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Isolate Actinomycetes SA32 Origin of Segara Anakan Mangrove Rhizosphere and its Capability in Inhibiting Multi-Drugs Resistant Bacteria Growth

Dini Ryandini[1]*, Ocky K Radjasa[1] and Oedjijono[2]

[1]*Department of Biology, Jenderal Soedirman University, Indonesia*
[2]*Department of Fisheries and Marine Science, Diponegoro University, Indonesia*

Abstract

The screening of antibiotic-producing actinomycetes from the mangrove environment is increasing to search for bioactive compounds capable of inhibiting the growth of Multi-Drugs Resistant (MDR) bacteria. The research aimed to isolate actinomycetes from mangrove mud rhizosphere in Segara Anakan lagoon that is capable of producing bioactive compounds inhibiting MDR bacterial growth, to characterize phenotypically along with 16S rRNA gene sequence, to cover out inhibition potencies toward MDR bacterial growth, and to characterize antibacterial compounds produced. Isolate actinomycetes SA32 was isolated from rhizosphere mud of *Rhizophora mucronata* from east Segara Anakan. It showed fragmented aerial mycelium similar to *Streptomyces* sp. NEAE-102; however, 16S rRNA gene sequence analysis yielded 96% similarity to *Streptomyces* sp. N56. In antagonism assay, it inhibited the growth of MDR bacteria *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas areuginosa*, *Enterococcus cloacae* and *Enterobacter* sp. The diffusion antagonism assay results in the highest antibacterial activity against *S. aureus* with clear zone diameter 20 mm. The result of Minimum Inhibitory Concentration (MIC) assay showed that 20% crude extract has been able to inhibit the growth of MDR bacteria characterized by the decrease of turbidity. The antibacterial compound produced was not known yet but has an Rf value of 0.7-0.9 in the TLC test. Isolate actinomycetes SA32 is potent to be developed as an MDR antibacterial substance source and it is proposed as a new strain of Streptomyces.

Keywords: Actinomycetes; East Segara Anakan; Multi drugs resistant bacteria inhibition; Inhibition diffusion method

Introduction

The use of large amounts of antibiotics resulted in pathogenic bacteria resistant to multiple drugs and developed Multi-Drugs Resistant (MDR) microbe [1]. The development of MDR bacteria causes difficulties in the treatment of bacterial infections. Based on these reasons, we searched for bioactive compounds that can inhibit their growth, either a new compound or antibiotic compounds that have greater control capabilities. The search for new antibiotics that is effective against multidrug-resistant pathogenic bacteria is presently an important area of antibiotic research [2].

The novel and broad spectrum substances are required to control or destroy resistant pathogen bacteria developed [3]. Bioactive compounds are heavily excavated from actinomycetes, which are considered as possible producers of novel bioactive compounds. Streptomyces, the most abundant actinomycetes, have 95.3% frequencies for an isolate known as a producer of 80% natural product and their capabilities in synthesizing secondary metabolite is unrivaled [4-7]. Actinomycetes group are the best sources of bioactive natural compounds used as antibiotics, pesticides, pharmaceuticals, herbicides, antiparasitics and enzymes [8,9]. Actinomycetes have a high rate of rediscovery of known compounds, and this has prompted a renewed interest in the development of new antimicrobial agents from rare and novel actinomycetes is urgently required to combat the increasing number of MDR human pathogens [10].

Unexplored marine environments are now a popular research area due to the potentially huge resources present within them, especially mangrove rhizosphere environment. Mangroves are a unique woody plant community of intertidal coasts in tropical and subtropical zones, located at the transition area between the land and the sea [11,12]. The mangrove environment functions as a source of microorganisms including actinomycetes capable of producing primary and secondary metabolites [13,14].

