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Antimicrobial Activity of *Streptomyces Albofaciens* against Methicillin Resistant *Staphylococcus Aureus* and Vancomycin Resistant *Enterococcus* Multi-Drug Resistant Species

Benita Mercy Rajan and Krishnan Kannabiran*

Division of Biomolecules and Genetics, School of Biosciences and Technology, VIT University, Vellore- 632014, India.

ABSTRACT

The aim of the study was to screen the antagonistic activity of *Streptomyces* species isolated from marine sediment samples against drug resistant Gram positive cocci. A total of 137 strains were isolated and 15% of the actinomycetes were found to be antibiotic producers. Out of which one potential isolate showed significant inhibitory activity against ATCC strains, Methicillin resistant *Staphylococcus aureus* (MRSA) and Vancomycin resistant *Enterococcus faecalis* (VRE) and compared with standard antibiotics. Ethyl acetate extract produced a zone of inhibition of 19 mm against *Staphylococcus aureus* (ATCC 29213) and 21mm against *Enterococcus faecalis* (ATCC 29212) and 21mm against the *Staphylococcus aureus* (MRSA) clinical isolate. Based on Nonomura's key for classification of *Streptomyces* and Bergey's Manual of Determinative Bacteriology, the isolate was identified as *Streptomyces albofaciens.* Culturing conditions including temperature, pH, sodium chloride concentration, carbon and nitrogen sources required were optimized. Based on the results of our study *Streptomyces albofaciens* is found to be a promising source for novel secondary metabolites active against drug resistant pathogens.

Keywords: Methicillin resistant *Staphylococcus aureus* (MRSA), Vancomycin resistant enterococci (VRE), *Streptomyces albofaciens*.

Corresponding Author

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INTRODUCTION

Among all known bacteria, *Staphylococcus aureus* is possibly the greatest concern of all health-care-associated pathogens due to its ability to cause a wide variety of life-threatening infections. *S. aureus* has the ability to rapidly adapt to different environmental conditions [1]. According to the NNISS report in 2004, *S. aureus* was one of the leading causes of health-care-associated infections globally. Numerous anti-staphylococcal agents are available in the market which includes linezolid, daptomycin, tetracyclines and fluoroquinolones, but these drugs are rapidly becoming of less value due to the resistance developed by the bacterium against these drugs. The resistance mechanism exhibited by *S. aureus* (MRSA) strains to methicillin is the most recognized one among all mechanism reported. *S. aureus* causes skin and soft tissue infections (SSTIs), endovascular infections, pneumonia, septic arthritis, endocarditis, osteomyelitis, foreign-body infections, and sepsis [1]. MRSA poses a therapeutic challenge in acute-care settings, as well as long-term skilled-nursing facilities [2]. Currently the drug of choice to treat MRSA infection is daptomycin or vancomycin. However resistance to these drugs have also been reported. The first report of an infection with a strain of *S. aureus* that had only intermediate susceptibility to vancomycin came from Japan in June 1996 [3]. More recently, two reports of VISA infections in the United States [4]. The history of new drug discovery processes shows that novel skeletons have, in the majority of cases, come from natural sources [5]. Among microorganisms, actinomycetes are one of the most attractive sources of all types of bioactive metabolites that have important applications in human medicine [6]. Streptomycetes and related actinomycetes continue to be useful sources of novel secondary metabolites with a range of biological activities that may ultimately find application as anti-infective, anti-cancer agents or other pharmaceutically useful compounds [7]. Hence a study was planned to isolate and to characterize potential actinomycetes that capable of producing potent antibiotic against a clinical isolate of MRSA.

MATERIALS AND METHODS

Sample collection and isolation of actinomycetes

Marine sediment samples were collected from coastal regions of Chennai, Pondicherry and Cuddalore, India at a depth of 400 cm. The sediment samples (1g) were diluted to 9.0 ml with sterile distilled water and 0.1ml of dilutions 10^{-3}, 10^{-4}, 10^{-5} was plated. The International Streptomyces Project (ISP) No. I media, with 25% sea water and 25% soil extract, was used for the isolation of actinomycetes. The growth media was supplemented with antibiotics, cycloheximide (25 mg/ml) and nalidixic acid (25 mg/ ml) (Himedia, Mumbai, India). Plates were incubated at 28°C for 7-18 days. The isolates were purified by repeated streaking and sub cultured and maintained in slant culture at 4 °C as well as at 20% (v/v) glycerol stock at -80°C [8]

