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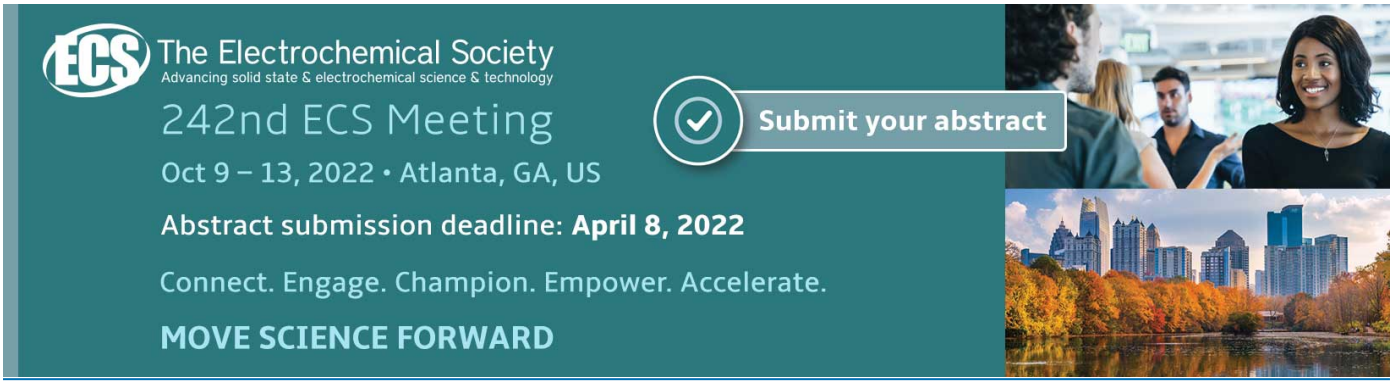

Bioactive compounds derived from *Streptomyces* sp. SA32: antibacterial activity, chemical profile, and their related genes

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Abstract. Microbes which are resistant to drugs and antibiotics as well as multi-drug resistant (MDR) microbes have developed due to the improper use of antibiotics and led to explore the microbial isolates as the sources of new antibiotics or those with highly effective and stabile attack. *Streptomyces* sp. SA32 was selected to inhibit the growth of MDR bacteria *Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa, Enterobacter cloaceae,* and *Enterococcus* sp. *E. coli* was sensitive to crude extracts of *Streptomyces* sp. SA32 at the concentration of 19 g.mL-1 with moderate strength against *E. cloacae*. The bioactive compounds analyzed using thin layer chromatography and phytochemical methods showed that the spot with Rf 0.63 and 0.68 was polyketide compound and that with Rf 0.74 was flavonoid compound. The bioautography assay on the TLC plate confirmed the absence of MDR bacterial growth on polyketide and flavonoid spots. The synthesis of antibacterial compounds was also confirmed by the successful analysis on both non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) gene sequences.
Keywords: anti bacteria, MDR bacteria, NRPS gene, PKS gene, *Streptomyces* sp. SA32.

1. Introduction

Improper use of antibiotics leads to the development of MDR microbes. According to the European Center for Disease Control (ECDC) and Center for Disease Control and Prevention (CDC), Atlanta, MDR properties are given to microbes resistant to at least one agent in three or more antimicrobial categories [1]. Exploration to the new types of antibiotics as a strategy to control MDR pathogens is needed [2, 3].

The new compounds for therapeutic applications can be explored from the natural sources, including actinomycetes. New types of antibiotics are greatly required because they have a specific mode of action, effective attack, and high stability. Actinomycetes are filamentous Gram-positive bacteria, which have hyphal structures, and are known as the most important microorganism in producing secondary metabolites, including antibiotics [4, 5]. These antibiotics are generally produced by Streptomyces and Micromonospora [5]. Streptomyces is a producer of 80% natural products which ability to synthesize secondary metabolites is unrivaled [6].

Streptomyces sp. SA32 has been isolated from mangrove rhizosphere mud which is capable of inhibiting the MDR bacterial growth including *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Enterobacter cloaceae*, and *Enterococcus* sp. [7]. Bioactive compounds were analyzed using a thin layer chromatography (TLC) method with chloroform: ethyl acetate : acetic acid (5:3:1) as eluent resulted in three spots with Rf values of 0.76, 0.8, and 0.9 indicating the presence of different metabolites. It was not known what compounds contained in the crude extract are able to inhibit the growth of MDR bacteria and the minimum concentration to inhibit the growth of MDR bacteria using a diffusion method.

