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Screening of *Actinomycetes* that Produce Antibiotics from Rhizosphere of Medicinal Plants in West Lombok , Indonesia as Anti-MRSA (*Methicilin Resistant Staphylococcus aureus*)

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Abstract : Infectious diseases caused by MRSA attract attention worldwide and become a great problem for the hospital clinician as a cause of nosocomial infection and its increased 20 % every year. Therefore, antibiotics that work specifically against MRSA is urgently needed. *Actinomycetes* is one of the most promising sources of antibiotics. This study aims to explore the potential of *Actinomycetes* from rhizosphere Suranadi Forest, Indonesia in inhibiting MRSA. In this study, 20 isolates were isolated from the rhizosphere Suranadi Forest, West Lombok then selected for their anti-MRSA activity. Antibacterial compounds from *Actinomycetes* isolates observed including inhibitory activity against pathogens, Minimal Inhibition Concentration (MIC) and spectrum analysis. VFD4 and LDE5 isolates showed the highest inhibition activity against pathogens. Inhibition zone formed by VFD4 against MRSA, *V. Cholera*, and EPEC sequentially are 38 ± 1 mm, 19.3 mm ± 1.53 and 1.3 ± 0.58 mm, whereas the inhibition zone formed by LDE5 were 10.5 ± 1.73 mm, 16.3 ± 1.53 mm, and 5 ± 1.73 mm respectively. Based on MIC test on five selected isolates, VFD4 and LDE5 showed great inhibitory activity, with 40% in inhibiting MRSA and *V. cholerae*. Spectrum analysis showed that ECM from LDD1, LDE3 and LDE5 have narrow spectrum that is specific to gram positive while LDE2 and VFD4 have broad spectrum. Based on this study, VFD4 and LDE5 can be concluded to have high antimicrobial activity against pathogens. Based on sequence analysis of 16S rDNA, LDE5 isolates identified as *Streptomyces aurantiacus* which is known to produce antibiotics aurantimycin.
Keywords : *Actinomycetes*, Anti-MRSA, Antibiotics,`Antimicrobes.

Introduction

Some antibiotics like methicilin and ampicilin used to be an effective treatment against infectious diseases, but these antibiotics now have become less effective because many bacterias have become resistant to such antibiotics. Resistant strain of *Staphylococcus aureus* that resistant to antibiotics such as vancomycin and methicilin causing an enormous threat to the treatment of serious infectious diseases worlwide[1]. To avoid this from happening, replacement of the existing antibiotic is very necessary immediately. *Actinomycetes* is one of microbial group that is still interestingly explore by researchers until now due to their potency to produce active

substances against various drug resistance and pathogen microbes. Filamentous *Actinomycetes* are well known for their ability in producing a wide variety of secondary metabolites, many of them have been explored in human and veterinary medicine and agriculture as antibiotics, herbicides, anticancer agents and others. Scientists are also exploring *Actinomycetes* from alternate ecological source as antiphytofungal[2]. Among *Actinomycetes*, Streptomyces has long been recognized as a major source of bioactive molecules covers around 70% of the total antibiotic products[3].

Antibiotic is the most important bioactive compounds for treating infectious diseases that have been spread worldwide. Emergencies of multi-drug resistant pathogen have been occured. Thus, due to this issued there has been increasing interest for searching and finding novel antibiotics from soil *Actinomycetes* in unexplored habitat. Scientists tried to investigate more about unexplored habitats for novel *Actinomycetes* as possible candidates of new antimicrobials substances against many resistant strain. *Actinomycetes* can be found in rizosphere. Positive rhizosphere effect and antagonistic effect on pathogens have been revealed[4]. Rizosphere from medicinal plant have many nutritients in it. The tree exudates contains organic compounds that can be used by the microbes in rizosphere to survive from pathogen in the natural environment. The exudate from roots can be different depend on the tree species[5]. Suranadi Forest is one of Forest at West Lombok, Indonesia which is rich of medicinal plants. Based on researchers identification there were 88 kinds of medicinal plant that grow there. *Actinomycetes* that inhabiting rizosphere not widely explore yet. The present research aims to study the potential of *Actinomycetes* from rizosfer of Suranadi Forest, Indonesia in dealing with pathogen MRSA.

