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Activity of Actinomycetes Isolated from Mangrove Segara Anakan Cilacap toward Methicillin-resistant *Staphylococcus aureus* (MRSA)

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major health concern because it causes numerous infections in both healthcare facilities and communities. The development of multiresistant against topical antibiotics has caused substantial difficulty in the management of Staphylococcus infection. Thus, this research was aimed to explore indigenous marine Actinomycetes isolated from Segara Anakan Cilacap for anti-MRSA activity. The methods used were screening anti-MRSA activity using agar-block method, production of anti-MRSA extract, extraction of the anti-MRSA extract with ethyl acetate, MIC determination of the ethyl-acetate extract, and species identification based on morphology and 16S rRNA genes. The results indicated that 14 out of 16 Actinomycetes have anti-MRSA activity. Three isolates, which were W-5B, W-5A, and P-7D, showed the highest anti-MRSA activity with the inhibition zone of 2.40 mm, 1.20 mm, and 0.80 mm, respectively. The minimum inhibitory concentration (MIC) of ethyl acetate extract from isolates W-5B, W-5A, and P-7D against MRSA were 2 mg/mL, 4 mg/mL, and 8 mg/mL, respectively. The species identification based on 16S rRNA genes indicated that Actinomycetes W-5B isolate has 92.51% similarity with *Streptomyces* sp. 2011. The W-5A has 94.69% similarity with Arthrobacter sp. HZ11. The P-7D has 94.79% similarity with *Streptomyces clavuligerus* strain A-ZN-05. The present study concludes that marine Actinomycetes from sediment mangrove in Segara Anakan Cilacap, Indonesia, has potency as anti-MRSA.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a *Staphylococcus aureus* bacterium that is resistant to β-lactam antibiotics such as penicillin and its derivatives namely methicillin, oxacillin, dicloxacillin, nafcillin and cephalosporin. *S. aureus* is found in many human epitheliums and is an opportunistic pathogen involved in nosocomial infections [1]. MRSA infections can cause metastatic or complicated infections such as infective endocarditis or sepsis and have fatal consequences for the sufferer. It begins to appear in moderate to acute atopic dermatitis patients and is resistant to multiple antibiotics [2]. In Asia, the prevalence of MRSA infections in healthcare facilities in the range of 28% (in Hongkong and Indonesia) to 70% in Korea [3]. The incidence of MRSA infections continues to increase throughout the world in the past decade and is endemic to hospitals in most regions of the world [4].

Changes in the epidemiology of MRSA and the widespread antibiotic resistance have become a concern for public health and are essential foundations for researchers to look for new bioactive compounds originating from natural sources. The spread of MRSA infection is no longer only in hospital infections but has reached the community; therefore, it is necessary to do microbial exploration with the potential of antagonistic compounds that can suppress MRSA problems. One of the microbes known to have the ability to produce the most

bioactive compounds compared to other bacteria is Actinomycetes [5].

Actinomycetes are Gram-positive mycelium bacteria widely distributed in natural environments [6]. Asnani *et al.* [7] have reported that Actinomycetes from the mangrove area of Segara Anakan Cilacap are potential sources for antibacterial compounds. Kannan *et al.* [8] have published an effort to produce anti-MRSA compounds from Actinomycetes isolated from Manakkudy's ecosystem mangrove Arabian coast India. Xu *et al.* [9] have reviewed 73 novel compounds and 49 known compounds isolated from mangrove Actinomycetes from Xiamen, Fujian Province, China. Genus Streptomyces has been considered as the richest source among mangrove Actinomycetes, which produced indole alkaloids, macrolides, and benzene derivatives as the main natural products. As an example, Streptomyces HKI0595 produced indolosesquiterpenes with strong antibacterial activities against MRSA and Vancomycin-resistant *Enterococcus faecalis* [9]. SR and Rao [10] also reported that Streptomyces sp. isolated from coastal marine land could inhibit Multi-Drug *Staphylococcus aureus* (MDRSA). Considering the possibility of mangrove Actinomycetes as an important source of bioactive compounds which could become new and effective anti-MRSA, thus this research was aimed to examine the antagonist activity of Actinomycetes from Segara Anakan Cilacap towards MRSA, to determine the minimum inhibition concentration of antibacterial extract, and to identify the potential Actinomycetes through morphology and 16S rRNA identification.