Bacterial strains

Gram positive bacteria *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212) and a MRSA clinical isolate were used to screen the antibacterial potential of actinomycetes isolates.The drug resistance profile of the MRSA clinical isolate was tested

against a spectrum of standard antibiotics, erythromycin (30 µg/disc), cefoxitin (5 µg/disc), ampicillin (10 µg/disc), gentamycine (10 µg/disc), ceftriaxone (30 µg/ disc), cefazolin (30 µg/disc), cefotaxime (30 µg/disc), Linezolid (30 µg/disc) and vancomycin (30 µg/disc) (Himedia, Mumbai, India). The zone of inhibition was interpreted in accordance with CLSI guidelines (2007).

Primary screening for antibiotic production

Primary screening was done by various methods to detect antibiotic producers. Comparison of screening methods was done for the following techniques, cross streak, cylinder plate method/agar plug; agar overlay method, well diffusion and disc diffusion method [9-12].

Assay of anti MRSA activity

The MRSA strains used in this study was sub cultured overnight at 37°C in Mueller Hinton Broth and adjusted to obtain turbidity comparable to 0.5 McFarland standards (1.0 x 108 CFU/ml) before MIC tests. The EA extract was assayed for the anti-MRSA activity using broth micro dilution method in sterile glass test tubes. Prior the MIC test, each EA extract was diluted in sterile ultra pure water. The EA extract was then diluted by doubling dilutions at concentrations ranging from 32-1024 µg/ml. The tested strains were added to sterile Mueller Hinton broth into the test tubes before the EA suspension prepared as described above were added. The bacterial suspension without the EA was used as positive control and extracts in sterile broth were used as negative control. The MIC values were taken as the lowest concentration that showed no turbidity after 24 hours of incubation at 37°C. The turbidity was interpreted as visible growth of the microorganisms. The minimum bactericidal concentration (MBC) was determined by subculture of the tube showing no apparent growth in a sterile Muller Hinton agar plate. The least concentration showing no visible growth on agar subculture was taken as MBC value.

Taxonomic identification of the actinomycetes

The classical method described in the identification key by Nonomura and Bergey's Manual of Determinative Bacteriology was used for the identification of the isolate [13, 14]. The morphological, cultural, physiological and biochemical characterization of the isolate was carried out as described in ISP [15]. The spore surface morphology and its orientation were studied using light microscope (1000x magnification) as well as scanning electron microscope (SEM) (Hitachi, S-3400N). Culturing media used were those recommended in the International Streptomyces Project (ISP) [16,17]. Carbohydrate utilization was determined by growth on carbon utilization medium (ISP 9) supplemented with 1% carbon sources at 28°C [18]. Temperature range for growth was determined on inorganic salts starch agar medium (ISP 4). Hydrolysis of starch and milk were evaluated by using the glucose starch agar and skim milk agar respectively. Reduction of nitrate and production of melanin pigment were determined by the method of ISP [19]. All cultural characteristics were recorded after 14 days.

Optimization of culturing conditions

To determine the optimal nutritional and culturing conditions and to identify the suitable media for growth, the strain was inoculated in different culture media (SCA, ISP 2, ISP 3, ISP 4, ISP 5, ISP 6, ISP 7, modified Bennett's agar, sucrose/nitrate agar, and nutrient agar) and the growth was investigated. The effect of different incubation temperatures (15, 25, 37 and 50 °C), different pH (5.0, 6.0, 7.4 and 9.0) and NaCl concentrations (2, 5, 7, 9 and 12%) on the growth of the isolate was also studied. The carbon and nitrogen sources required were also studied by inoculating the isolates into mineral salt agar with different sugars substituted to starch (D-glucose, sucrose, starch, D-xylose, D-galactose, maltose, L-arabinose, fructose, lactose, and glycerol), organic nitrogen sources like peptone, yeast extract, casein and inorganic sources like ammonium sulphate, ammonium nitrate and urea. The concentrations of carbon sources and carbon utilization tests were done as described earlier [20]. After incubation the dry weight of the mycelium was measured and correlated with the growth of the isolate. Based on the growth of the isolate in different media the cultural conditions were optimized.