The inhibition assay using a diffusion method can be influenced by an active compound type. To avoid the repeating results, a polymerase chain reaction (PCR) method was required to determine the genes encoding the synthesis of antibacterial bioactive compounds, consisting of non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) genes. The presence of NRPS and PKS genes indicated that the isolates are able to synthesize the secondary metabolites [8]. Marine microbes produce metabolites mainly through metabolic pathways type I polyketide synthases (PKS-I), type II polyketide synthases (PKS-II), NRPS and PKS-NRPS [9]. Several isolates of marine actinomycetes isolated from corals exhibited antimicrobial properties and possessed biosynthetic genes NRPS and PKS [10].

Based on the descriptions above, the purpose of this study was to determine the class of compounds produced by *Streptomyces* sp. SA32, to observe the minimum inhibitory concentration using a diffusion method, and to identify the profiles of NRPS and PKS genes related to genes encoding the bioactive compounds.

2. Materials and methods

2.1. Production of antibacterial compounds

The procedure was based on [7], in which the inoculum was prepared from the *Streptomyces* sp. SA32 culture on starch casein nitrate agar (SCNA, composition: 10g soluble starch, 1g casein hydrolysate, 1g KNO_3, 0.5g $KHPO_4$, 0.5g $Mg_2SO_4.7H_20$, 0.5g NaCl, 0.01g $FeSO_4.7H_20$, 20g agar, and distilled water up to 1000mL), incubated for 7 days, then 5 plugs were inoculated (cork drill size 5 mm) for every 150 mL of starch nitrate broth (SNB, composition: as SCNA without casein hydrolysate and agar) and starch casein nitrate broth (SCNB, composition: as SCNA without agar) media. Fermentation was performed at the temperature of 28±2^{o}C for 21 days with the agitation at 120 rpm. Then, the mycelium biomass was separated from the medium by filtration using filter paper of Whatman number 1. The filtrate was extracted using ethyl acetate in a ratio of 1:1 (v/v), and then shaken for 1 hour. The ethyl acetate phase was evaporated at the temperature of 70^{o}C using a rotary evaporator to obtain crude extract. The extract obtained was weighed and used to determine its antibacterial activity through a disc diffusion method.

2.2. Antibacterial assay of crude extract

The procedure was based on [7], in which MDR bacteria of *Escherichia coli* (EC), *Staphylococcus aureus* (SA), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA), *Enterococcus* sp. (ETC), *Enterobacter cloacae* (ETB), obtained from Kariadi Hospital, Semarang was prepared on Mueller Hinton Agar (MHA) medium using a pour plate method, in which 15 mL of melting MHA medium was inoculated with 1 mL (10^6 cell.mL^{-1}) of MDR bacterial broth culture, homogenized, and then poured into sterile petri dishes. The culture was allowed to solidify and dry. A 6 mm diameter paper disc was placed on the bacterial lawn surface, slightly pressed and tended to adhere perfectly, then 30 µL of crude extract was added. The culture was then incubated at the temperature of 37°C for 24 hours. Each assay was repeated twice. Observation on the formation of a clear zone around the disc was conducted since 6 hours of incubation. The diameter of the formed inhibition zone was measured and the average diameter was then calculated. *E. coli* and *S. aureus* bacteria were used as control.

Observation on the lowest concentration of crude extract inhibiting the growth of MDR bacteria was conducted using the diffusion method (7, modified). The extract was diluted using distilled water in the

dilution series of 50%, 25%, 12.5%, 6.25%, 3.125%, and 1.625%. The assay was conducted by pouring MDR bacteria on NA medium and the extract was dripped into 6 mm paper discs. The test cultures were incubated at the temperature of 37℃ for 24 hours. Observation on inhibition was performed for 6 to 24 incubation hours. The formed inhibition zone diameter was measured and the average diameter was then calculated.