Mangrove rhizosphere in Segara Anakan Cilacap is a source of actinomycetes isolates producing bioactive compounds. The area is a complex lagoon with various mangrove vegetations. Rhizophora usually grows in the middle or behind the coastal line, specific with their raising roots; they grow dominantly in east Segara Anakan. Previously, you can find Rhizophora in the middle, but their density decreases due to sedimentation covering the plant seed [15]. There have been no reports of actinomycetes information in the mangrove environment Segara Anakan Cilacap antagonist to MDR bacteria. The objective of this research is to isolate actinomycetes from Segara Anakan mangrove environment that is capable of producing bioactive compounds inhibiting MDR bacterial growth. The identification of isolate actinomycetes is based on 16S rRNA gene sequence and phenotypic characteristics, to conceal the inhibitory potencies towards the bacterial growth of MDR, to characterize antibacterial compounds produced.

Materials and Methods

Isolation of actinomycetes

Actinomycetes were isolated from mud and soil of mangrove (*Rhizophora* sp.) rhizosphere in east Segara Anakan, Cilacap, at locations

***Corresponding author:** Dini R, Department of Biology, Jenderal Soedirman University, Indonesia, Tel: 62247460012; E-mail: dini_ryandini@yahoo.com

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E16, E40, E46, E47, and E44. The map of site sampling referred to Hinrichs et al. [15] (Figure 1).

The soil and mud sample was cultivated on Starch Casein Nitrate Agar (SCNA) medium through the dilution series by pour plate method. Pretreatments before the cultivation of the soil and mud sample were by heating the sample (70°C, 30 min), air dry, heating the sample suspension (85°C, 30 min) and treating it with antibiotic nystatin. After that, the incubation of the cultures at room temperature for 5-7 days.

Morphology and biochemical observation of actinomycetes isolates

Morphology characters observed involved aerial and substrate mycelium, colony size and form, colony surface, mycelium color and pigments that diffused into the medium. Morphology observation was conducted using stereo microscope (Olympus), light microscope (Olympus) and scanning electron microscope (SEM, TM3000 Hitachi) [16].

Biochemistry/enzymatic characters assayed were catalase, oxidase, amylase, protease, lipase, carbohydrase of xylose, inositol, sucrose, raffinose, fructose, rhamnose, arabinose and mannitol.

DNA isolation

One mL SA32 broth culture in SCNB medium, age 48-hours was placed in a microtube, centrifuged 8000 rpm for 5 min [17]. The expunged supernatant and the pellet were mixed with 400 µl TE 1x with a micropipette and then centrifuged again at 5000 rpm for five min. After that, the supernatant removal. 400 µl of pH 8 STE solutions and 50 µl of 50% tenderizer solution was added to the pellets, incubated at 37°C for 1 h and occasionally homogenized by slowly flipping the tube. 50 µl of 10% SDS solution was added and reincubated at 65°C for 2 h, occasionally homogenized, then centrifuged at 11,000 rpm at 4°C for 10 min. After that, the transfer of the supernatant to a new tube. Cold chloroform-isoamyl alcohol (24:1) was added as much as 400 µl, incubated at 37°C for 30 min, again centrifuged at 11,000 rpm at 4°C for 10 min. After that, the transfer of 400 µl supernatant into a new tube and 400 µl of isopropanol was added, then incubated overnight at -20°C. After that, it was centrifuged 11,000 rpm at 4°C for 10 min. Then, the removal of the supernatant; we added 100 µl of cold 70% ethanol and vortex for 10 s. After that, it was centrifuged 11,000 rpm at 4°C for 10 min. The supernatant was then removed, we evaporated the ethanol and 50 µl TE 1x was added then vortex for 10 s. The DNA samples were visualized using 1% agarose gel.

