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The Activities of Streptomyces W-5A as Antibacterial and Antibiofilm towards Methicillin-resistant *Staphylococcus aureus* 2983

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Abstract: Methicillin-resistant *Staphylococcus aureus* (MRSA) causes nosocomial infection worldwide. MRSA can defend itself by forming a biofilm layer, thereby increasing the virulence factor. WHO categorizes MRSA as high risk on the Priority Pathogen List for searching for new antibiotics. Recently, we have reported the potency of Streptomyces W-5A as anti-MRSA based on qualitative screening. Thus, this research aimed to analyze the potency of Streptomyces W-5A as antibacterial and antibiofilm towards MRSA 2983. Cultivation of Streptomyces W-5A used Starch Casein Nitrate (SCN) agar, and anti-MRSA extract production used SCN broth. Samples were taken at incubation times of 0, 3, 6, 9, 12, and 15 days. Each sample was tested for antibacterial activity with the Kirby-Bauer method, whereas inhibition of biofilm formation and biofilm degradation with microtiter method. The results showed that the optimum antibacterial activity was achieved after nine days of incubation with an inhibition zone of 9.589 ± 0.521. The optimum % inhibition of biofilm formation was 77.806 ± 13.595% after 12 days of incubation. The optimum % degradation of biofilm was 80.465 ± 7.586% after nine days of incubation. These findings suggest that Streptomyces W-5A has the potency to produce antibacterial and antibiofilm compounds against MRSA 2983.

1 INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an *S. aureus* bacterium resistant to methicillin and several other beta-lactam antibiotics (Boucher & Corey, 2008). MRSA is an infection-causing bacterium that can defend itself by forming a protective layer called a biofilm. S. aureus biofilms develop rapidly and form colonies on moist and nutrient-rich surfaces (Tarver, 2009). The ability to form biofilms is one of S. aureus' virulence factors, which causes antibiotic resistance to bacteria (Høiby et al., 2010; Lee et al., 2013).

Infection by microbes is estimated to be 80% related to the formation of biofilms, which contribute to the nature of antibiotic resistance (Archer et al., 2011). Biofilms consist of microbial cells and a matrix of extracellular polymeric substance (EPS). As much as 50-90% of the EPS matrix's main ingredients are organic carbon consisting of polysaccharides, proteins, nucleic acids, lipids, phospholipids, and humic substances, and 15% are bacterial cells (Deshpande & Joshi, 2011). These biofilms cause antibiotics cannot reach the target, bacterial cells; hence the bacteria that cause infection cannot be inhibited or destroyed using antibiotics. Therefore, exploration of antibiofilm compounds becomes necessary. The antibiofilm compounds are expected to inhibit biofilm formation and degrade biofilms (Konai & Haldar, 2017). The inhibitory activity of biofilms occurs because these compounds have antibacterial activity, while biofilms' degradation activity occurs because they can depolymerize complex compounds in the biofilm matrix.

Actinobacteria, particularly the genus Streptomyces, has been reported to produce bioactive compounds with anti-MRSA activity. Streptomyces albus, granaticin B from *Streptomyces violaceoruber*, and streptorubin B from Streptomyces sp. MC11204 has been reported to inhibit biofilm formation and

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damage the *S. aureus* biofilm (Oja et al., 2015; Suzuki et al., 2015). Bhakyashree and Krishnan (2018) reported that Streptomyces sp. Strain VITBKA3 showed anti-MRSA activity. Balasubramanian et al. (2017) also reported that ethyl acetate extract from Streptomyces sp. strain SBT343 could reduce biofilm formation of several Staphylococcal species, including MRSA USA300.

Recently, Asnani et al. (2020) have reported the potency of Streptomyces W-5A as anti-MRSA based qualitative screening. Streptomyces W-5A was isolated from mangrove areas in Segara Anakan Cilacap, a source of indigenous marine actinobacteria (Asnani et al., 2016). Following that report, this research aimed to analyze the potency of W-5A isolates as antibacterial and antibiofilm towards MRSA 2983.

2 MATERIALS AND METHODS

The research was conducted from January to September 2020 in the Biochemistry Laboratory and Research Laboratory in NSOED, Purwokerto. Streptomyces W-5A was isolated from mangrove sediment in Segara Anakan Cilacap. The MRSA 2983 is known as a bacterium capable of producing biofilm matrix. It was isolated from a clinical specimen from the pus of female patient in Prof. Dr Margono Soekarjo Hospital, Banyumas regency, Indonesia.