Fermentation and extraction of secondary metabolites

Spores at 10^7/ml of the strain were used to inoculate 1000 ml Erlenmeyer flasks containing 200 ml of ISP 1 broth supplemented with 1% (w/v) of glucose and magnesium. After incubation at 30 °C for 24 h in an orbital incubator shaker at 200 rpm, this pre-culture was used to inoculate (5% v/v) a total volume of 15 L culture medium having the same composition as the pre-culture. After seven days of incubation the culture broth was filtered to separate mycelium and supernatant. The supernatant was extracted twice with equal volume of ethyl acetate and the combined organic layers were evaporated. The brown gum obtained from the extract was dissolved in 100ml ethyl acetate [21]. The ethyl acetate (EA) extract was tested for antimicrobial activities against drug resistant strains.

RESULTS

Isolation of actinomycetes

A total of hundred thirty seven strains were isolated from the three sampling sites. Out of which 20 actinomycetes strains were found to be antibiotic producers (Figure.1). The organism which produces white powdery and dried colonies suspected to be actinomycetes were sub cultured on ISP-1 agar with sea water. Microscopic identification was also carried out to confirm the identity. The screening methods employed showed variable efficacy in detecting antibiotic producers and agar well diffusion method was found to be more effective (Figure. 2). The strain which showed antagonistic activity against MRSA was chosen for further studies

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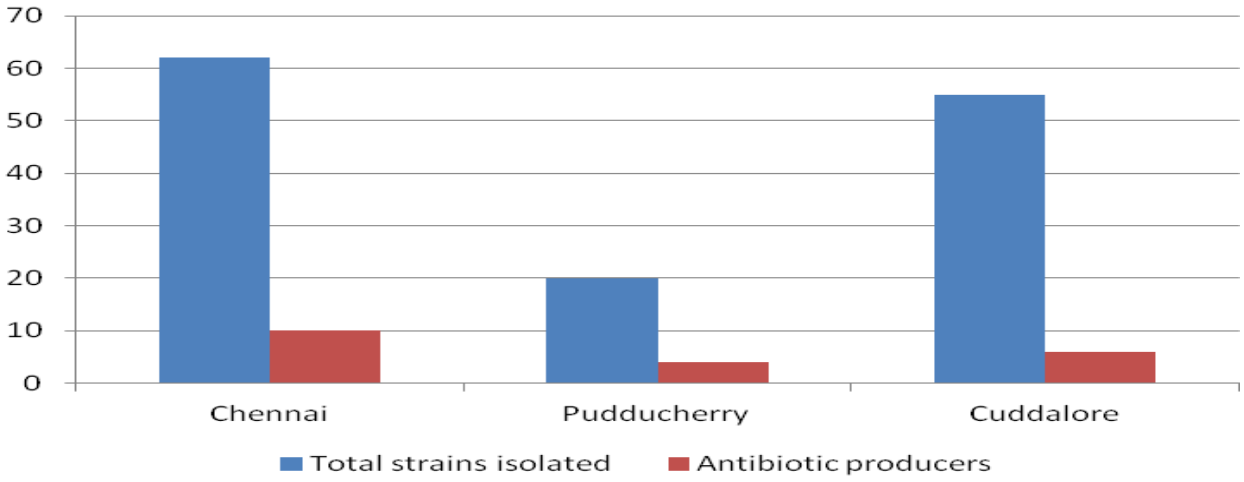

Figure 1. Number and distribution of the Antibiotic producer from different sampling sites