Amplification and analysis of 16S rRNA gene sequence

The isolate 16S rRNA gene region was amplified from genomic DNA through Polymerase Chain Reaction mechanism, using two universal primers for bacteria: pA 5 'AGA GTT TGA TGG CTC AG 3' (8-28) for forward and pH 5 'AAG GAG GTG ATG CAG CGG CA 3' (1542-1522) for reverse primers. The composition of PCR Reaction (volume 50 µl): Aquabidest (ddH2O) 19 µl, Thermo Kit PCR mix 2x 25 µl, DNA template two µl, forward primer two µl, reverse primer two µl [18].

All ingredients were mixed in orderly, homogenized. The amplification cycle starts from the beginning of denaturation at 94°C for 3 min followed by 40 cycles starting from the DNA denaturation stage at 94°C for 30 seconds. The annealing stage at temperature 52°C for 60 s and the extension stage at 72°C for 1 min 30 s. After completion of the PCR cycle, we continued with the post-extension stage at 72°C for 10 min. Then the storage was continued at 8°C for 5 min. The PCR product was performed by electrophoresis using 1% agarose and viewed under UV transilluminator. The sequencing process was done by sending samples to First Base, Singapore.

Sequencing sequence analysis using BioEdit application and sequencing alignment using Local Basic Alignment Search Tool (BLAST) program at National Center for Biotechnology Information (NCBI) page at http://www.ncbi.nlm.nih.gov. Phylogenetic tree construction using MEGA application. The construction of the evolution distance in the degree of confidence using the Neighbor-joining method using the bootstrap value in the NJ plot program with Bootstrap 1000.

Inhibition assay of actinomycetes isolates toward MDR bacterial growth

Clinical MDR bacteria isolates, obtained from Kariadi government hospital Semarang, were *Escherichia coli* (EC), *Enterococcus* sp. (ETC), *Enterobacter cloacae* (ETB), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA) and *Staphylococcus aureus* (SA). We used Diffusion method for this assay in Plate Count Agar (PCA) medium.

Actinomycetes isolate cultured in 100 mL Starch Casein Broth (SCB) and Starch Nitrate Broth (SNB) media, incubated during 7, 14 and 21

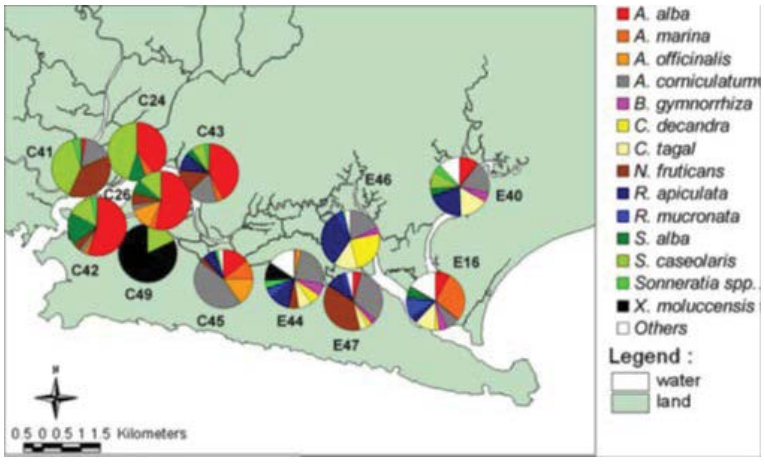

Figure 1: Sampling location in Segara Anakan lagoon, Cilacap.

days at room temperature. At the end of incubation period, the culture was filtered then mycelium dry weight and the pH was measured. The filtrate was then extracted using ethyl acetate and methanol. Then, we got the crude extract after evaporating it at 70°C for 2 h.

The crude extract was assayed against MDR bacteria through dropped extract as much as 20 µL onto 6 mm disc paper. The disc was placed on the lawn of 10^8 cels/mL MDR bacteria, incubated at 37°C for 24 h. Clear zone (CZ) surrounding the disc was observed and measured.