2.1 Cultivation of Isolate W-5A

Streptomyces W-5A was cultivated using continuous streak on Starch Casein Nitrate (SCN) following the procedure described by Asnani et al. (2016). Starch Casein Nitrate (SCN) agar [starch, casein, KNO_3, KH_2PO_4, $MgSO_4.7H_2O$, NaCl, $FeSO_4.7H_2O$, agar] was added 1 µL nystatin for every 10 mL of SCN medium. The cultures were incubated for seven days at room temperature.

2.2 Production of Anti-MRSA Extract

Production of anti-MRSA extract used SCN broth following the procedure described by Asnani et al. (2020). A total of 10 plugs (6 mm diameter) of Streptomyces W-5A were inoculated into 100 mL of SCN broth. The culture was incubated at 90 rpm and room temperature until it reached the exponential phase for inoculum. Next, 10% of the inoculum was inoculated into a new SCN broth to produce anti-MRSA compounds. The cultures were incubated using an orbital shaker at a speed of 90 rpm. Samples were taken at incubation times of 0, 3, 6, 9, 12, and 15 days. Each sample was separated by centrifugation at 4.000 rpm for 10 minutes at 4°C, then filtered to obtain the crude extract. Each extract was tested for antibacterial activity, inhibition of biofilm formation, and biofilm degradation.

2.3 Antibacterial Test

The extract's antibacterial activity was evaluated using the disc paper diffusion method on the Mueller Hinton Agar (MHA) medium following CLSI (2019). A total of 30 µL of the extract was added to disc paper (6 mm), then placed on the MHA medium that had been inoculated by MRSA 2984 using the spread plate method. The test culture was incubated at 37°C for 24 hours. A clear zone around the disc paper indicated a positive result of antibacterial activity against MRSA. Hence, the parameter observed was the diameter of the inhibition zone.

2.4 Biofilm Formation Inhibition Test

The potency of the extract to inhibit biofilm formation was tested using the microtiter plate method described by Suzuki et al. (2015) with modification. The MRSA 2983 was inoculated in Brain Heart Infusion (BHI) medium with 1% glucose (BHI-Glu) and incubated at 37°C for 24 hours. Then, the culture was adjusted for its turbidity level using a 0.5 McFarland standard and diluted in BHI-Glu with a ratio of 1:100. The inhibition of biofilm formation was carried out by adding a total of 10 µL of diluted MRSA and 100 µL of extract to the microplate and incubated at 37°C for 24 hours. After incubation, planktonic cells were removed carefully, washed twice with PBS, stained with 0.1% crystal violet solution, and incubated for 30 minutes. Then, the microplate was washed with water to remove excess crystal violet and dried. HCl in isopropanol (1:20) was then added, and the optical density (OD) value was measured using a microplate reader at a wavelength of 595 nm. The percentage of biofilm formation inhibition was calculated using the following formula (Pratiwi et al., 2015).

$$\%\text{Inhibition} = \frac{OD_{control} - OD_{test}}{OD_{control}} \times 100\% \quad (1)$$

2.5 Biofilm Degradation Test

The potency of extract to degrade biofilms was tested using the microtiter plate method described by Suzuki

et al. (2015) with modification. The MRSA 2983 was prepared as the previous procedure. The biofilm degradation test was carried out by inoculating diluted MRSA in each microplate well and incubated at 37°C 24 hours. After incubation, planktonic MRSA cells were carefully removed, then the extract was added into each well. The mixtures were incubated at 37°C for 24 hours, exposing the extract to biofilm formation at the bottom of the well. After incubation, the microplate was treated similarly to the previous procedure, and the OD value was measured at 595 nm. The percentage of biofilm degradation was be calculated using the following formula (Pratiwi et al., 2015).

$$\%\text{Degradation} = \frac{OD_{control} - OD_{test}}{OD_{cpntrol}} \times 100\% \qquad (2)$$

2.6 Data Analysis

The research data were the diameter of the inhibition zone, %inhibition, and %degradation. All data were analyzed using one-factor analysis of variance (ANOVA) at a 95% confidence level. If the ANOVA analysis results have a significant effect, it continued with the Duncan test.

3 RESULTS

3.1 Antibacterial Activity

The extract was tested for antibacterial activity using the paper disc diffusion method. This method is expected to determine the sensitivity of a microbe to various antibiotics. The experimental parameters were the presence or absence of a clear zone formed around the disc paper, which showed MRSA's growth inhibition. The research result indicated that the extract has antibacterial activity (Figure 1). The antibacterial activity of Streptomyces W-5A's extract increased from day 0 and reached the highest activity on day 9th with the diameter of the inhibition zone was 9.589 ± 0.521 mm. The zone of inhibition showed potent inhibition of extract against MRSA 298.

