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Antibacterial Activity of *Streptomyces* SAE4034 Isolated from Segara Anakan Mangrove Rhizosphere against Antibiotic Resistant Bacteria

✉Dini Ryandini, Hendro Pramono, Sukanto

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Laboratory of Microbiology, Faculty of Biology, Universitas Jenderal Soedirman

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Abstract

Actinomycetes SAE4034 isolates was isolated from *Rhizophora apiculata* rhizosphere mud showed some antibacterial properties. The antibacterial ability of this isolate has not been tested on antibiotic resistant bacterial pathogens. However, there was no research has been reported regarding actinomycetes from Segara Anakan mangrove area resulting compounds inhibit the growth of antibiotics-resistant bacteria. Therefore, it is important to investigate its capability against antibiotics resistant bacteria or multi drug resistant bacteria (MDR bacteria). The research aimed to know the ability of actinomycetes SAE4034 in inhibit MDR bacteria and to identify the species profiles. The research methods included isolate characterization involving morphology, physiology/enzymatic and molecular properties. MDR bacterial inhibition assay, antibacterial compound extraction and antibacterial compound test using thin layer chromatography (TLC) method, observation of morphological and biochemical properties, DNA isolation, amplification and analysis of 16SrRNA sequence, and phylogeny tree analysis. The methods of this study included MDR anti-bacterial assay and antibacterial compound test. Subsequent step was isolate characterization including observation of morphological and physiological / enzymatic properties, and 16S rRNA gene sequence. The results showed that culture extract was able to inhibit the growth of MDR bacteria i.e. *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterococcus* sp., but no inhibition to *Enterobacter cloacae*. The bioactive compound showed 4 spots with Rf values of 0.36; 0.45; 0.54; and 0.6. Based on morphology, physiology / enzymatic and 16S rRNA gene sequences characteristics, actinomycetes SAE4034 isolate is *Streptomyces* sp. This research showed new *Streptomyces* strain that serves as a source of MDR antibacterial compounds and useful in development of antibiotic for combating infectious diseases caused by MDR bacteria..

How to Cite

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✉ Correspondence Author:
Jl. HR Boenyamin 708, Grendeng, Purwokerto Banyumas, Jawa Tengah 53122
E-mail: dini_ryandini@yahoo.com

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INTRODUCTION

Actinomycetes is prokaryotic which most plays a role in producing secondary metabolites, including antibiotics (Bhat *et al.*, 2013). About two-thirds of natural antibiotic for the medical benefit is obtained from actinomycetes (Okami & Hotta, 1988). Various types of antibiotics produced by actinomycetes include aminoglycosides, anthracyclines, glycopeptides, beta-lactams, macrolides, nucleosides, peptides, polyene, polypeptides, actinomycin, and tetracyclines (Kekuda *et al.*, 2010; Bhat *et al.*, 2013). Most antibiotics are produced by *Streptomyces* and *Micromonospores* (Bhat *et al.*, 2013). *Streptomyces* is an unrivaled species in producing secondary metabolite natural products (Radjasa, 2013).

Actinomycetes SAE4034 was isolated from *Rhizophora apiculata* rhizosphere mud, they have powdery colony and showed antibacterial activity in the isolation in initial process. Its antibacterial potency and species characteristics have not known yet. Conventional identification is not sufficient to show the diversity of species, due to visual limitations in observation. The morphological performance of colonies is also strongly influenced by the nutritional conditions of the growth medium. Therefore, further testing is required using the Polymerase Chain Reaction (PCR) method and the 16S rRNA gene sequencing analysis. Sequence 16S is a sequence of genes in the prokaryotes that have conserved nucleotide sequences, so it can be used as a guide to compare the identity of actinomycetes species.

Mangrove rhizosphere in Segara Anakan Cilacap, as a source of actinomycetes SAE4034, have an opportunity as a source of actinomycetes isolates producing bioactive compounds. The mangrove environment is a potential source of the isolation of antibiotic-producing actinomycetes (Das *et al.*, 2006, Hu *et al.*, 2012). Soil sample collected nearer to the root region of the mangrove *Rhizophora apiculata* also produced totally 22 actinomycetes through dry heat (70°C) pretreatment method on SCA medium and was subjected to antimicrobial activity selection (Janaki, 2016). There have been no reports of actinomycetes information in the Segara Anakan Cilacap mangrove environment against to MDR bacteria. The objective of this research was to investigate the actinomycetes SAE4034 isolate in inhibiting MDR bacterial growth, including the characterization of antibacterial compounds produced. The subsequent target was to identify species profiles based on phenotypic and genotypic characteristics. These informations usefully contribute in new drug investigation for combating diseases caused by MDR bacteria.