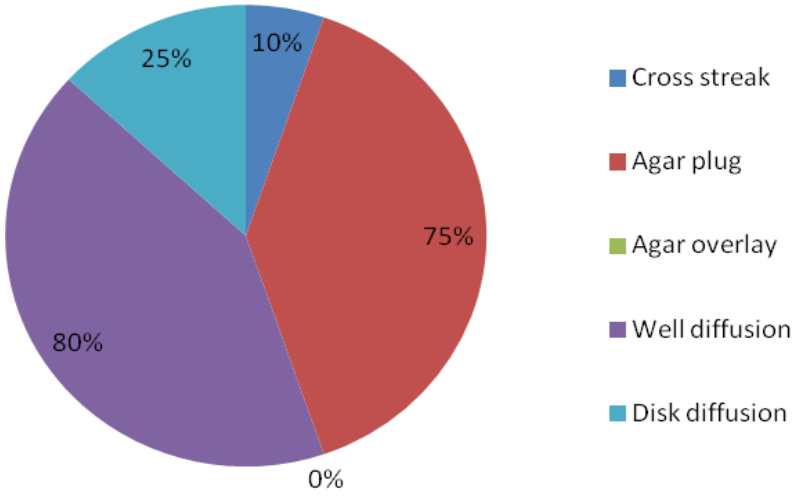

Figure 2. Efficacy of the different screening methods employed.

Antibacterial activity

The MRSA strain was resistant (6mm) to all the antibiotics tested Erythromycin (30 µg/disc), Cefoxitin (5 µg/disc), Ampicillin (10 µg/disc), Gentamycine (10 µg/disc), Ceftriaxone (30 µg/ disc), Cefazolin (30 µg/disc), Cefotaxime (30 µg/disc), except vancomycin (30 µg) 18 mm zone of inhibition and linezolid (30 µg) 23 mm zone of inhibition (Figure 3 A). The antagonistic activity of the EA extract of the potential strain is given in Figure 3 B.

Assay Anti- MRSA activity

The anti MRSA activity of the EA extract of *Streptomyces albofaciens* is given in Table 1. The EA extract (21 µg/ml) showed an inhibition zone of 21 mm against MRSA (clinical isolate). Determination of minimum inhibitory concentration (MIC) by broth dilution method was performed by using the two fold concentration increments of the extract incorporated into the broth. The method is a modification of the protocol in the CLSI M7-A4 document. The positive and negative control for the experiment was satisfactory .The concentration at which complete inhibition of visible growth was taken as the MIC for the MRSA strain. The MIC of the organism to the antibiotic was recorded as was at

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512µg/ml. The least concentration 1024 µg/ml) showing no visible growth on agar subculture was taken as MBC value (Table 2).

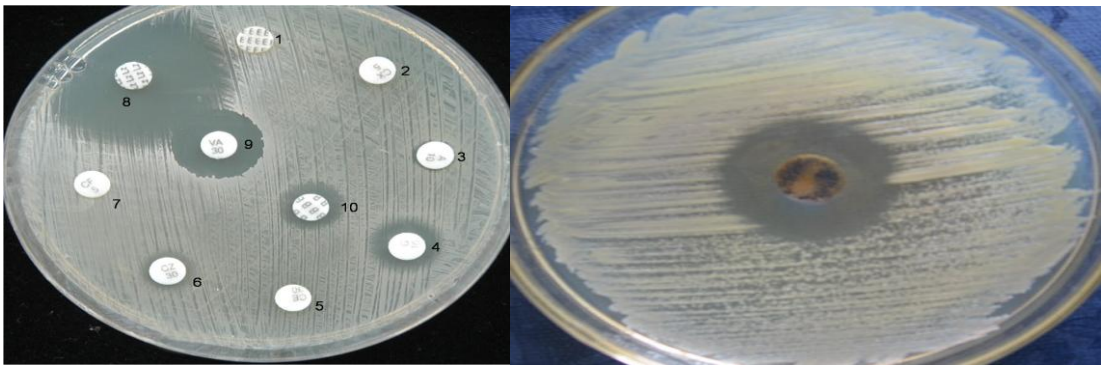

Figure 3. A) The drug resistance profile of the MRSA clinical isolate against a spectrum of standard antibiotics 1- Erythromycin (30 µg/disc), 2- Cefoxitin (5 µg/disc), 3-Ampicillin (10 µg/disc), 4- Gentamycine (10 µg/disc), 5-Ceftriaxone (30 µg/ disc), 6-Cefazolin (30 µg/disc), 7- Cefotaxime (30 µg/disc), 8- Linezolid (30 µg/disc), 9-Vancomycin (30 µg/disc). **B)** Anti MRSA (clinical isolate) activity of ethyl acetate extract of *Streptomyces albofaciens*..