The crude extract was then diluted to 20%, 40%, 60%, 80% and 100% and subjected to Minimum Inhibitory Concentration (MIC) test toward MDR bacteria. Two mL of Nutrient Broth (NB) medium was inoculated by 0.25 mL MDR bacterial culture and 0.25 mL diluted extract. We incubated the culture for 24-48 h at 37°C. Observation put on inhibition extract concentration (marked by a decrease in turbidity) and lethal concentration (characterized by clear culture).

Data Analysis

The isolate identification and antagonist capability data were analyzed descriptively, Sequencing sequence analysis using BioEdit application and sequencing alignment using Local Basic Alignment Search Tool (BLAST) program at the National Center for Biotechnology Information (NCBI) page at http://www.ncbi.nlm.nih.gov. Phylogenetic tree construction using MEGA application. The construction of the evolution distance in the degree of confidence using the Neighbor-joining method using the bootstrap value in the NJ plot program with Bootstrap 1000.

Results

Isolation and morphology identification of actinomycetes isolates

Various isolates of actinomycetes have been isolated from the mangrove (*Rhizophora* sp.) rhizosphere that varies in those morphologies. The specific characters of actinomycetes were exhibited by their mycelium structures (aerial mycelium and/or substrate mycelium), slow growth, hard texture colony and pigment diffusion into the medium by some isolates. Their structures showed that actinomycetes have the characteristics of both bacteria and fungi. The sampling location was a dry land environment, wet soil, mud, and flooded soil. The environmental condition of the sampling site has an ambient temperature of 28-31°C, soil pH 5.8-7 and salinity 5-41 ppm. The soil sample was collected nearer to the root region of the mangrove *Rhizophora apiculata* which also produced 22 actinomycetes through dry heat (70°C) pre-treatment method on SCA medium and was subjected to antimicrobial activity selection. The mangrove environment is a potent source for the isolation of antibiotic-producing actinomycetes.

The morphology of colonies and aerial mycelial supports in the identification of actinomycetes since the mycelium has a specific performance. The specific morphology allows it to distinguish between Streptomyces and rare actinomycetes. Specific shapes are, for example, represented by a straight or spiral shape. The hypha structure can be either straight (stable) or fragmented into coccoid or rod shapes. The mycelium supports spores or conidias chain; the long spore chain type supports more than 20 spores, and short spore chain type supports less than 20 spores [19]. Based on colony and mycelium characters, isolate actinomycetes SA32 was predicted as a rare Streptomyces.

Isolate SA32 grew with aerial mycelium on the colony surface, substrate mycelium under colony and no pigment was diffused into the medium. The colony has cream color; the surface showed smooth powdery colony. The aerial mycelium looks straight, no spiral form, thin, fragmented into coccoid, twisting fragment hypha, and supporting long chain spores (Figures 2A and 2B).

The results of physiological and biochemical assays showed that isolate SA32 was able to use sugars fructose, sucrose, raffinose, rhamnose, inositol, mannitol and negative test in arabinose and xylose sugars. The isolate also exhibited positive result in lipolytic, amylolytic, proteolytic, catalase tests.

Amplification and sequencing of 16S rRNA gene

The result of the amplification using a universal 16S primer and sequencing of 16S gene showed a band with nucleotide base sequence, which was most closely related to *Streptomyces* sp. N56 with 96% similarity index(access number HQ132789.1) as shown in Figure 3.