2. Methodology

In the present study, 16 marine Actinomycetes were isolated from the mangrove ecosystem in Segara Anakan Cilacap and analyzed for their anti-MRSA activities. The sediment samples were collected from twenty spots in two different locations, namely Kalipanas (P) and Kutawaru (W). The samples were pre-treated with dry heating at 90°C for 15 min, 1.5% phenol, and olive oil separation methods. Then, the colonies were cultivated on starch-casein-nitrate (SCN) agar (10 g starch, 1 g casein; 1 g KNO_3; 0.5 g K_2HPO_4; 0.5 g $MgSO_4.7H_2O$; 0.5 g NaCl; 0.01 g $FeSO_4.7H_2O$; and 20 g Bacto agar). The colonies which showed morphology characteristics to Actinomycetes were further purified. The 16 pure isolates obtained were kept at SCN agar at 28°C for further study [7].

2.1. Screening of Actinomycetes with anti-MRSA activity

Sixteen marine Actinomycetes were primary screened for anti-MRSA activity by the agar-block method [11]. The strain MRSA 2983 was a methicillin-resistant bacterium and available from Research Laboratory of Medicinal Faculty, Universitas Jenderal Soedirman, Purwokerto, Indonesia. Each Actinomycetes was cultivated in SCN agar medium by tight streaks on the plate surface and incubated at 38°C for seven days. The MRSA was cultivated separately in Nutrient Agar medium using a pour plate. The experiment was carried out by culturing an agar-block (6 mm in diameter) of Actinomycetes on the surface Nutrien Agar which was previously inoculated by MRSA. The plates were incubated at 38°C for 24 hours. The antagonism was observed based on the clear zone around the agar-block. The inhibition zone was measured using the following formula.

$$Inhibition\ zone = \frac{d_2 - d_1}{d_2}$$

In which the d_1 was diameter of agar block (mm) and d_2 was the diameter of the clear zone (mm). The inhibition zone measurements were done in five replications. Three potential Actinomycetes with the highest inhibition zone were selected and subjected to fermentation process and ethyl acetate extraction.

2.2. Fermentation process and ethyl acetate extraction

The inoculum was prepared by inoculating three potential Actinomycetes in SCN broth for eight days at 38°C. 10% of inoculum was subsequently cultivated in new SCN broth and incubated in various incubation times (7, 14, 21, and 28 days). After each incubation time, the mixture was centrifuged at 4.000 rpm for 20 minutes at 4°C. The clear supernatant was tested for anti-MRSA activity using the disc diffusion method [12]. The incubation time, which produced the highest anti-MRSA activity, was used as the optimum incubation time for fermentation to produce anti-MRSA extract.

The fermentation process was started with inoculating 10% of inoculum Actinomycetes in SCN broth and incubated with optimum incubation time to produce anti-MRSA compound with the highest activity. After fermentation process, the culture was centrifuged, and the supernatant obtained was extracted three times with ethyl acetate (1:1, v/v). The combined organic layers were evaporated at 40°C to yield the ethyl acetate extract (EA extract).

The minimum inhibition concentration (MIC) of ethyl acetate extract was determined by the disc diffusion method [12]. The MRSA was cultivated in Nutrient Broth (Oxoid) and incubated at 37°C for 24 hours. 0.1 mL of MRSA culture was lawn cultured on Nutrient Agar plates. Each blank disc (6 mm diameter, Oxoid) was impregnated with 15 μL of EA extract with various concentrations (2, 6, 8, and 10 mg/mL). All discs were allowed to dry in room temperature and place onto NA with tested MRSA. The growth of MRSA was evaluated after incubation at 37°C for 24 hours. The MIC was defined as the lowest concentration of EA extract that showed growth inhibition of MRSA.

2.3. Identification of potential Actinomycetes

Morphology observation. The colonies of three potential isolates were microscopic observed after seven

days of incubation. The observation was based on the known criteria such as the colony shape, size, surface, elevation, margin, the color of air mycelium, substrate mycelium, and pigmentation. The morphology of the mycelium was observed by preparing Henrichi's Slide Culture [7].