2.3. Identification of MDR antibacterial compounds

The characteristics of antibacterial compounds were analyzed using the thin layer chromatography (TLC) and phytochemical methods. TLC was performed using the stationary phase TLC aluminum sheet silica gel of 60 F254 and the mobile phase methanol: ethyl acetate: distilled water (7:3:1). The stationary phase was previously activated by heating in an oven at the temperature of 100℃ for 15 minutes. The mixture of mobile phase composition was filled into the chamber and then tightly closed to reach the mobile phase saturated inside the chamber.

Extract was spotted 3 µL on a silica gel plate, then immersed in a chamber saturated by the mobile phase. After the eluted extract reached the end point, the plate was removed from the chamber and air-dried. The eluted spots were detected with UV light of 366 nm in a UV cabinet. The spots showing certain colors were marked with a pencil and then the Rf value was measured by measuring the distance of the formed spot divided by the distance traveled by the solvent.

Identification of polyketide compound was performed by observing the spots on UV light at λ254 nm which appeared not shining but showing a certain color at λ 366 nm. Identification of alkaloid compound by spraying the spots with Dragendroff reagent, heated in an oven at the temperature of 100℃ for 5 minutes, then yellow orange or orange spot was formed. Identification of terpenoid compound, by spraying the spot with vanillin-sulfuric acid reagent, heated in an oven at the temperature of 100℃ for 10 minutes, then produced blackish purple spot. Identification of the flavonoid compound by spraying the spot with citroborate reagent, heated in an oven at the temperature of 100℃ for 5 minutes, then a yellow spot was formed and seen under UV light of λ 366 nm

2.4. Bioautography assay

A liquid culture of MDR bacteria was prepared in 10 mL NB medium, added with 0.1 mL of 2,3,5-*triphenyltetrazolium chloride* (TTC), and then dripped into a TLC plate containing bioactive spots. The TLC plate was incubated at the temperature of 37°C for 24 hours. After incubation, the plates were observed for the growth inhibition zone of MDR bacteria or where there was no red color in the spot. Bacterial growth was indicated by the red color of bacterial colonies**.**

2.5. Identification of NRPS and PKS genes sequence

Identification of NRPS and PKS gene sequences begins with DNA isolation [11] and amplification by PCR method [12] with several modifications in the working stages. DNA was isolated from 3-day-old vegetative cell pellets in SCNB medium. The isolated DNA was visualized by 1% agarose gel electrophoresis.

Amplification of NRPS gene with a total volume of 25 µL used the primers of A3F (GCSTACSYSATSTACACSTCSGG) and A7R (STACCGSACSGGBGACSTS) with the target amplicon size of 700 bp. PKS gene amplification used the primers of K1F (TSAAGTCSAACATCGGBCA) and M6R (TACTGGTAC SGSAACCTGCG) with the target amplicon size of 1200-1400 bp based on [13]. The PCR composition (volume and concentration) consisting of DNA template, primer, NZY taq II 2x Green Master Mix, and dd H_2O was prepared as shown in table 1. The amplification results were visualized using agarose gel 1%.

2.6. Data analysis

The data of antibacterial compounds and bioautographic were descriptively analyzed by comparing the Rf spot values and the results of bioautographic test. The analysis of NRPS and PKS gene sequence data

was conducted using the BioEdit application and then continued using the AntiSmash platform version 5.0.

Table 1. Composition of PCR mix and PCR condition for the amplification of NRPS and PKS genes (volume 25 µL).

Composition and stages of amplification	Volume/concentration and amplification conditions	
	NRPS Gene	PKS Gene
dd H_2O	9.5 µL	9.5 µL
Master Mix	12.5 µL	12.5 µL
Primer Forward	1 µL (0.01 µM)	1 µL (0.016 µM)
Primer Reverse	1 µL (0.01 µM)	1 µL (0.016 µM)
DNA Template	1 µL (10 ng.µL^{-1})	1 µL (10 ng.µL^{-1})
PCR condition		
- Pre-Denaturation	95°C; 2’	95°C; 2’
- Denaturation	95°C; 30”	95°C; 30”
- *Annealing*	57°C; 30”	57°C; 30”
- Extention	72°C; 1’30”	72°C; 1’30”
- Cyclus	40 cyclus	40 cyclus
- Post extention	72°C; 5’	72°C; 5’
- Storage	8°C; 3’	8°C; 3’

3. Results

3.1. The inhibition of Streptomyces sp. SA32 crude extract against MDR bacteria

The results of antibacterial test on crude extract against MDR bacteria by diffusion method showed that the extract was able to inhibit all MDR bacteria. The inhibition zone diameter yielded by 100% extract obtained from SNB medium of EC: 8 mm, SA: 12.5 mm, KP: 10 mm, PA: 10.5 mm, ETC: 12.5 mm, and ETB: 0 mm (figure 1). The presence of an inhibition zone indicated that the *Streptomyces* sp. SA32 produced the bioactive compounds with antibacterial properties.