Capability of actinomycetes SA32 isolate against MDR bacteria growth

Isolate actinomycetes SA32 grown on SCB and SNB media have

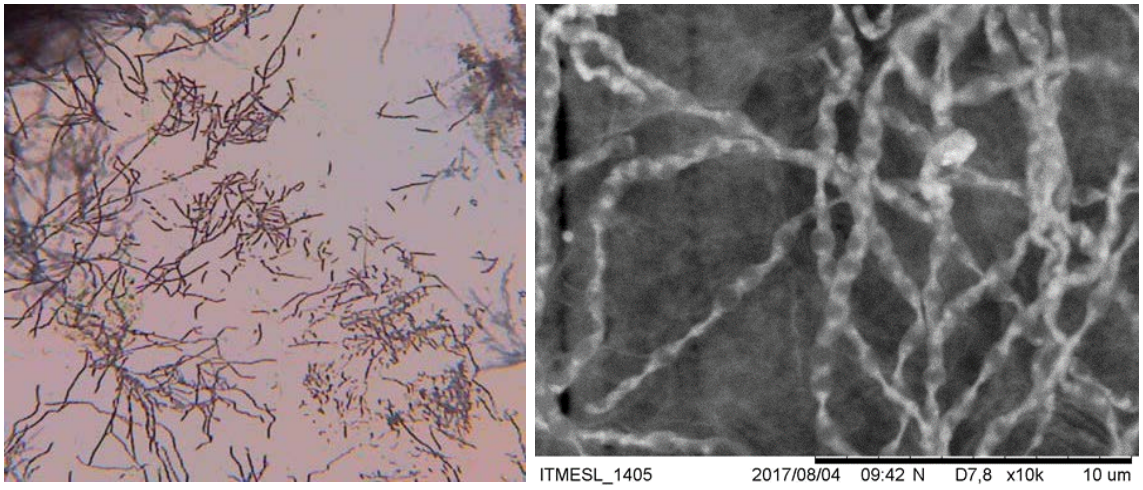

Figure 2: The appearance of the aerial mycelial isolate SA32 at magnification 400x (A) and 10.000x (B).

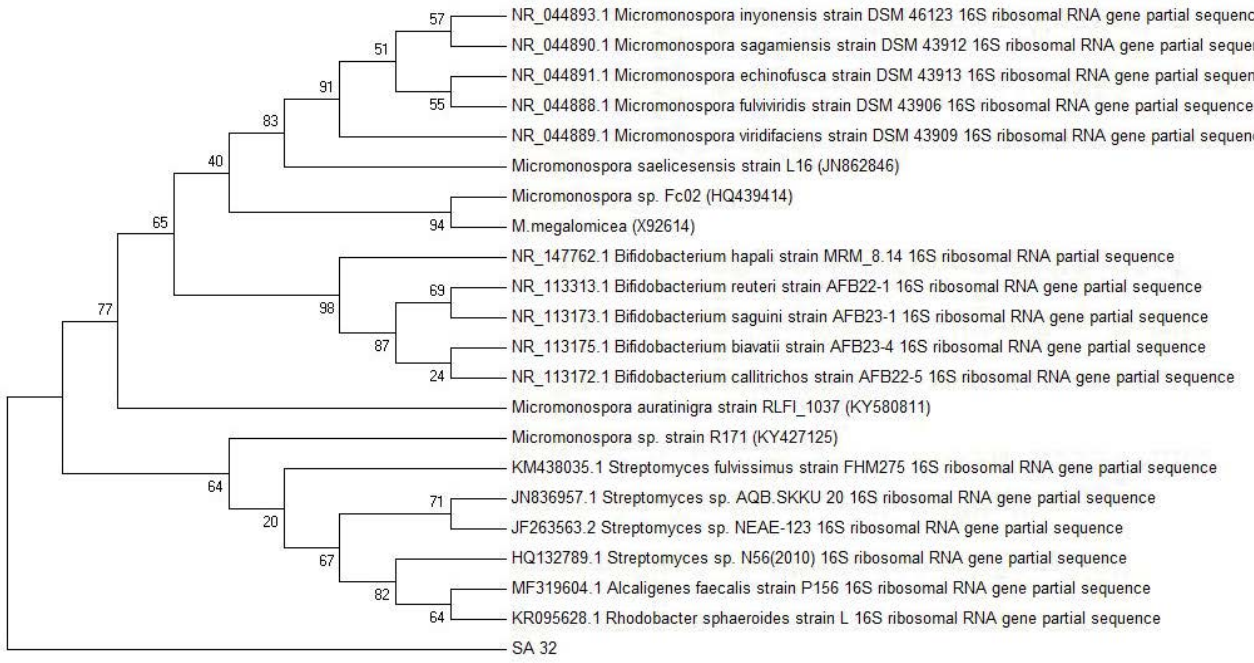

Figure 3: Phylogenetic tree of isolate SA32 and other actinomycetes or bacterial isolates.