DNA extraction and 16S rRNA gene amplification. The DNA of Actinomycetes was extracted following the protocol described by Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan). The concentration and purity of isolated DNA were measured with Spectrophotometer (Shimadzu IP1600i) at λ 260 nm and λ 280 nm, respectively. The isolated DNA was amplified with PCR (Thermo primus 25 advanced peQlab). The PCR reaction was performed in a total volume of 50 μL which contained 25 μL of KAPPA Taq Extra Hotstart ReadyMix with dye (Merck, Germany), 50 ng DNA template, 2 μL of 0.5 μM 27F (AGAGTTTGATCMTGGCTCAG) primer, 2 μL of 0.5 μM 1492R (TACGGYTACCTTGTTACGACTT) primer, and free-nuclease water. PCR condition was as follows, first heating at 95°C for 3 minutes, denaturation at 94°C for 15 seconds, annealing at 55°C for 15 seconds, and the final extension at 68°C for 1 minute. The cycle was repeated 30 times [7]. The PCR products were observed using electrophoresis on 1% (w/v) agarose gel submerged in 1x TAE buffer and visualized using UV transilluminator (DL-UV312).

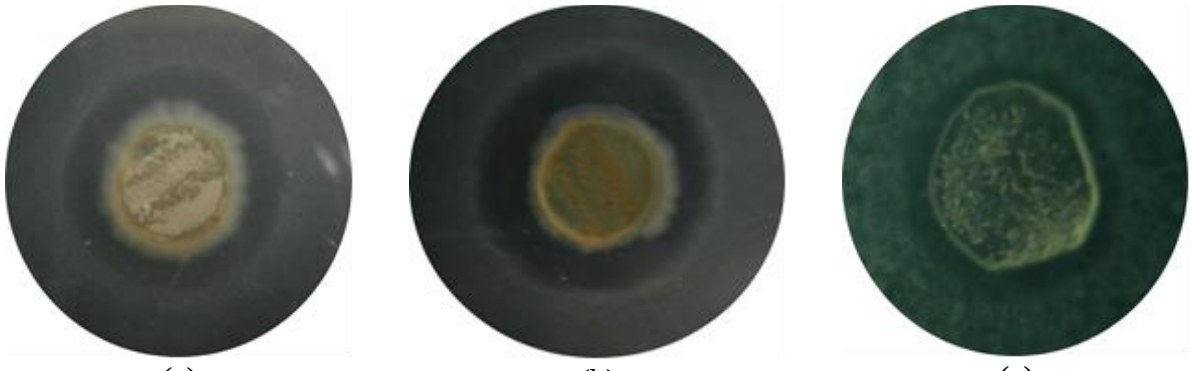
16s rRNA sequencing and phylogenetic construction. The PCR products were sequenced by 1st Base DNA Sequencing Services (Singapore). The sequences obtained were analyzed and edited using BioEdit 7.2 program. Sequence Alignment used Clustal W. The aligned results of 16S rRNA genes were compared to other sequences in GenBank by using BLAST on the National Centre for Biotechnology Information (NCBI). The phylogenetic tree was constructed with the Neighbor-Joining method and 1000 bootstrap replications using the Molecular Evolutionary Genetics Analysis (MEGA) version 6:0 [13].

3. Results and Discussion

3.1. Screening of Actinomycetes with anti-MRSA activity

Screening for anti-MRSA activity was carried out using the agar-block method [11]. The actinomycetes were cultivated on their appropriate culture medium by tight streaks on the plate surface. The cultures were incubated for seven days to provide enough time to produce active metabolite substance, which diffused into the agar medium. After incubation, an agar-block of Actinomycetes was placed on the agar surface of another plate previously inoculated by the MRSA. During incubation, the active substances diffused from the agar-block to the medium. Then, the anti-MRSA activity of the Actinomycetes secreted molecules was detected by the appearance of the inhibition zone around the agar-block. The screening results indicated that 14 out of 16 Actinomycetes tested have anti-MRSA activity (Table 1). Three isolates with the highest inhibition zone were Actinomycetes W-5B, W-5A, and P-7D with the inhibition zone of 2.40 mm, 1.20 mm, and 0.80 mm, respectively (Figure 1).