METHODS

Preparation of actinomycetes SAE4034 isolate

Actinomycetes SAE4034 isolate was cultured (streak culture) on Starch Casein Nitrate Agar (SCNA) medium, incubated at room temperature for at least 5 days. This isolate was subjected to inhibition assay, morphological and biochemical observation, and 16SrRNA analysis.

Inhibition assay of actinomycetes SAE4034 isolate against MDR bacterial growth

Clinical MDR bacterial 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). Diffusion method was used for this assay on Plate Count Agar (PCA) medium and Mueller Hinton (MH) medium.

Actinomycetes isolate was cultured in 100 mL Starch Casein Broth (SCB) and Starch Nitrate Broth (SNB) media, incubated for 7, 14 and 21 days at room temperature. At the end of each incubation period, the culture was filtered then mycelium dry weight and pH were measured. The filtrate was then extracted using ethyl acetate and methanol. The crude extract was obtained after being evaporated at 70°C for 2 hours.

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

The extract then was subjected to chromatography assay. The chromatographic plate (TLC aluminum sheet silica gel 60 F254) was marked to determine the starting point and 10 µl of crude extract was dropped on the plate. The plates were then immersed in an eluent solution chloroform, ethyl acetate and acetic acid (3: 3: 1) mixture, removed and dried at 80°C for 2 hours. The plate was observed under UV light (λ 366 nm). Spots formed were measured for the Rf value.

Morphology and biochemical observation of actinomycetes isolate

Morphological characteristics observed included aerial and substrate mycelium, colony size and shape, colony surface, mycelium color and pigments that diffused into the medium. Morphology observation was conducted using

the stereo microscope (Olympus), light microscope (Olympus) and scanning electron microscope (SEM, TM3000 Hitachi). Biochemistry/enzymatic characters assayed were catalase, oxidase, amylase, carbohydrase of xylose, inositol, sucrose, raffinose, fructose, rhamnose, arabinose, and mannitol.

The assay procedures based on Cappuccino and Sherman (1987); Sharma (2014), Khanna (2011) and determination guidance using Holt *et al.* (2000) and Miyadoh (1997).

DNA isolation (Song *et al.*, 2004 modified)

One mL SAE4034 broth culture in SCNB medium, age 48-hours was placed in a microtube, centrifuged at 8000 rpm for 5 min. The supernatant then was removed, and the pellet was mixed by 400 µl TE 1x using a micropipette and then centrifuged again in 5000 rpm for 5 min. The supernatant then was removed. 400 µl of pH 8 STE solution and 50 µl of 50% tenderizer solution was added to the pellets, incubated at 37°C for 1 hour and occasionally homogenized by slowly flipping the tube. 50 µl of 10% SDS solution was added and re-incubated at 65°C for 2 hours, occasionally homogenized, then centrifuged at 11,000 rpm at 4 °C for 10 min. The supernatant was transferred into 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. 400 µl supernatant was transferred into a new tube, and 400 µl of isopropanol was added, then incubated overnight at -20°C. Next, the solution was centrifuged 11,000 rpm at 4°C for 10 min. The supernatant was removed, 100 µl of cold 70% ethanol was added and vortexed for 10 seconds. Next, the solution was centrifuged 11,000 rpm at 4°C for 10 min. The supernatant was removed, ethanol was evaporated 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 (Ayari *et al.*, 2016 modified, Nurkanto & Agusta, 2015)

The isolate 16S rRNA gene sequence was amplified using two universal primers for bacteria: pAF 5 'AGA GTT TGA TGG CTC AG 3' (8-28) and pHR 5 'AAG GAG GTG ATG CAG CGG CA 3' (1542-1522). The composition of reaction (volume 50 µl): double-distilled water (ddH2O) 19 µl, Thermo Kit PCR mix 2x 25 µl, DNA template 2 µl, forward primer 2 µl and reverse primer 2 µl.