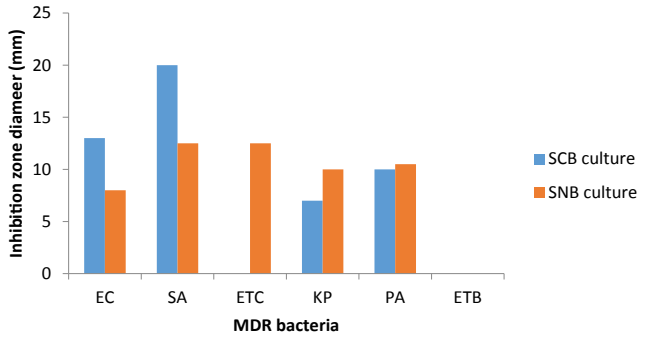

Figure 4: Inhibition zone diameter measurement (mm) of starch casein broth (SCB) and starch nitrate broth (SNB) culture extract to *Escherichia coli* (EC), *Staphylococcus aureus* (SA), *Klebsiella pneumonia* (KP), *Enterococcus* sp. (ETC), *Pseudomonas aeruginosa* (PA) and *Enterobacter cloacae* (ETB).

different inhibitory capabilities against MDR bacteria. The filtrate of SCB medium showed no inhibition to MDR bacteria growth. While the filtrate of SNB medium showed inhibition to the growth of *S. aureus* (clear zone/CZ diameter was 14 mm) and *P. aeruginosa* (CZ diameter was 10 mm). The 21 day filtrate yielded a diameter of 18 mm inhibition zone to *S. aureus* and 10 mm to *P. aeruginosa*.

The crude extracts of antibacterial compounds obtained from the extraction using methanol and ethyl acetate solvents showed different inhibitory capabilities against the test bacteria. The highest inhibiting activity was shown by SCB extract to *S. aureus* (IZ dia=20 mm) and low inhibitory activity was exhibited by SCB extract to *K. pneumoniae* (IZ dia=7 mm). The inhibitory activity of the extract on MDR bacteria also showed that SNB culture extract was higher (inhibited five isolates) than that of SCB culture extract (inhibited four isolates) (Tables 1 and 2 and Figures 4 and 5).

The antibacterial compounds produced by the SA32 mycelium

Culture Medium	MDR bacterial inhibition zone diameter (mm)					
	EC	SA	ETC	KP	PA	ETB
SCB	13	20	0	7	10	0
SNB	8	12.5	12.5	10	10.5	0

Table 1: Inhibition zone diameter of starch casein broth (SCB) and starch nitrate broth (SNB) culture crude extract to *Escherichia coli* (EC), *Staphylococcus aureus* (SA), *Klebsiella pneumonia* (KP), *Enterococcus* sp. (ETC), *Pseudomonas aeruginosa* (PA) and *Enterobacter cloacae* (ETB).

MDR bacteria	Concentration of crude extract					
	0%	20%	40%	60%	80%	100%
E. coli	+++	+	+	+	+	+
S. aureus	+++	++	++	-	-	-
E. cloacae	+++	-	-	-	-	-
Enterococcus sp.	+++	+++	++	++	++	++
P. aeruginosa	+++	+	-	-	-	-

Figure 5: MIC test result of SA32 isolate extract cultured on SNB medium.
+++ High growth
++ Moderate growth
+ Low growth
- No growth

culture biomass grown during the incubation period of 7-21 days. The dry weight biomass and pH of the medium during the incubation period showed culture development growth, through growth phases until culture synthesized and produced antibacterial bioactive compounds (Table 2).

