growth on the basal medium plus glucose; +, when the isolated growth on tested carbon in the basal medium was significantly better than in basal medium without carbon, but somewhat less than on glucose; -, when the isolated growth was similar to or less than growth in basal medium without carbon.

Isolate 2310 was slow growth compared with the other colonies due to appearing after two months of incubation time. Morphological colony isolates 2310 was a tough and wrinkle colony and orange on the ISP2 agar

medium. It was similar to the morphological and color colony to *Micromonospora gifhornensis* on GYM Streptomyces medium (https://bacdive.dsmz.de/strain/8029 accessed on 18/06/2021) and Verrucosispora sp. MS100047 on VER01 medium. Compositions of GYM Streptomyces medium is ISP2 medium added with 2 g/L of $CaCO_3$ but different with VER01 (1 % starch, 1 % glucose, 1 % glycerol, 0.25% corn steep powder, 0.5 % peptone, 0.2 % yeast extract, 0.1 % NaCl, 0.3 % $CaCO_3$, 2 % agar, pH 7.0) [20]. Isolate 2310 produced yellow diffusible pigment (Table 1). No diffusible pigment is detected on *M. gifhornensis* on GYM Streptomyces medium and *C. Verrucosispora* sp. MS100047 on VER01 medium [20].

Characterization of isolate 2310 was conducted based on morphological, physiological, biochemical, and molecular traits (Table 1). The colony color of isolate 2310 was aerial and orange on ISP4 agar but showed good growth on ISP2 agar. Isolate 2310 could grow at a broader range of pH from 5 to 10, categorized as an alkali tolerant actinobacterium. Isolate 2310 can grow from 0 to 12.5% of NaCl. Most tested carbohydrates were not utilized by isolate 2310 except D-arabinose and dextrin. Isolate 2310 can hydrolyze starch but no tween.

Isolate 2310 was genetically determined by sequencing of a partial 16S rDNA sequence. The sequencing result of 16S rDNA of isolate 2310 was 873 bp. The partial 16S rDNA sequence of isolate 2310 was used as a query to search the other 16S rDNA sequences from the NCBI 16S rDNA using BLASTn search. The result exhibited 100% to 99.77% of identity toward *Micromonospora* sp. and *Verrucosispora* sp. Some 16S rDNA from the NCBI database was downloaded. Then, all sequences were analyzed similarity using Omega Clustal program with ClustalW 2.1 with character counts as output format. The similarity was presented as the pairwise percent identity. The result showed that isolate 2310 share the highest sequences similarity with *Micromonospora gifhornensis*/*Verrucosispora gifhornensis* (https://bacdive.dsmz.de/strain/8029 accessed on 18/06/2021), and *Micromonospora craterilacus* with percent identity reached 100% identity (Table 2). The percent identity less than 98.7% means that the isolate is novel [21]. Isolate 2310 was categorized as a known species due to more than 98.7% of the percent identity.

A phylogenetic tree described evolutionary relationships between the isolates or among taxa. Isolate 2310 showed a share common ancestor with *Verrucosispora maris*, *Micromonospora lutea*, *Verrucosispora rhizophaerae*, *Micromonospora fiedleri*, *Micormonospora maris*, *M. gifhornensis*/*V. gifhornensis* (Figure 2). Finally, isolate 2310 based on morphological, pairwise percent identity, and phylogenetic tree belonged to *Micromonospora* sp. and called *Micromonospora* sp. 2310.