It is interesting to note that the three potential Actinomycetes, W-5A, W-5B, and P-7D were originally isolated from mangrove sediment around *Rhizophora apiculata* trees in Kutawaru (W-5) and Kalipanas (P-7) area. Differently, Retnowati *et al.* [14] reported that Actinomycetes with the highest antibacterial activities were found at the rhizosphere of *Avicennia marina* and *Xylocarpus* sp trees in the mangrove forest of Torosiaje, Gorontalo. Indeed, the type of mangrove vegetation significantly influences the Actinomycetes occurrence due to variation in the physical, chemical, and biological factors in mangrove ecosystems [15]. The unique mangrove environment based on pH, temperature, salinity, and availability of nutrient differs considerably in different regions so that mangrove-derived Actinomycetes offer distinctive antibacterial activities [10, 14, 16, 17, 18].



(a) (b) (c)

Figure 1. The inhibitory zones of Actinomycetes (a) W-5A, (b) W-5B, and (c) P-7D against MRSA using the agar block method

Table 1. Inhibition zone of Actinomycetes against MRSA

No.	Sediment Sources	Isolate	Average Inhibition zone (mm)
1.	*Rhizophora apiculata*	W-5B	2.40 ± 0.55
2.	*Rhizophora apiculata*	W-5A	1.20 ± 0.45
3.	*Rhizophora apiculata*	P-7D	0.80 ± 0.45
4.	Bottom of *Kali Panas*	P-3A	0.70 ± 0.27
5.	*Nypa fruticans*	W-7A	0.70 ± 0.27
6.	*Xylocarpus moluccensis*	W-6A	0.60 ± 0.22
7.	*Sonneratia alba*	W-1A	0.60 ± 0.00
8.	*Avicennia marina*	P-6B	0.50 ± 0.00
9.	*Avicennia marina*	P-6E	0.50 ± 0.00
10.	*Rhizophora apiculata*	P-7I	0.50 ± 0.00
11.	*Sonneratia alba*	W-1A	0.50 ± 0.00
12.	*Nypa fruticans*	W-8A	0.50 ± 0.00
13.	*Sonneratia sp*	W-3A	0.50 ± 0.00
14.	*Rhizophora sp.*	W-9A	0.50 ± 0.00
15.	*Xylocarpus moluccensis*	W-6B	0.00 ± 0.00
16.	*Avicennia marina*	P-6D	0.00 ± 0.00

3.2. Fermentation process and ethyl acetate extraction

Three potential Actinomycetes, W-5B, W-5A, and P-7D, were further used for fermentation to produce anti-MRSA metabolite. It is known that the high production of active metabolites correlates with the growth phase of the microbe. Thus, at first, the fermentation of potential Actinomycetes was carried out in various incubation times to analyze the correlation of incubation time with anti-MRSA activity. The research indicated that the optimum incubation time with the highest anti-MRSA activity was obtained after 14 days of incubation for all isolates W-5B, W-5A, and P-7D W (Figure 2).

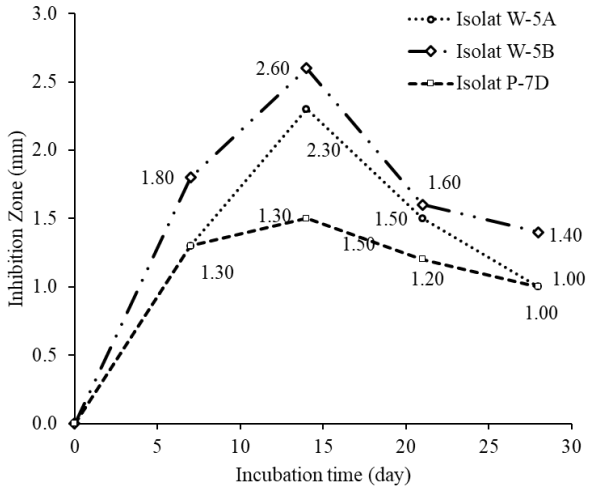


Figure 2. Inhibition zone of isolates W-5A, W-5B, and P-7D at various incubation times to determine the optimum incubation time for the fermentation process