The result of Minimum Inhibitory Concentration (MIC) assay showed that 20% crude extract of SA32 has been able to inhibit the growth of MDR bacteria characterized by the decrease of turbidity. SA32 isolate extracts are known to inhibit the isolates of *E. coli, S. aureus, Enterococcus* sp. and *P. aeruginosa*, but less inhibit *E. cloacae*.

Analysis of SA32 isolates active compound using thin layer chromatography (TLC) method with eluent chloroform: ethyl acetate: acetic acid 5:3:1 exhibited three spots on silica plate. The three spots have different Rf sizes, i.e., Rf value 0.76, 0.8 and 0.9.

Discussion

Isolate SA32, isolated from *Rhizophora mucronata* rhizosphere mud, in E16 location, saline environment, has a smooth powdery colony surface with cream color and slow growth. SEM (10.000 times) investigation showed that aerial mycelium looked thin like a ribbon, fragmented into coccoid or irregular rod, no spiral pattern appears, elongated oval spores (Figures 2A and 2B). While common aerial mycelium of Streptomyces is tube-like rather than flat and based on spore chains, it may be divided into rectiflexibiles, retinaculiperti and spirals [20,21].

The SA32 aerial mycelium type was highly similar to that of *Streptomyces* sp. NEAE-102. This actinomycete was isolated from Egypt soil, produced long, straight spore-chains with elongated, irregular and smooth-surfaced spores [22]. Isolate SA32 performed some different characters to *Streptomyces* sp. NEAE-102, indicating that both isolates were a different strain/species of Streptomyces (Table 3).

Morphological identification is difficult enough to obtain results up to the species level. Therefore 16S rRNA studies help determine the phylogenetic relationship and make possible the recognition up to species level.

Based on sequencing analysis of 16S rRNA gene, isolate SA32 has a 96% similarity to *Streptomyces* sp. N56 that isolated from soil samples of wheat cropping system from Indo-Gangetic Plains of India [23]. *Streptomyces* sp. N56 was isolated from land crop with low salinity rather than wet soil with high salinity. Further study, morphological as well as biochemical characterization of SA32 isolate compared to strain *Streptomyces* sp. N56 was significant different. This investigation showed actinomycetes diversity and may contribute a novel actinomycetes list. In agreement with Malviya et al. [23] isolate SA32 was predicted to be new species then needs to be validated by DNA-DNA hybridization and (%) GC content for its identification was up to species level. A microbe with the similarity of 16S rRNA sequence <97% require further analysis since it is known that microbes with 16S rRNA sequence similarity up to 97% identical should be considered as members of the same genus [23]. It also could represent new species, considering a limit of similarity of 97% in the 16S rRNA gene sequences [24]. The sequence cannot be compared to the 16S sequence of *Streptomyces* sp. NEAE-102, due to the sequence was not registered yet in genebank and was still being proposed as novel species of Streptomyces [22]. Since of the phenetic and phylogenetic characters of isolates, SA32 are not similar as those of the existing actinomycetes isolates; this leads to its proposal as new species of Streptomyces.

Antibacterial capability of isolate SA32 was higher shown by the extract because it contained concentrated compounds than that in filtrate for a similar volume treated. The extract concentration of 20% has been able to inhibit the MDR bacteria growth. Therefore this isolates promoting to be developed as an antibacterial compound source.

Incubation time (day)	Dry weight biomass (mg/100 mL)		Medium pH	
	Culture in SNB medium	Culture in SCB medium	Culture in SNB medium	Culture in SCB medium
7	23	88	6.13	5.51
14	60	118	6.9	5.86
21	55	180	6.7	5.87

Table 2: Dry weight biomass and pH culture medium of isolate SA32 during 7-21 days incubation.