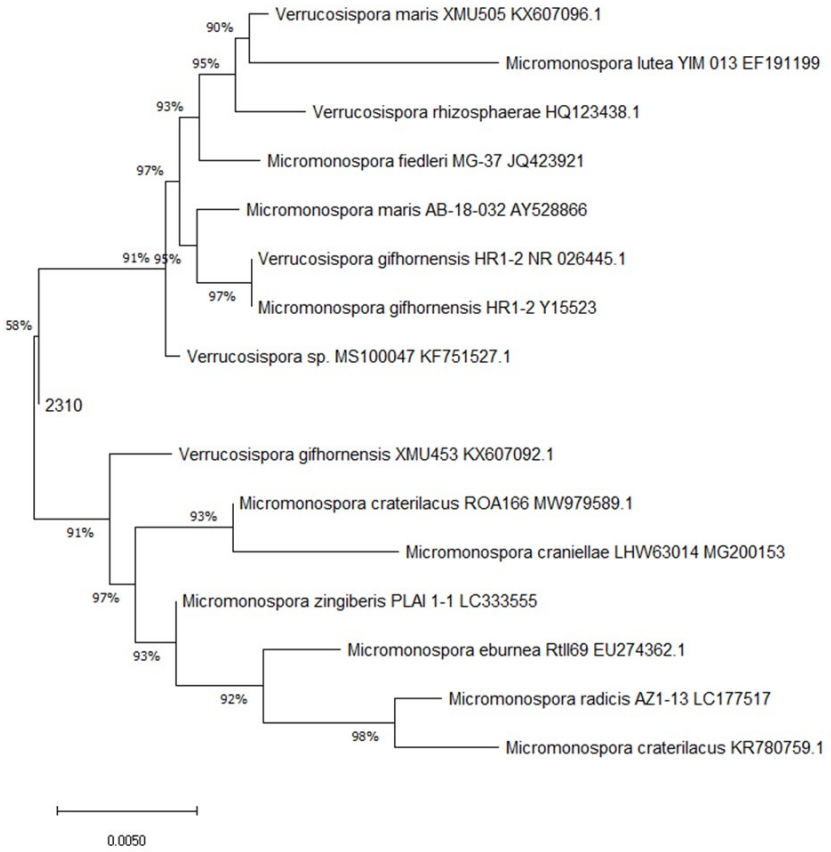

Figure 2. Phylogenetic tree of the 16S rRNA gene showing relationships between isolate 3210 using MegaX. Bar 0.0050 substitutions per nucleotide position. Percentage presented the percent data coverage for the internal node. This analysis involved 15 nucleotide sequences. All ambiguous positions were removed for each sequences pair (pairwise deletion option)

Table 2. Pairwise percent identity of each isolate

Bacteria	*M. lutea* YIM013 EF191199	*M. maris* AB18032 AY528866	*M. craniellae* LHW63014 MG200153	*M. fiedleri* MG 37 JQ423921	*M. zingiberis* PLAI11 LC333555	*M. eburnea* RtII69 EU274362.1	*M. craterilacus* KR780759.1	*M. radicis* AZ113 LC177517	*V. maris* XMU505 KX607096.1	*V. rhizosphaerae* HQ123438.1	*M. gifhornensis* HR12 Y15523	*V. gifhornensis* HR12 NR026445.1	*M. craterilacus* ROA166 MW979589.1	*V. gifhornensis* XMU453 KX607092.1	2310	*Verrucosispora* sp. MS100047
M. lutea YIM013 EF191199	100.00															
M. maris AB18032_AY528866	97.03	100.00														
M. craniellae LHW63014_MG200153	96.25	98.67	100.00													
M. fiedleri MG37 JQ423921	96.69	99.15	98.07	100.00												
M. zingiberis PLAI11 LC333555	97.60	98.98	99.79	98.37	100.00											
M. eburnea RtII69 EU274362.1	96.53	98.05	98.84	97.57	99.06	100.00										
M. craterilacus KR780759.1	96.18	98.61	99.25	98.16	99.43	98.77	100.00									
M. radicis AZ113 LC177517	96.75	98.75	99.38	98.45	99.72	99.13	99.46	100.00								
V. maris XMU505 KX607096.1	98.01	98.90	97.17	99.25	97.32	97.42	99.17	97.46	100.00							
V. rhizosphaerae HQ123438.1	97.38	99.30	98.64	99.00	98.65	97.90	98.50	98.77	98.33	100.00						
M. gifhornensis HR12 Y15523	96.96	99.65	98.76	99.08	98.93	97.98	98.70	98.70	98.04	99.38	100.00					
*V. gifhornensis*_HR12 NR026445.1	96.96	99.65	98.76	99.08	98.93	97.98	98.70	98.70	98.04	99.38	100.00	100.00				
M. craterilacus ROA166 MW979589.1	97.70	98.89	99.71	98.26	99.78	98.96	99.42	99.64	97.22	98.71	99.06	99.06	100.00			
V. gifhornensis XMU453 KX607092.1	96.91	98.23	97.97	98.42	98.12	98.01	97.68	97.83	97.90	97.10	97.24	97.24	98.03	100.00		
2310	98.17	99.77	99.77	99.89	99.89	99.89	99.89	99.89	99.89	99.89	100.00	100.00	100.00	100.00	100.00	
Verrucosispora sp. MS100047	97.38	98.98	98.04	99.19	98.25	97.31	97.82	97.96	99.56	98.62	98.91	98.91	98.14	98.97	100.00	100.00