The fermentation process to produce antimicrobial compounds from marine Actinomycetes is quite diverse. Valan *et al.* [16] fermented marine Actinomycetes from Bay of Bengal India in modified nutrient glucose agar (MNGA) medium for five days to produce novel polyene metabolite. Marine Actinobacteria were fermented for seven days in soluble starch (SS) media to produce MDRSA [10]. Similarly, antimicrobial metabolites from Streptomyces sp. SN0280 was produced after seven days of fermentation in the F medium [19]. Fermentation of Streptomyces strain M7 for ten days in SCN broth produced antibacterial compound against MRSA [20]. On the contrary, Sangkanu *et al.* [17] reported three weeks (21 days) of incubation time in International Streptomyces Project-2 (ISP-2) broth to produce antibacterial compound from mangrove sediment-derived Actinomycetes. The differences in fermentation time might arise due to various fermentation medium used and the typical metabolite produced.

The extraction of antimicrobial compounds from Actinobacteria mostly employs different polarity of organic solvents. Among other organic solvents, ethyl acetate was repeatedly reported as the best solvent to extract secondary metabolites from Actinomycetes [21]. The ethyl acetate extracts of marine Actinobacteria SRB25 showed maximum antimicrobial activity against MRSA compare to chloroform and butanol extracts [10]. The ethyl acetate extracts compare to chloroform, hexane, butanol, and diethyl ether were reported to have the maximum recovery of active metabolites from fermentation process of Streptomyces M7 [20]. Solvent ethyl acetate was also produced the highest inhibition zone against MDR *S. aureus* compared to acetone and methanol extracts. Hence, this research also used ethyl acetate to extract the anti-MRSA compound.

The minimum inhibitory concentration (MIC) of ethyl acetate extracts of W-5A, W-5B, and P-7D against MRSA was 4 mg/mL, 2 mg/mL, and 8 mg/mL. These results indicated that ethyl acetate extract of W-5B has the best antibacterial activity in inhibiting MRSA. Similarly, antimicrobial from *Nocardiopsis prasina* against MRSA has MIC of 2.5 mg/mL [6], whereas antimicrobial from *Streptomyces* sp against MRSDA has MIC of 1 mg/mL [10].

3.3. Identification of potential Actinomycetes

The colony observation of three potential Actinomycetes showed that all strains have a small size with a circular or irregular shape. The colony surface was powdery which is typical of Actinomycetes. Initially, colonies were a relatively smooth surface, but later after 14 days of incubation, they developed aerial mycelium that appears powdery. The aerial mycelium and substrate mycelium have distinctive colors. Pigmentation to the medium was also observed in those three isolates (Table 2). The morphology of isolates showed that aerial mycelium formed chains of three to many spores. Isolate W-5A produced branches mycelium, P-7D has a short chain of spores, and W-5B has long chains in spiral form that typical Streptomyces (Figure 3).