Table: 1. Antagonistic activity of *Streptomyces albofaciens* against drug resistant bacterial strains

Bacterial Pathogens	Zone of inhibition (mm)		
	EA extract (20µg/ml)	Vancomycin (30 µg/disc)	Linazolid (30 µg/disc)
Staphylococcus aureus ATCC 29213	19	17	_*
Enterococcus faecalis ATCC 29212	21	18	_*
Staphylococcus aureus (MRSA)	21	18	23

*Since Vancomycin is the drug of choice, Linazolid was not tested.

Table 2: Anti MRSA activity of ethyl acetate extract of *Streptomyces albofaciens* against MRSA clinical isolate

Ethyl acetate extract (µg/ml)	Growth/turbidity
Positive control	Present
Negative control	Absent
32	Present
64	Present
128	Present
256	Present
512	Absent
1024	Absent

Taxonomy

The isolate is Gram positive, non acid fast, non motile and aerobic actinomycetes. The gram stain morphology showed filamentous forms with abundant spores. It has branched aerial mycelium, greyish white in colour. The substrate mycelium is branched and

did not produce any pigment. At maturity, the aerial mycelium formed long, straight to rectiflexibile spiral spore chains. The spores were non-motile and elliptoid in shape (Fig. 4: a, b). The biochemical and physiological characteristics of *Streptomyces albofaciens* are given in Table 3. The strain developed well on several media. With temperature range for growth from 15–37 °C and NaCl tolerance was 5%, with an optimal temperature at 28 °C. The strain assimilated arabinose, xylose, inositol, mannitol, fructose, sucrose and raffinose, however the strain did not utilize rhamnose. The strain utilized 0.1% of L-asparagine, L-phenylalanine, L-histidine and L-hydroxyprolone as nitrogen source. The strain was halophylic in nature tolerated NaCl concentrations between 2% and 12%. The strain showed β-haemolysis on blood agar containing 5% sheep blood. Based on the results of physiological, biochemical and cultural characterization as well as matching the keys given for classification of 458 species of actinomycetes included in International Streptomycetes Project the isolate was identified as *Streptomyces albofaciens* and designated as *Streptomyces albofaciens* VITBRK2.

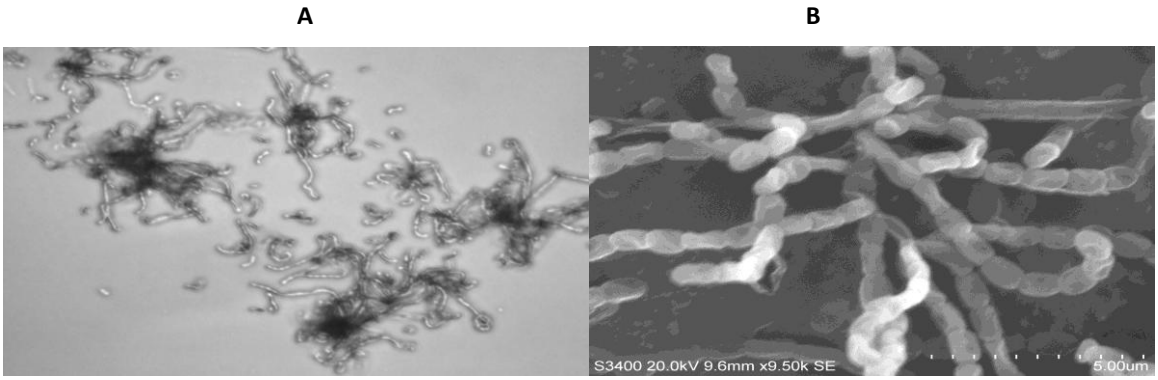

Figure 4: (a) Spiral spore chain and surface morphology of *Streptomyces albofaciens*
Figure 4: (b) Scanning electron micrographs of the spore chains showing the smooth spore surface of strain VITBRK2 grown on ISP 1 agar for 12 days at 28 °C, Bars 5µm.

Table 3. Biochemical and physiological characteristics of *Streptomyces albofaciens*.