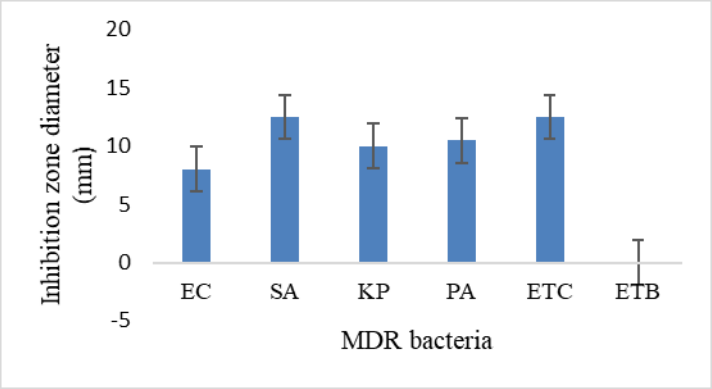

Figure 1. The inhibition zone diameter produced by *Streptomyces* sp. SA32 crude extract.

The antibacterial testing of crude extract with different dilutions (50%, 25%, 12.5%, 6.25%, 3.125%, and 1.625%) in solid culture showed that the inhibition was influenced by the concentration of extract and incubation time (figure 2.). Inhibition can be observed since the incubation period was 6 hours. The results of observations for 6-24 hours showed the stability of inhibitory ability. Inhibition stability against EC, ETB, and SA was indicated by a clear zone and a small decrease of inhibition zone diameter.

The crude extract sample at 50% dilution had a concentration of 19 µg. mL^{-1}. The positive control used was chloramphenicol with a concentration of > 30 µg/mL, while the negative control was sterile distilled water. Chloramphenicol with those concentrations inhibited *E. coli* and other MDR bacterial growth. In the resistance test, chloramphenicol (concentration of 10 µg. mL^{-1}) did not inhibit *E. coli*,

Enterococcus sp., and *P. aeruginosa*. The inhibition made by chloramphenicol resulted in a high diameter of inhibition zone (15-30 mm), while the distilled water did not show an inhibition zone. The inhibition zone produced by crude extract with various dilutions (50% to 1.625%) showed a lower ability than that produced by chloramphenicol. Based on [14], the inhibition produced by the *Streptomyces* sp. SA32 crude extract belonged to the strong criteria since having a diameter of 10-20 mm. Inhibition is classified into very strong when the diameter of inhibition zone is > 20 mm, classified into medium when the diameter of inhibition zone is 5-10 mm, and shows no response when the diameter of inhibition zone is 0-5 mm. The lowest concentration of SA32 crude extract which still inhibited the growth of MDR bacteria was 6.25% (concentration 2.375 µg. mL^{-1}).