Materials And Methods

Sample collection

Soil samples were collected from three different medicinal trees at Suranadi Forest of West Lombok, Indonesia (Lat. 8° 34'0" N ; Long. 116° 13'0" E). The portion of 6-10 cm soil was taken and transferred to sterile bag and transported to the laboratory.

Isolation

The sample that were collected were kept at room temperature. *Actinomycetes* were isolated by performing pre-treated soil sample at 50^{o}C for 15 minutes in waterbath, serially diluted up to 10^{-6} and 1 ml of each diluted samples were placed on starch casein agar medium suplemented with cyclohexamide to retard the growth of fungi respectively. After incubated at 28^{o}C for 8 days, suspected typical *Actinomycetes* colonies observed based on morphological characteristics were chosen, purified and preserved at 4^{o}C for stock. The isolated *Actinomycetes* strains then screened to their potential in producing bioactive compound as antimicrobes[6].

Screening of strains for antagonistic activity

Anti-MRSA activity

Anti-MRSA assay of the isolates was performed using paper disc methods. The supernatan cell free of isolates were tested against MRSA. MRSA were spreaded on starch casein agar plates at the concentration 10^{-7} respectively. When the agar surface dried, each paper disc of 6 mm diameter were dipped into supernatan cell free of test samples. Ampisilin used as positive control. The plates were incubated at 28^{o}C for 24 hours and the inhibition zone that formed around each disc were measured. The assay was repeated three times and the results were described as diameter (in mm) of inhibition zone[7].

Spectrum bioassay

Supernatan cell free of *Actinomycetes* were tested using 2 gram positive bacteria (*S. aureus*, *S. epidermidis*) and 2 gram negative bacteria (*E.coli*, *Pseudomonas aeruginosa*). Spectrum assay performed using paper disc methods. Pathogens were spreaded on Nutrient agar plates at the concentration 10^{-7} respectively. When the agar surface dried, each paper disc of 6 mm diameter were dipped into supernatan cell free of test samples. The plates were incubated at 28^{o}C for 24 hours and the inhibition zone that formed around each disc were measured. The assay was repeated three times and the results were described as diameter (in mm) of inhibition zone.

Minimum Inhibitory Concentration (MIC)

MIC values were observed following modified methods[8] to determine the lowest concentration of extract at which the pathogen test organism can be inhibited. MIC values of the crude extracts were 0%, 20%, 40%, 60%, 80% and 100% in 3 mL test tubes containing Nutrient Broth media. A total of 20 mL of pathogens (MRSA) with a density of 10^7 cfu / mL inserted into each test tube and incubated in a shaker incubator for 24 hours at a temperature of 28 ^oC. After incubation, the MIC value is measured by inoculating 0.1 mL suspension in media Nutrient Agar and counted the cell density values.

Molecular identification

Molecular identification and phylogenetic analysis were assessed for 3 *Actinomycetes* isolates with highest activity against pathogen following modified published method[9]. The isolates were grown for 4 days at 28^o C in SCA medium. Biomass was harvested by centrifuged 3 ose of isolates in double-distilled water at 10.000 rpm for 2 minutes. The pellet was used for DNA extraction following modified Ausuble methods[9]. The pellet was dispersed in 500 ml of TE buffer and 20 mg/ml lysozyme then incubated in 37^o C for 1 hour. Then 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K were added and incubated at 37^{o}C for an hour. After that, 100 µl of % M NaCl and 80 µl of CTAB were added and reincubated at 65^o C for 10 minutes. The lysate was extracted with an equal volume of phenol/Chloroform/Isoamyl alcohol (50—50%, v/v) and centrifuged at 10.000 rpm for 5 min and then the aqueous layer was reextracted with Chloroform/Isoamyl (50—50%, v/v). DNA was presipited using ethanol absolute. The precipitated DNA was cleaned with 500 µl of 70% ethanol, centrifuged at 10.000 rpm for 5 min, and the pellet were resuspended in 50 ml of TE buffer (10 mM Tris—HCl, pH 7.4;1 mM EDTA, pH 8), and stored at 20^o C. The purity of DNA solution was checked spectrophotometrically at 260 and 280 nm, and the quantities of DNA were measured at 260 nm. Polymerase chain reaction then performed using universal primer 27F-5'-GCCTAACACATGCAAGTCGA-3' and 1495R CGTATTACCGCGGCTGCTGG-5'[10]. The amplified 16S rDNA amplicon was sequenced by Macrogen Laboratories and the sequences analysed using BLAST software.