Tests	Results
Gram stain	+
Endospore staining and motility	-
Aerial mycelium	White
Aerial spore mass colour	White grey
Colony colour	White
Production of diffusible pigments and melanin	-
Reverse side pigments	-
Spore chain	Spiral
Spore surface	Smooth
Range of temperature for growth	25-28°C
Optimum temperature for growth	28°C
Range of pH for growth	6-8
Optimum pH	7.4
Starch hydrolysis	+
Haemolysis of 5% sheep blood	β-haemolytic
Catalase	+
Amylase	+
Production of amylase	+
Utilization of carbon source (1% w/v)*	
Arabinose	+

Xylose	+
Inositol	+
Mannitol	+
Fructose	+
Rhamnose	-
Sucrose	+
Raffinose	+

+ Positive; - Negative

DISCUSSION

Streptomycetes species having antagonistic activity against drug resistant MRSA was isolated from marine sediment samples collected at the Chennai coast of Bay of Bengal, India. The EA extract obtained from the isolate exhibited significant activity against standard bacterial pathogens as well as multidrug resistant MRSA clinical isolate (Table 1). The EA extract (21 µg/ml) showed 21mm zone of inhibition which is higher inhibition than vancomycin (30µg/disc) with only 18mm zone of inhibition but lesser than linezolid (30µg/disc) which showed 23mm zone of inhibition.

Few reports are available about the strains isolated from marine sediments exhibited antagonistic activity against drug resistant bacterial strains, a new actinomycetes strain designated as BT-408 producing polyketide antibiotic SBR-22 having antibacterial activity (20mm) against methicillin resistant *Staphylococcus aureus* have been reported [22]. The EA extract of *Streptomyces albofaciens* not only inhibits MRSA but also vancomycin resistant *Enterococci* (VRE), forming 20mm and 16mm inhibition zones respectively. It was reported that an active compound, laidlomycin obtained from *Streptomyces* sp. CS684 showing antibacterial activity against MRSA and VRE strain [23]. Neocitreamicins I and II, novel antibiotics with activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci* have been reported from *Nocardia* [24]. In a recent report a compound, 2-(2',4'-Dibromophenoxy)-4,6-dibromophenol isolated from the marine sponge *Dysidea granulosa* (Bergquist) collected at the coast of Lakshadweep islands, Indian Ocean, exhibited potent and broad spectrum *in-vitro* antibacterial activity, especially against MRSA, methicillin sensitive *Staphylococcus aureus* (MSSA), VRE, vancomycin sensitive *Enterococci* (VSE) and *Bacillus* species [25]. Another study reported that *Streptomyces* sp. exhibiting activity against MRSA and the compound was water soluble, thermally stable and showed an inhibition zone of 22mm at the concentration of 100mg/ml of partially purified compound [26]. *Streptomyces albofaciens* exhibited a broad spectrum of activity against bacterial pathogens (ATCC cultures) and standard MRSA. Multidrug resistance is a growing threat, however discovery of new antimicrobials have been dwindling in the past years. Basic exploratory research, like the present study is of at most importance to identify the antimicrobial lead compounds from natural sources. The novelty of this study is that it is the first study undertaken in South India that focuses entirely on anti MRSA and VRE compounds from marine *Streptomyces.* Recently few compounds extracted from marine actinomycetes have been shown to possess anti MRSA and anti VRE activity. It was reported that dichloromethane extracts of 3 isolates (I-400A, B1-T61, M10-77) showed strong inhibitory activity against MRSA (ATCC 43300) and vancomycin-resistant *E. faecalis* (ATCC 51299) [27]. A new thiopeptide antibiotic with rare aminoacetone moiety designated as TP-1161 extracted from *Nocardiopsis* has been shown to possess significant antibacterial and

anti-VRE and anticandidal (multidrug resistant) activity [28]. A streptogramin antibiotic etamycin extracted from actinomycete strain CNS-575 was reported to be active against hospital- and community-associated methicillin-resistant Staphylococcus aureus (HA- and CA-MRSA) [29]. A compound 1-Acetyl-β-Carboline and β-Lactams extracted from marine *Streptomyces* was reported to be active against MRSA [30]. The results of this study showed that *Streptomyces albofaciens* is a potential actinomycetes strain capable of producing a lead antibacterial compound against MRSA strains.

CONCLUSION

The results of this study showed that *Streptomyces albofaciens* species is one of the potential actinomycetes isolate capable of producing bioactive antibacterial compounds against MRSA strains. Identification and characterization of the antagonistic secondary metabolite from the isolate is under progress from the author's laboratory.

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