The one-way ANOVA test analysis results showed a significant effect (Sig. <0.05) between the incubation time and the inhibition zone diameter (Table 1). Further tests using the Duncan test showed that the 9-day incubation time had an antibacterial activity significantly different from other incubation times. This finding indicated that the optimum incubation time for producing antibacterial compounds was nine days.

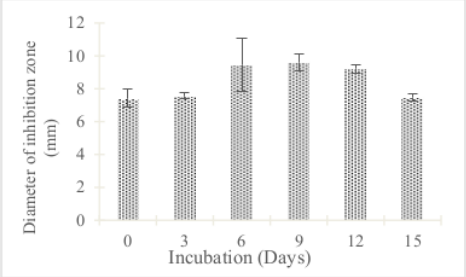

Figure 1: Antibacterial activity of Streptomyces W-5A extract against MRSA 3983.

3.2 Inhibition of Biofilm Formation

The inhibition of biofilm formation aimed to determine the potency of extract to inhibit MRSA biofilms' formation. Compounds with antibacterial activity against microorganisms that form biofilms can be used to inhibit biofilm formation (Memariani et al., 2019). The tests used a microplate and BHI medium with glucose. BHI medium is often used to grow Gram-positive and Gram-negative bacteria, whereas glucose to the BHI medium aimed to induce MRSA biofilms formation (You et al., 2014).

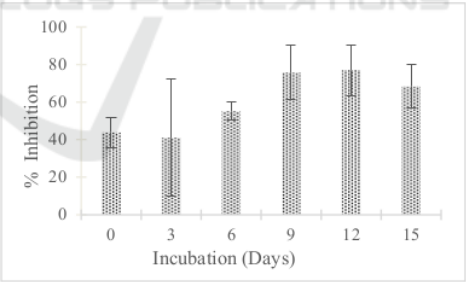

Figure 2: The inhibition formation of biofilm MRSA 2983 by Streptomyces W-5A extract.

The research result is presented in Figure 2. The extract inhibited the formation of biofilm MRSA 2983. The percentage inhibition of biofilm formation by the extract increased from 0 days until it reached the optimum incubation time, 12 days, with a per cent inhibition of 77.806% ± 13.595%.

Table 1: Results of one-way ANOVA for antibacterial and antibiofilm activities

	Sum of Squares	df	Mean Square	F	*Sig.
(1) Antibacterial Activity					
Between Groups	17.209	5	3.442	6.307	0.004
Within Groups	6.549	12	0.546		
Total	23.758	17			
(2) Inhibition of biofilm formation					
Between Groups	3766.922	5	753.384	2.856	0.630
Within Groups	3165.493	12	263.791		
Total	6932.415	17			
(3) Biofilm degradation					
Between Groups	4655.138	5	931.028	3.084	0.051
Within Groups	3622.848	12	301.904		
Total	8277.986	17			
*Significant value = 0.05					

After it reached the optimum incubation time, the rate of inhibition of biofilm formation decreased.

The analysis results with the one-way ANOVA test showed no significant effect (Sig. <0.05) between the incubation time treatment on the percentage of inhibition of biofilm formation (Table 1). However, 50% inhibition of biofilm formation was achieved after six days of incubation and reached a maximum of 12 days of incubation.

3.3 Biofilm Degradation

The research result indicated that the extract could degrade the biofilm of MRSA 2983 (Figure 3). The percentage of biofilm degradation increased from day 0 until it reached the optimum incubation time, which was the 9th day, with the percentage of biofilm degradation was 80.465% ± 7.586%. After it reached the optimum incubation time, the rate of biofilm degradation decreased.

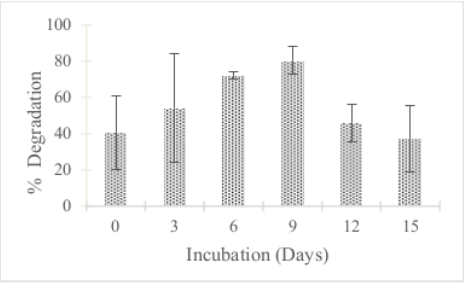

Figure 3: The degradation of biofilm MRSA 2983 by Streptomyces W-5A extract.

The analysis results using the one-way ANOVA test showed no significant effect (Sig. <0.05) between the incubation time treatment on the percentage of biofilm degradation (Table 1). However, 50% of biofilm degradation was achieved after three days incubation and reached a maximum at nine days incubation.