Results And Discussion

Anti-MRSA activity of the extracts obtained from isolated strains

In the previous study primary screening of twenty isolates were done using cell culture and five isolates that have highest activity against MRSA were chosen for secondary screening.

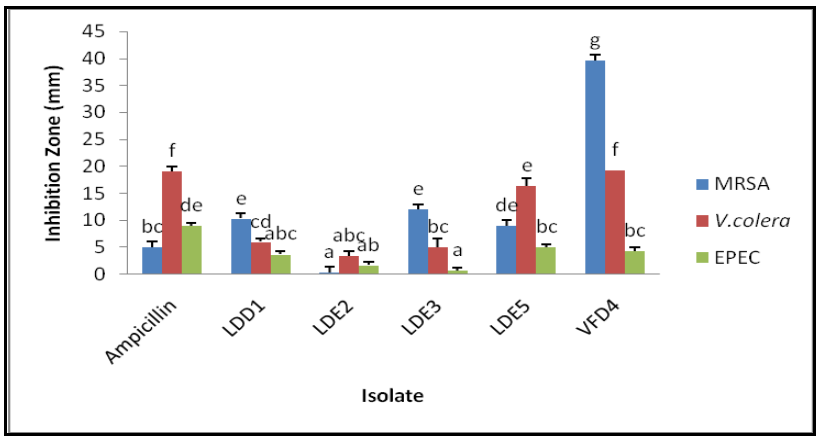

Fig. 1 Antimicrobial Activity of Isolated strains against Pathogen

The test results of Extracellular metabiltes (ECM) selected isolates showed that the five isolates have inhibitory activity against pathogenic bacteria test (MRSA, EPEC, and *Vibrio cholerae*) but with varying levels of inhibition (Figure 1). Each of these isolates showed 'trend' of its own against the pathogen; LDE2 isolates had low activity against the pathogen MRSA, isolates LDE3 have low activity against pathogenic EPEC, while isolates LDD1, LDE5, and VFD4 showed antimicrobial activity against all pathogenic specially VFD4.

Inhibition zone formed by VFD4 isolates of patoghen MRSA, *V. Colera* and EPEC sequentially were 38 ± 1 mm, 19.3 mm ± 1.53 and 1.3 ± 0.58 mm. Inhibition zone by LDD1 sequentially were 10.3 ± 0.58 mm, 6 mm ± 1.0 and 3.7 ± 1.15 mm. Inhibition zone by LDE2 were 0.33 ± 0.58 mm, 3.33 ± 0.58 mm, and 1.67 ± 0.6 mm. Inhibition zone by LDE3 sequentially were 12 ± 1.0 mm, 5 ± 1.0 mm, and 0.67 ± 0.58 mm, whereas the inhibition zone by LDE5 respectively were 10.5 ± 1.73 mm, 16.3 ± 1.53 mm, and 5 ± 1.73 mm. Based on the inhibition test has been conducted on the five isolates, VFD4 showed the highest antimicrobial activity ($p<0.05$) compared to other isolates (Figure 1) against MRSA (38 ± 1 mm) and *V. cholerae* (19.3 ± 1 , 53 mm), while isolates LDE5 was the most sensitive to pathogenic EPEC (5 ± 1.73 mm) compared to other isolates. The antimicrobial activity of LDE 5 equivalent to ampicillin in overcoming pathogenic *V. cholerae*. LDD1 isolates showed their antimicrobial activity against all pathogens test but not significantly different, as well as isolates LDE2. LDE3 isolates showed high activity against MRSA pathogens and showed lower activity against *V. cholerae* and EPEC. Based on the test results is known that the five isolates have the low activity against pathogenic EPEC (*Entero-pathogenic Escherichia coli*) compared to other pathogens. Other research revealed other strain of *Streptomyces* has bioactivity against pathogenic Gram negative bacterial strain, one of them is *Streptomyces* sp MA7[11]. The Bioactivity against pathogens seems differ between strain of *Streptomyces* .