Characterization	Isolate SA32	*Streptomyces* sp. NEAE-102
Colony of inorganic salt-starch-agar/SCNA medium	Substrate and aerial mycelium: Cream color, Smooth powdery colony	Substrate mycelium: ivory color, aerial mycelium: faint yellow. Aerial mycelium was abundant
Diffusible pigment	Non-pigmented	Non-pigmented
Aerial mycelium	Looked thin like a ribbon, fragmented into the coccoid or irregular rod and its fragment looked twisting, no spiral pattern appears	Long, straight spore-chains with elongated, irregular and smooth-surfaced spores
Spores	Elongated oval spores	Elongated
Spores chain morphology	Rf	RF
Amilolitic	+	+
Lipolytic	+	
Proteolitic	+	+
NaCl tolerance	Grow in 40 ppm salinity	Tolerance to 8% salinity
The use of glucose	+	+
Fuctose	+	+
Sucrose	+	+
Raffinose	+	
Rhamnose	+	
Inositol	+	
Mannitol	+	
Arabinose	-	
Xylose	-	
Catalase	+	

Table 3: Some characters of isolate SA32 compared to *Streptomyces* sp. NEAE-102.

Bioactive compounds produced by actinomycetes biomass were affected by the incubation time. The exponential phase growth of isolate SA32 was at 7-10 days incubation time. Incubation time 14 days produced higher biomass than other incubation time. At the end of the incubation time (21 days), the biomass weight decreases, however still showed inhibitory activity, since the antibacterial compound was synthesized at the end exponential growth phase and accumulated until the end of the incubation period. The maximum antibacterial activity of five actinomycetes isolated from mine soil samples collected from Salem, Tamil Nadu toward multi drugs resistant bacteria wound isolates was also observed on 14th-day incubation [25]. While production of tetracycline optimum during 4-7 days, the longer the incubation time, decreased the antibiotic produced [26]. While *Streptomyces kanamyceticus* M27 showed no direct relation between the growth of the organism and antibiotic formation. The pH of the medium might be an important factor for antibiotic formation, as media giving high antibiotic yields showed an alkaline pH [27].

Inhibition activity observed higher come from SNB culture than that from SCB medium. SCB medium is commonly used as cultivation medium for actinomycetes that contain casein as a nitrogen source for supporting growth. As a growth medium, it supported growth rather than secondary metabolite synthesis unless the isolate has starvation condition. When nutrient become limited, it stimulates secondary metabolite synthesis and results in antibacterial activity. SNB medium contained inorganic salt nitrate that functioned as inorganic nitrogen source and cofactor that supporting secondary metabolite synthesis [27]. The maximum antibiotic biosynthesis by Streptomyces isolates J12 was obtained in medium supplemented with 10 g/l starch as a sole carbon source and 2.5 g/l potassium nitrate in addition to 0.3 g/l casein as nitrogen sources at pH 7.2 after six days of incubation [28]. Starch was also the best carbon source for actinomycin-D production [29].

Inhibition activity was higher to Gram-positive bacteria *S. aureus* than to Gram-negative bacteria. This distinct response due to the sensitivity of Gram-positive and Gram-negative bacterial cell wall is different. Gram-negative cell wall has outer membrane structure composed of lipopolysaccharide beside thin peptidoglycan, while Gram-positive cell wall is only composed of thick peptidoglycan. This composition makes the cell wall impermeable to lipophilic solutes; The gram-positive should more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier [25,30-33].

The antibacterial compound produced by isolate SA32 was not known yet but has an Rf value of 0.7-0.9 in the TLC test. It requires testing with other methods to determine the compounds type contained in the extract, in addition to testing on various culture media to determine higher antibacterial compounds synthesis.

Conclusion

Isolate actinomycetes SA32 is distinct from other phylogenetically and phenotypically related Streptomyces species, therefore it is predicted as a new strain of Streptomyces that is capable of inhibiting MDR bacteria growth, It has the potential for further investigation and development as a new source of antibacterial compounds.

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