The tests used the microplate method with BHI-Glu medium. The clinical MRSA 2983 was added to a well-filled medium, then incubated for biofilm formation. After incubation, planktonic cells formed on the surface, while biofilms formed and stuck tightly to the well-walls. This adhesion was accompanied by a build-up of organic materials covered by an extracellular polymer matrix. This matrix was a structure of threads crossed with each other and acted as an adhesive for the biofilm. The planktonic cells formed were removed by washing, then the Streptomyces W-5A extract was added to the well. After incubation, all wells were washed to remove planktonic bacteria and added crystal violet to colour the biofilm. Besides using crystal violet, biofilm quantification can also use resazurin, safranin, and tryptan blue dyes (Peeters et al., 2008; Sandasi et al., 2010). In this research, crystal violet dye was used because it was easy to obtain and economical in price.

4 DISCUSSION

Actinobacteria have been reported to have antibacterial activity against MRSA. Bister et al. (2004) wrote that Abyssomicin C obtained from actinobacteria has potential as anti-MRSA. León et al. (2011) reported that the dichloromethane extract from actinobacteria isolates (I-400A, B1-T61, M10-77) have high antibacterial activity against MRSA ATCC 43300 and VRE ATCC 51299. Rajan & Kannabiran (2014) also reported 2,4-dichloro-5-sulfamoyl benzoic acid (DSBA) extracted from marine

Streptomyces sp. VITBRK2 has anti-MRSA activity. Furthermore, Bhakyashree & Krishnan (2018) wrote that Streptomyces sp. VITBKA3 is potential as an anti-MRSA compound against MRSA strains ATCC43300 and ATCC700699.

Isolate at a particular incubation time that produces the highest inhibition zone is considered the optimum time for making antibacterial compounds. The duration of the production phase of each microbe varies depending on genetic factors and environmental conditions. Susilowati et al. (2007) reported that actinobacteria produced optimal antibacterial compounds at the optimum incubation time of 72 hours (isolate A3.5) and 96 hours (isolate F6.1) against Enteropathogen bacteria. Dhananjeyan et al. (2012) used an incubation time of five days to produce antibacterial compounds from actinobacteria against *Escherichia coli* MTCC 50, *Pseudomonas aeruginosa* MTCC 424, and *Bacillus subtilis* MTCC 441. Bhakyashree & Krishnan (2018) used an incubation time of seven days to produce antibacterial compounds from actinobacteria against MRSA strains ATCC 43300 and ATCC 700699.

The clinical isolate MRSA 2983 is known as a biofilm producer. This research used an initial MRSA concentration of 1.5 × 108 CFU/mL (0.5 McFarland standard). According to Skogman et al. (2016), biofilms can be formed with an initial bacterial concentration of 106-108 CFU/mL. MRSA was added to the well containing the medium, and then the extract was added so that the biofilm growth would coincide with the presence of extract. If the extract has inhibitory activity, there will be inhibition of MRSA biofilm formation.

After incubation, the well was washed to remove planktonic bacteria, then stained with crystal violet. Crystal violet can bind to proteins and polysaccharides in the bacterial extracellular matrix (Peeters et al., 2008). In this research, if, after staining with crystal violet, a purple colour was formed, the amount of binding dye was assumed to be the same as the number of biofilm matrices in the well (O'Toole, 2011).

Microbes capable of producing biofilms generally have the potential for resistance to antibiotics. Bacteria in the biofilm can withstand antibiotics because the antibiotics fail to penetrate to destroy the biofilm. Biofilm degradation test is known as an approach to dealing with biofilms by adding bioactive compound that can trigger biofilm destruction (Belbase et al., 2017; Bjarnsholt et al., 2013; Schierle et al., 2009). Thus, the biofilm degradation test aims to determine the potency of extract to degrade biofilm MRSA.

Bioactive compounds to inhibit biofilm formation were highly produced at 12 days of incubation in this research, whereas bioactive compounds to degrade biofilm were highly made at nine incubation days. Indeed, the production of anti-MRSA compounds has various optimum incubation time. Oja et al. (2015) reported that *Streptomyces violaceoruber* (DSM-40701) produced antibiofilm compounds against S. aureus with an incubation time of four days. Suzuki et al. (2015) said that Streptomyces sp. strain MC11024 made antibiofilm compounds against *S. aureus* and MRSA N315 with an incubation time of three days. Balasubramanian et al. (2017) reported that Streptomyces sp. SBT343 produces antibiofilm compounds against Staphylococcal bacteria with an incubation time of ten days.

5 CONCLUSIONS

Streptomyces W-5A isolated from Segara Anakan Cilacap indicated the potency as anti-MRSA. To our knowledge, this is the first report of indigenous Streptomyces with three major anti-MRSA activities, including antibacterial, biofilm formation inhibition, and biofilm degradation. This finding highlights Segara Anakan Cilacap as an essential source of indigenous microbe for pharmaceutical purposes. Further research is necessary, particularly for the isolation and characterization of bioactive compounds.

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