Some research suggests that *Actinomycetes* widely explored as a source of new antibiotics, antimicrobial as well as antifungal. Antimicrobial activity produced by isolates VFD4 (38 ± 1 mm) against MRSA showed a higher yield compared to some of the research that has been done before[12], isolates strain ZZ027 has an activity 29 ± 2.1 mm against MRSA ATCC 33592, while research conducted by Bizuye[1], which examines the soil *Actinomycetes* in Ethiopia had activity 20 ± 1 mm to MRSA2, and it's had inhibition zone formed 17 mm by *Nocardia caishijiensis* which is one of the non-Streptomyces species[13]. This shows that the secondary metabolites produced by VFD4 and LDE5 potential as an antibiotic in dealing with MRSA infections.

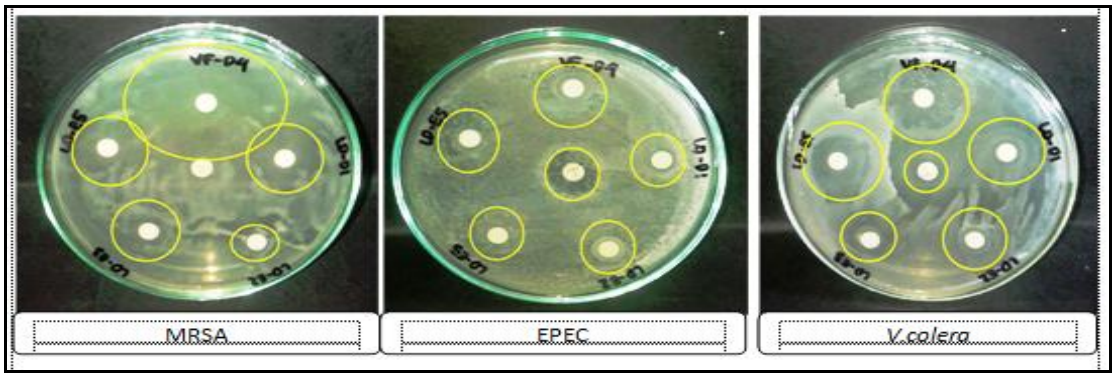

Fig. Zone inhibition formed of Isolated strains against Pathogen

Spectrum test of the extracts obtained from isolated strains.

Spectrum test conducted against Gram-positive bacteria (*S. aureus and S. epidermidis*) and Gram-negative (*E. coli and P. aeruginosa*). Antimicrobial activity by each of the isolates showed a significant difference ($p <0.05$) as shown in Figure 3. Based on the inhibition test, the most promising isolates to have broad-spectrum antimicrobial character are VFD4 and LDE5. Based on the test results can be seen that the five isolates of *Actinomycetes* have high activity in inhibiting Gram positive bacteria and have a low activity against Gram negative especially against *E.coli* as shown in Figure 3. Therefore, Extracellular metabolites in the form of antibiotics produced by the five isolates were classified as specific in inhibiting Gram positive bacteria and is did not supress Gram negative bacteria. *Streptomyces misioensis* isolated from Egyptian soil reported produced substance that active against Gram positive bacteria[14]. Metabolite produced by LDE5 and VFD4 did not suppress the growth of *E.coli* which is the microflora of the digestive tract so it is safe to use in the gastrointestinal tract. One of the problems being faced currently about antibiotic is unspecified commercial antibiotic targets that have been used. The results of this study offer the potential of antibiotics that has specifications in the treatment of Gram positive bacteria without destructtion of non-target bacteria.

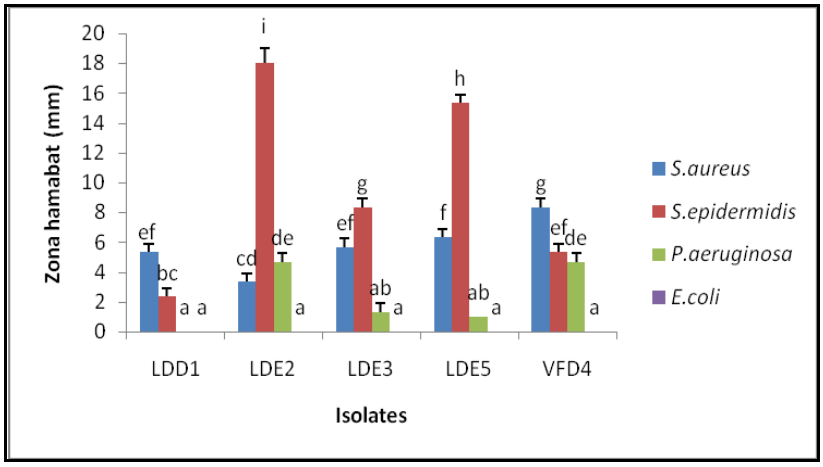

Fig. 3 Antimicrobial Activity of Isolated strains against gram positive and gram negative bacteria