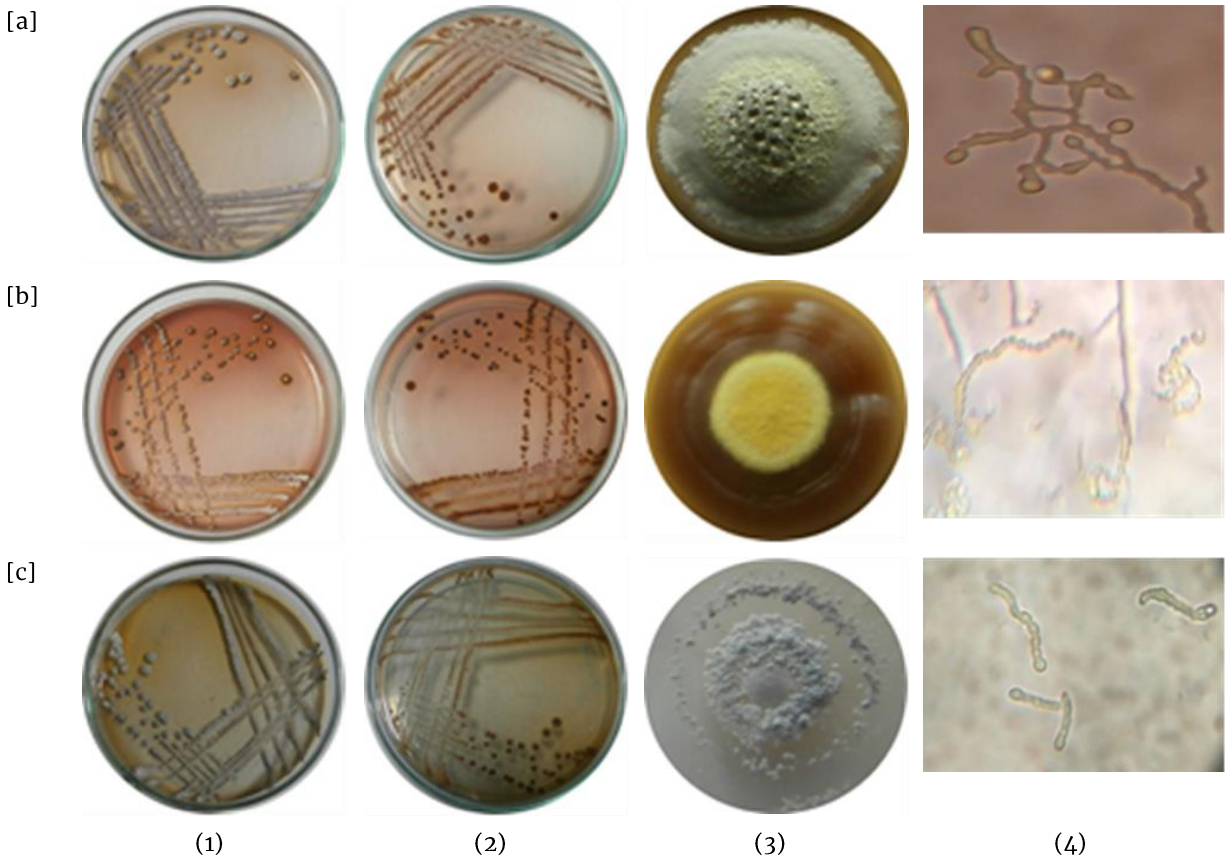


Figure 3. The colony and morphology of isolates (a) W-5A, (b) W-5B, and (c) P-7D; (1) the top appearance, (2) the bottom appearance, (3) a single colony, and (4) the mycelium.

The spore chain morphologies of Streptomyces are extensive. However, the International Streptomyces Project (ISP) recognized three categories of spore chains which were (a) straight to flexuous, (b) hooks, loops, or spirals; and (c) spirals [22]. The gram staining results further confirmed that isolates W-5A, W-5B, and P-7D were Gram-positive bacteria.

Table 2. Colony observation of Actinomycetes W-5A, W-5B, and P-7D

Characteristics	W-5A	W-5B	P-7D
Shape	circular	circular	irregular
Size	small	small	small
Surface	dry powdery	shrink	dry powdery
Elevation	umbonate	raised	Umbonate
Margin	entire	entire	undulate
Aerial mycelium	white	yellowish-white	light grey
Substrate mycelium	dark brown	dark brown	dark brown
Pigmentation	pigmented	pigmented	pigmented

Assigning unknown Actinomyctes to genera based on 16S rRNA gene sequence analysis is standard practice [22]. Thus, isolated DNAs were amplified using 27F and 1492R primers. PCR amplification yielded a single amplicon around 1400 bp for W-5A, W-5B, and P-7D, respectively (Figure 4). These results were expected from DNA amplification with universal primers.