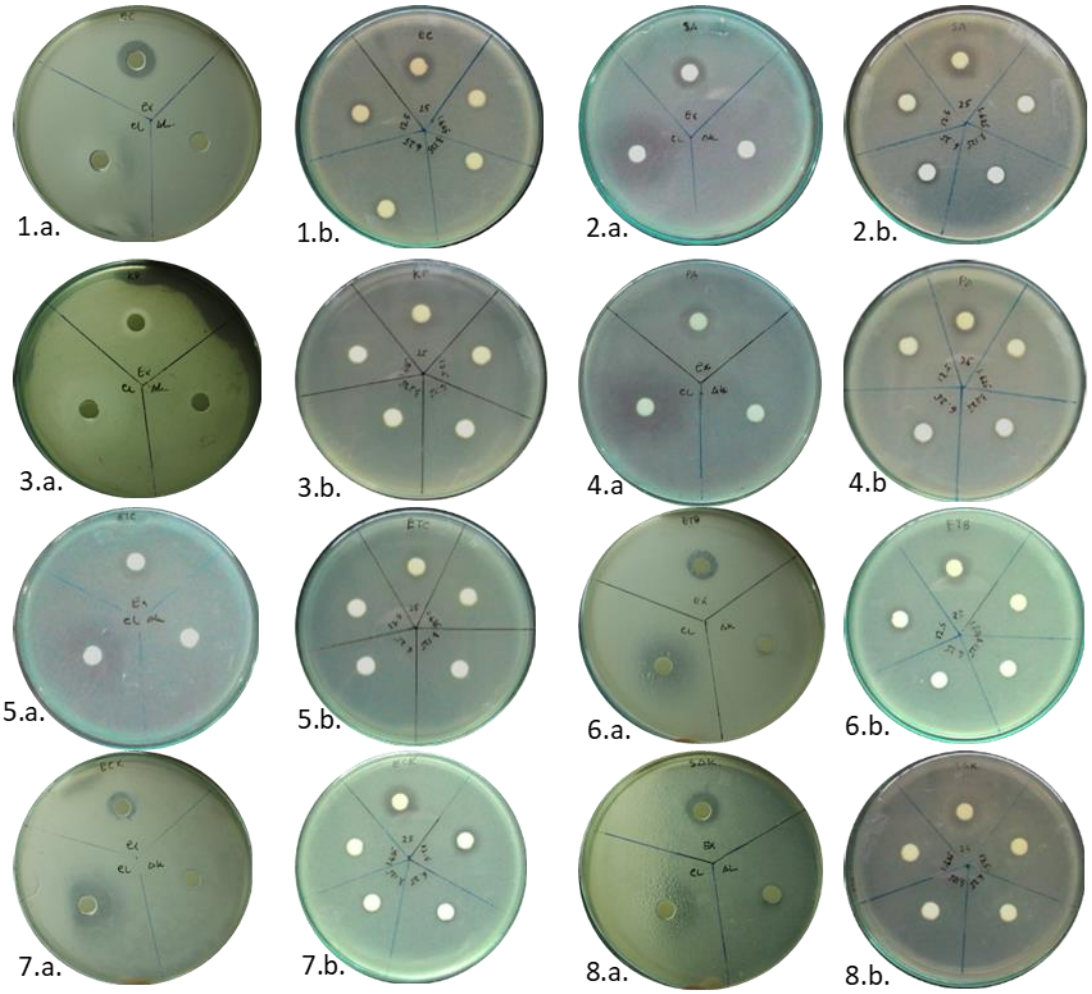

Figure 2. Inhibition zone of bacterial growth by Streptomyces sp. SA32 crude extract. Detail: (a) extract dilution 50%, chloramphenicol as positive control and distilled water as negative control; (b) extract dilution 25%, 12.5%, 6.25%, 3.125%, and 1.625%; (1) *E. coli*; (2) *S. aureus*; (3) *K. pneumonia*; (4) *P. aeruginosa*; (5) *Enterococcus* sp.; (6) *E. cloacae*; (7) *E. coli* control; (8) *S. aureus* control.

3.2. Characterization of antibacterial compounds produced by Streptomyces sp. SA32

Antibacterial bioactive compounds were obtained through extraction using ethyl acetate as a solvent. Ethyl acetate was also the best solvent in extracting antibacterial compounds produced by the isolate SVG-07-15 in inhibiting the growth of *Staphylococcus aureus* [15]. Based on the results of TLC and phytochemical analysis, it was found that the *Streptomyces* sp. SA32 crude extract contained 7 polyketide and flavonoid compounds.

The eluent methanol, chloroform, and water produced 3 spots of polyketide compounds with Rf values of respectively 0.63, 0.68, and 0.74. Meanwhile, the other eluent composition (ethyl acetate : methanol 9:1) produced 4 polyketide compounds with Rf values of respectively 0.55, 0.64, 0.66, and 0.81. Many polyketides or polyketide-like antibacterial compounds are produced by actinomycetes, particularly Streptomyces [16].

3.3. The antibacterial compounds assay

The results of bioautography assay showed that the bioactive compound spots showed no bacterial growth. Bacterial growth was indicated by the presence of fine red bacterial colonies. The results of bioautography test confirmed that the polyketide and flavonoid compounds contained in the bioactive compounds of *Streptomyces* sp. SA32 were MDR anti bacteria.