Minimum inhibition concentration of the extracts obtained from isolated strains

The isolates were tested for MIC value were observed to determine the lowest concentration of extract at which the growth of pathogen is inhibited following modified published methods[7]. Other Research[12] have different results with MIC values obtained ranged from 0.6 to 1.25 mg / mL in inhibiting the pathogen MRSA (*methicillin resistant Staphilococcus aureus*) and MSSA (*methicillin sensitive Staphilococcus aureus*). The concentration required to kill pathogens MRSA is vary compared to other pathogens such as *S. cereviseae* which has been explored[15], MIC of metabolite of *Actinomycetes* isolates MG-500 and SR-2-2 against *S.cereviseae* are 128 ug / mL and 256 mg / mL. The same concentrations known to be able to inhibit the growth of pathogenic *Candida albicans*. The results obtained in this study require higher concentrations to inhibit pathogens compared with a previous study[12,15]. This is because in this study we are using crude extracts and the Extracellular metabolites of *Actinomycetes* that we used has not been purified.

Table 2. MIC value of the isolates agains pathogen

Isolates	**Minimum Concentration Inhibition (%)**		
	MRSA	***V. colera***	**EPEC**
LDD1	40	40	60
LDE2	60	40	80
LDE3	60	60	60
LDE5	40	40	60
VFD4	40	40	60

Based on the test we got variety results. Isolates LDD1, LDE5 and VFD4 have high inhibitory activity whereas isolates LDE2, and LDE3 have a low inhibitory activity. These groupings are referred from published research[16] which says that an antimicrobial compound is said to have high activity when the value of the minimum inhibitory concentration (MIC) occurs at low concentrations or low levels. Other research reported that *Streptomyces* sp. SRF1 also active against *B. Cereus* with low MIC value (0.39 mg/ml)[17].

Molecular Identification

Streptomyces is a bacteria in group of *Actinomycetes* and known to have similarities with the group of fungi that produce various metabolites and enzymes. *Streptomyces aurantiacus* known to have the ability as an antifungal as it has been investigated[18] in which the *Streptomyces aurantiacus* showed antifungal activity against *Fusarium* sp. *Streptomyces aurantiacus* strain ST 39, ST 61 and ST 62 of the study are known to produce *L. Asparginase* both intracellular and extracellular (Figure 3).

Streptomyces aurantiacus is known as a producer of antibiotic Aurantimycin. Aurantimycin produced by *Streptomyces aurantiacus* has high activity in overcoming gram-positive bacteria. Aurantimicyn divided into

four, namely A, B, C, and D, and at the same group as Azinothricin. These secondary metabolites produced together with pamamycin by *Streptomyces aurantiacus*. Aurantimycin D indicates a moderate level of antibacterial activity against a narrow spectrum of gram-positive bacteria (*Bacillus subtilis* ATCC 6633, MIC 62.5 pg / ml; Micrococcus flaws 10249, MIC 31.3 pg / ml; Micrococcus luteus 125, MIC 12.5pg / ml; and Staphylococcus aureus 511, MIC 62.5 pg / ml). Based on molecular identification, the antibiotics produced by isolate LDE5 can be suspected as aurantimycin[19].