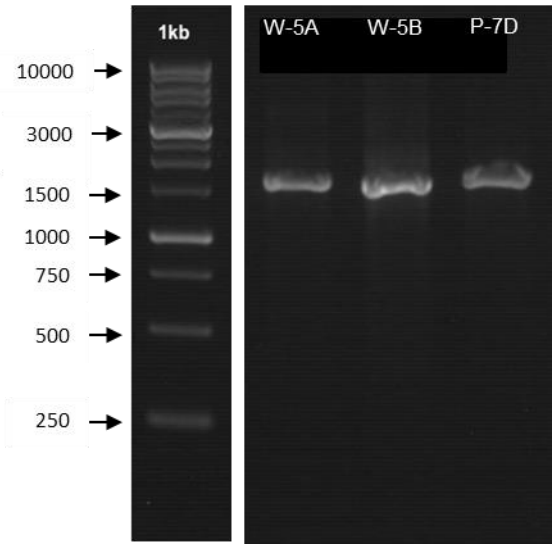


Figure 4. Electrophoresis of PCR amplicon of Actinomycetes W-5A, W-5B, and P-7D.

The 16S rRNA sequencing determined the closest homologues sequences of query Actinomycetes. The results from BLAST analysis of 16S rRNA genes (Table 3) designated that W-5A isolate has 94.69% similarity with Arthrobacter sp. HZ11 (KY064075.1). The W-5B isolate has 92.51% similarity with *Streptomyces* sp. 2011 (JF751041.1). The P-7D has 94.65% similarity with *Streptomyces*

clavuligerus strain A-ZN-05 (MF083720.1). The query covers of all three isolates were very high that indicated the high alignment of the query sequences with sequences in GenBank.

Table 3. BLAST analysis of 16S rRNA genes from isolates W-5A, W-5B, and P-7D

Code	Isolate name	Max Score	Total Score	Query Cover	E value	Percent Identity	Accession No.
W-5A	Arthrobacter sp. HZ11	2058	2058	93%	0.0	94.69%	KY064075.1
W-5B	Streptomyces sp. 20111	2076	2076	99%	0.0	92.51%	JF751041.1
P-7D	Streptomyces clavuligerus strain A-ZN-05	2259	2259	99%	0.0	94.79%	MF083720.1

The phylogenetic analysis using the neighbor-joining method further validated the relation of Actinomycetes W-5B, and P-7D with genus *Streptomyces* (Figure 5). Evolutionary analyses were conducted in MEGA6 [13]. The analysis involved 27 nucleotide sequences in which all positions containing gaps and missing data were eliminated. Bootstrap values were expressed as the percentage of 1000 replications.

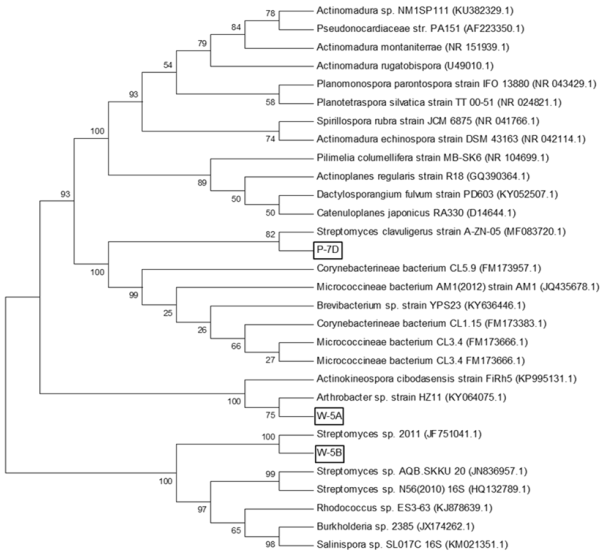


Figure 5. Phylogenetic tree obtained by the Neighbor-Joining method of 16S rRNA gene sequences from Actinomycetes W-5A, W-5B, P-7D, and related species. Numbers on branch nodes are bootstrap values, which were expressed as a percentage of 1.000 replications.

4. Conclusion

Mangrove ecosystem is rich in biological diversity. The results of the present study revealed the potency of marine Actinomycetes from Segara Anakan, Cilacap, Indonesia, as anti-MRSA. The ethyl acetate extract of W-5B should be purified further to obtain the bioactive compound. The anti-MRSA mechanism by bioactive compounds from marine Actinomycees should also be investigated for a better understanding of MRSA treatment.

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