3.4. The amplification of NRPS and PKS genes sequence

The isolation of *Streptomyces* sp. SA32 DNA used Zymo Research Quick DNATM Fungal/Bacteria Minirep Kit. The result showed a good DNA quality, which was clearly visualized, thick, single band on agarose gel and has a size of 10,000 bp. The DNA concentration of 171 ng. μL^{-1} and the level of DNA purity showed good results (A260/A280: 1.893 and A260/A230: 1.516).

The amplification of NRPS and PKS genes produced amplicons with band sizes in accordance with Ayudo-Sacido & Genilloud [13], 700 bp and 1200 bp. Based on the analysis of NRPS gene sequence, *Streptomyces* sp. SA32 had a 90.70% similarity to *Streptomyces koyangensis* strain of VK-A60T with access number of CP031742.1. The results of BGC analysis, *S. koyangensis* strain of VK-A60T has one region (region 4) of NRP-polyketide involved in the synthesis of secondary metabolites.

The results of PKS gene analysis using the AntiSmash program showed that *Streptomyces* sp. SA32 had PKS gene with a similarity to *Nocardia asteroides* strain of NCTC11293 and *Streptomyces dengpaensis* strain of XZHG99 (87%) with the access numbers of LR134352.1 and CP026652.1. The region 6 of PKS gene *Nocardia asteroides* strain of NCTC11293 was involved in the synthesis of antibiotic nocobactin NA, the nrps-t1pks gene with the similarity of 87%. Meanwhile, region 10 of *S. dengpaensis* strain of XZHG99 contained the similarity of 27% to *Streptomyces* sp. SA32 in synthesizing the RK-682 compound.

4. Discussion

The bioactive compound of *Streptomyces* sp. SA32 crude extract contained polyketide and flavonoid compounds which possibly inhibit the growth of MDR bacteria. The inhibition ability was indicated by the presence of a clear zone around the disc in the diffusion test and the presence of an area with no bacterial growth in the TLC spot in the bioautography assay. The diameter of clear zone appeared slightly different between the test bacteria since the lower the extract concentration, the smaller the diameter is performed. The lowest extract concentration still giving the inhibition was 6.25% or equivalent to the crude extract concentration of 2.375 g. mL^{-1}. The diameter of the widest inhibition zone was indicated by the crude extract with a dilution of 50% (concentration of 19 µg. mL^{-1}, volume of 20 µL), which was 14 mm with a very clear inhibition zone and a clear edge of zone since the incubation time was 6 hours. At the same incubation time, the inhibition zone produced by the positive control was not clearly visible, but the resulting inhibition zone was larger than that produced by the sample's crude extract due to the higher concentration of chloramphenicol (> 30 µg. mL^{-1}) used than the concentration of the sample's crude extract. The clear effect produced by the crude extract showed that the active compound contained in the extract worked quickly and effectively in killing the test bacteria.

The inhibition zone formed a decreasing diameter with a longer incubation time, yet a clear zone remained until the incubation period reached > 96 hours, especially against MDR bacteria of *E. coli, S. aureus*, and *P. aeruginosa*. These results indicated that the active compound of *Streptomyces* sp. SA32 consistently inhibits the growth of MDR bacteria. Antibacterial activity resulted by the diffusion method can be influenced by several factors, including test medium, number of bacteria, concentration of

antibacterial compounds, and incubation time [17]. Incubation time provides time for the test bacteria to grow more, which can cause the antibacterial effect invisible. The antibacterial compounds also showed a broad spectrum based on its ability to inhibit Gram-positive and Gram-negative MDR bacteria.

According to the European Committee on Antimicrobial Susceptibility Testing [18], microbes are sensitive when inhibited at MIC less than 16 µg. mL^{-1}, intermediate when inhibited at MIC 16 – 128 µg. mL^{-1}, and resistant when inhibited at MIC more than 128 µg. mL^{-1}. Referring to [19], *E. coli* was sensitive to crude extract of *Streptomyces* sp. SA32 with a crude extract concentration of 19 ug. mL^{-1}, and medium strength effect for *E. cloacae*.

Polyketide compound is a secondary metabolite compound including antibiotics, antifungals, antitumors, and anticholesterol. Some polyketide antibiotics are classified into macrolide antibiotics (erythromycin, pycromycin), polyenes (amphotericin), and tetracyclines. Polyketide compounds are non-ribosomal peptides formed by PKS and NRPS [20].