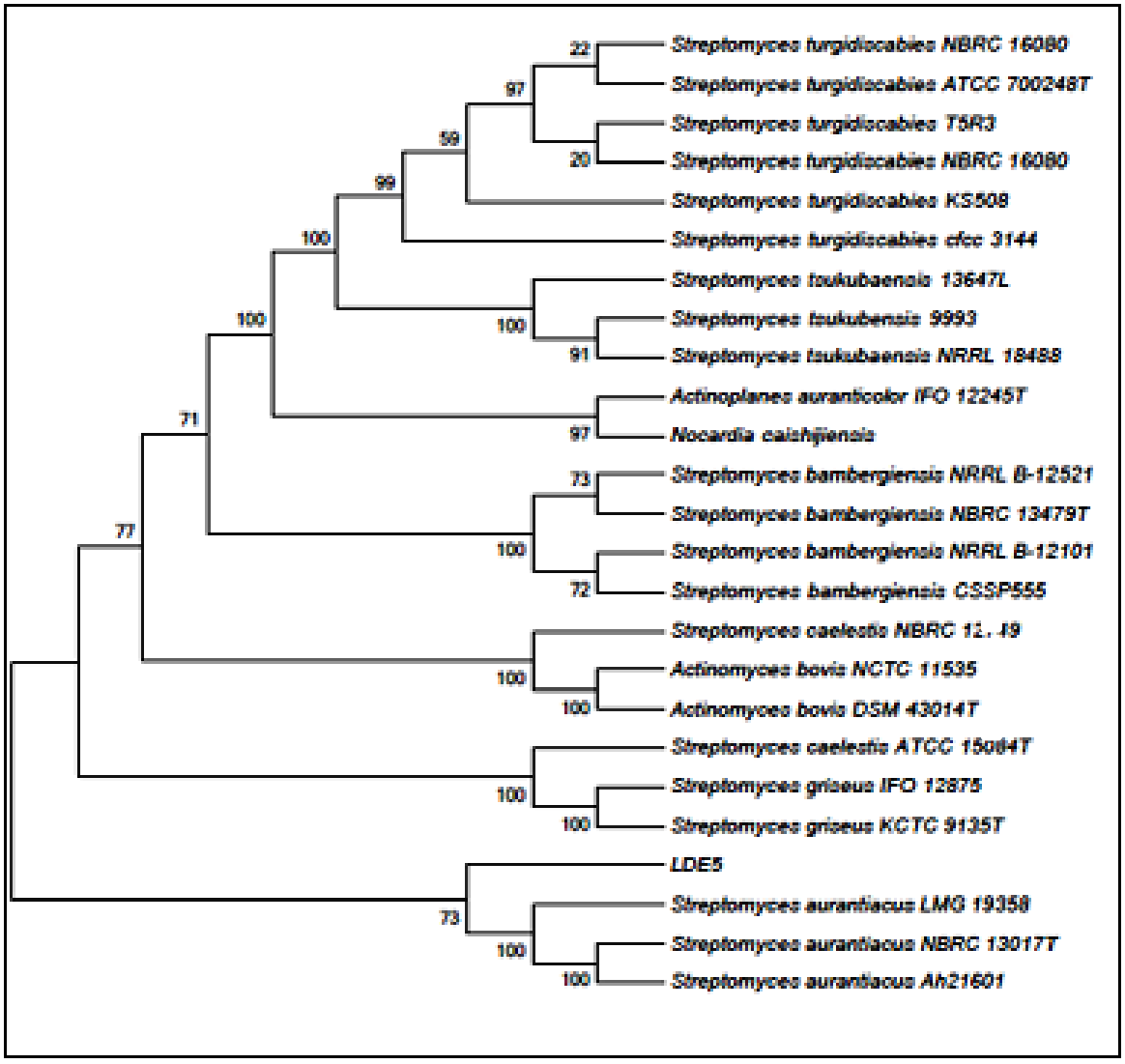

Fig. 3 Phylogeny tree of LDE5 based on 16S rDNA sequences, methods of construction using Neighbor-joining algorithm Tamura-Nei bootstrap 1000 by MEGA program 5.

Conclusions

In conclusion, the crude extract of isolated *Actinomycetes* showed good activity against test pathogen. *Actinomycetes* isolates LDE5 and VFD4 has antimicrobial activity equal to ampicillin against MRSA and pathogenic *Vibrio cholerae*. *Actinomycete*s isolates of LDD1, LDE5 and VFD4 have high activity in overcoming infection by pathogen MRSA, *Vibrio cholerae* and EPEC based on the test with MIC value 40 % and did not antagonistic to the normal microflora *E.coli* so it safe for use in the gastrointestinal tract. The present results suggest that the isolated *Actinomycetes* can be used as potent antimicrobial agent against the tested human infectious pathogen MRSA and work spesifically toward gram positive bacteria. Further studies on purification and identification of metabolite that produced by the isolated *Actinomycetes* are needed. Based on sequence analysis of 16S rDNA, LDE5 isolates identified as *Streptomyces aurantiacus* that produced antibiotic aurantimicyn while VFD4 and LDDI could not be identified yet.

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