M. Micromonospora; V. Verrucosispora

3.2. Screening of Antibacterial Activities

All extracts of *Micromonospora* sp. 2310 prepared from various media (ISP1, AM, MB+ASW) was active against *E. coli*, *S. aureus*, *S. enterica*, *B. subtilis* but no *M. smegmatis* (Table 3). *P. aeruginosa* was only inhibited by extract *Micromonospora* sp. 2310 prepared from AM media. It was interesting because most antimicrobial compounds were not active against *P. aeruginosa.* It needed a higher dose (400 µg/well) than the other extract (200 µg/well) against the bacteria even though the inhibition zone diameter for each extract showed almost similar. The extract *Micromonospora* sp. 2310 prepared from the AM medium likely contained a low concentration or titer of active compounds than the other extract or different active compounds.

Table 3. Screening of antibacterial activities from extract isolate 2310

Medium	Incubation time, days	Dose, µg/well	Diameter of inhibition zone, cm					
			MS	EC	SA	SE	BS	PS
ISP1	7	200	-	0.2	0.4	0.3	0.6	-
ISP1	10	200	-	0.6	0.4	0.4	0.6	-
AM	7	400	-	0.8	0.4	0.3	0.4	0.4
MB+ASW	7	200	-	0.6	0.4	0.4	0.8	-
Tetracycline		1		1.2	1.6	1.2	1.6	ND

-. No antibacterial activities; EC. *E. coli*; SA. *S. aureus*; SE. *S. enterica*; BS. *B. subtilis*; PS. *P. aeruginosa*. ND. no determined

A phylogenetic tree described evolutionary relationships between the isolates or among taxa. Isolate 2310 showed a share common ancestor with *Verrucosispora maris*, *Micromonospora lutea*, *Verrucosispora rhizophaerae*, *Micromonospora fiedleri*, *Micormonospora maris*, *M. gifhornensis*/*V. gifhornensis* (Figure 2). Finally, isolate 2310 based on morphological, pairwise percent identity, and phylogenetic tree belonged to *Micromonospora* sp. and called *Micromonospora* sp. 2310.

In addition to different media, *Micromonospora* sp. 2310 was cultivated on ISP1 broth medium was incubated at different times, 7 and 10 days. Antibacterial activities for the different incubation times showed no activity changes, such as the number of bacteria or inhibition zone diameter (Table 3). However, the best incubation time for this case was chosen, a shorter time of 7 days.

A microorganism, i.e., actinobacteria, can produce various secondary metabolites, but its production depends on condition, environment, media, or substrates. The different media may affect secondary metabolites, including antibacterial compounds. Isolate *Nonomuraea kuesteri* E3N418 is active against 4 of 15 bacteria when the extract grows on Bennet medium compared with the ISP2 and GYEA media [9]. Extract *Streptomyces* sp. RS6 prepared on MB+ASW medium can inhibit five of six bacteria, while ISP1+ASW+TE and AM are 1 and 2 of the bacteria, respectively [22]. Extract *Streptomyces* sp. RC5 prepared on AM was active against four of six bacterial tests while inactive on the ISP1+ASW+TE medium.

4. Conclusion

A rare actinobacterium, *Micromonospora* sp. 2310, was successfully isolated from marine sediment at Buru Island, West Kalimantan. Extract *Micromonospora* sp. 2310 showed a broad spectrum of antibacterial activities due to active toward both Gram-positive and negative bacteria when grew on ISP1, AM, and Bennet broth media.

The best medium for the antibacterial production of *Micromonospora* sp. 2310 was AM broth medium with 7 days of incubation time. The best incubation time for the antibacterial production of *Micromonospora* sp. 2310 was seven days on the ISP1 medium *M. Micromonospora* sp. 2310 showed no different antibacterial activities with different incubation time on the ISP1 broth medium. Therefore, *Micromonospora* sp. 2310 had potency as a lead compound with antibacterial activities.

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