Flavonoids are a group of compounds with 15 carbon atoms (C6-C3-C6 carbon framework) commonly found in plants and fungi. These natural products can be divided into 3 classes consisting of flavonoids (2-phenylbenzopyrans), isoflavonoids (3-benzopyrans), and neoflavonoids (4-benzopyrans) [21]. *Streptomyces* sp. BT01, an endophyte in *Boesenbergia rotunda*, produces several antibacterial flavonoid compounds [22]. Flavonoids are known as antibacterial agents against various pathogenic microorganisms. With the increasing prevalence of untreatable infections caused by antibiotic-resistant bacteria, flavonoids have the potential to substitute antibiotics. The activities of flavonoids are (a) as direct anti bacteria, (b) in synergy with antibiotics, and (c) to suppress the bacterial virulence factors in various in vitro studies and a limited number of in vivo studies. The antibacterial mechanisms of flavonoids are the inhibition of nucleic acid synthesis, cytoplasmic membrane function, energy metabolism, attachment and biofilm formation, porins in cell membranes, changes in membrane permeability, and attenuation of pathogenicity [23]. Flavonoids have the antibacterial activity against Gram-positive and Gram-negative bacteria [24]. Flavonoids are added to the list of natural compounds found active against antibiotic-resistant bacteria.

The results of analysis on NRPS and PKS gene sequences supported those of phytochemical and bioautographic tests confirming that the isolates produced secondary metabolites of polyketide and peptide groups inhibiting the MDR bacteria. The results of analysis on bioactive compounds, based on the NRPS gene, showed that there was similarity of 100% with the natural compound of SGR PTM produced by *Streptomyces koyangensis* strain of VK-A60T, similarity of 85% with the frontalamide compound. Polycyclic tetramate macrolactam (PTM) is a natural antibiotic compound. Frontalamide is a polycyclic tetramate macrolactam antibiotic including dihydromaltophyllin, maltophyllin, cylindramide, ikarugamycin, alteramide and discodermide [25]. The presence of a single type 2 polyketide synthase biosynthetic gene cluster in the *S. formicae* genome produced MRSA and VRE inhibitory polyketide compounds [26]. Along with the antibacterial activity, the presence of PKS gene can serve as an efficient screening method for extracting the pharmaceutical strains and compounds [15]. The confirmation of SCA-1isolate of PKS gene inhibiting *Enterococcus* sp. showed a complete resemblance to *Streptococcus felleus* and was comparable to its proximity to *S. vietnamensis*, *S. blastmyceticus*, *S. hygroscopicus* and *Micromonospora* as the producers of polyketide antibiotics [27].

Based on PKS gene, *Streptomyces* sp. SA32 produced the antibacterial compound of nocobactin NA produced by *Nocardia asteroides* strain of NCTC11293 and *Streptomyces dengpaensis* strain of XZHG99. Nocobactin NA is a fat-soluble iron-chelating compound with a specific UV absorption spectrum, isolated from *Nocardia asteroides* cultured under iron-deficient conditions. Nocobactin NA resembles the structure of mycobactin Min, except for the presence of an oxazole ring replacing the oxazoline ring, and a short side chain on the cobactin fragment [28]. The siderophore nocobactin NA biosynthetic gene group, the nbt cluster, is a gene with a highly homologous to the mycobactin biosynthetic gene by *Nocardia farcinica* IFM 10152, consisting of 10 separate genes located in two genomic regions. The nbt gene is regulated by the iron-dependent IdeR protein [29].

5. Conclusion

Streptomyces sp. SA32 produces the secondary metabolites of polyketide and flavonoid compounds which possibly inhibit the MDR bacteria, especially *E. coli* and *Enterobacter cloacae*. The ability to produce antibacterial compounds was confirmed by the NRPS and PKS gene sequences. The research has excellent prospects for the development of isolates and products. Thus, several stages of further research are greatly needed to optimize the synthesis of bioactive compounds in obtaining products with high yields, purification and characterization of bioactive compounds equipped with sensitivity and stability tests using test isolates, standardized compound concentrations, and inhibition mechanism. In addition, cluster analysis of NRPS and PKS genes can be developed with various primers and other potential tests.

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