All ingredients were mixed in orderly until the solution became homogenized. The amplification cycle starts from pre-denaturation at 94°C for 3 minutes followed by 40 cycles starting from the DNA denaturation stage at 94°C for 30 seconds. Annealing stage at temperature 52°C for 60 seconds. Extension stage at 72°C for 1 minute 30 seconds. After completing the PCR cycle, the process continued by the post-extension stage at 72°C for 10 min, followed with storage at 8°C for 5 minutes. The product PCR was performed by electrophoresis using 1% agarose and viewed under UV transilluminator. The sequencing process was conducted in First Base, Singapore.

Data analysis

Antibacterial capability data were analyzed descriptively. Morphological and biochemical characteristic were analyzed based on *Bergey's Manual Determinative Bacteriology* and Atlas of Actinomycetes. Sequencing analysis was conducted by using BioEdit application and was subjected to sequence alignment through Local Basic Alignment Search Tool (BLAST) program on National Center for Biotechnology Information (NCBI) page at http://www.ncbi.nlm.nih.gov. Phylogenetic tree construction was based on MEGA application. The construction of the evolution distance in the degree of confidence was conducted by using the Neighbor-joining method with the bootstrap value in the NJ plot program with bootstrap 1000.

RESULTS AND DISCUSSION

Inhibition capability of actinomycetes SAE4034 isolates against MDR bacterial growth

Actinomycetes SAE4034 broth culture grown in SCB and SNB media have different inhibitory capabilities against MDR bacteria. SCB culture filtrate showed inhibition to *S. aureus* (ϕ 9 mm) and no inhibition showed to other MDR bacterial growth. While, the 14 days-filtrate of SNB culture showed inhibition to the growth of *S. aureus* (ϕ CZ was 10 mm) and *Enterococcus* sp (ϕ CZ was 8 mm). The 21 days-filtrate yielded a diameter of the 9 mm inhibition zone to *S. aureus* and 10 mm to *P. aureuginosa*.

The results of antibacterial assay from SAE4034 actinomycetes liquid culture extract using diffusion method showed the ability of inhibition against 5 MDR bacteria, both Gram-positive and Gram-negative bacteria. Liquid culture extract on SCB medium inhibited highest to *S.aureus* bacteria (φ12 mm) and lowest to *E. coli* (φ 6,5 mm). While, the liquid culture extract of

SNB medium showed the inhibitory ability to 5 MDR bacteria on PCA medium higher than the test on Mueller Hinton medium (Figure 1). The widest clear zone diameter was shown by *S. aureus* (21.5 mm) and the lowest (12.5 mm) by *E. coli* and *K. pneumoniae* (Figures 1, 2 and 3). *Streptomyces* SAE40.3.4 isolate inhibition ability was stable, observed from the test results up to more than 48 hours which was still showing a consistent inhibitory capability. The antagonist assay was conducted by using PCA and Mueller Hinton media rather than NA due to a fear of contamination by other bacteria. Sipriyadi *et al.* (2016) successfully assayed antagonist test of actinomycetes isolated from CIFOR forest against *E. coli* and *Bacillus* sp. by pouring bacteria on the top of nutrient media, and the actinomycetes colony was placed on the medium in the upside-down position.

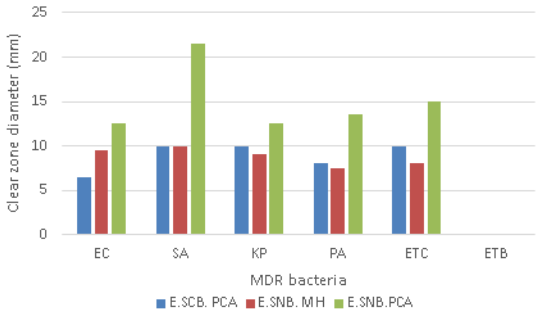

Figure 1. The clear zone diameter produced by the crude extract of SAE4034 culture cultivated in Starch Casein Broth and Starch Nitrate Broth on the PCA and Mueller Hinton media against *E. coli* (EC), *S. aureus* (SA), *K. pneumoniae* (KP), *P. aureoginosa* (PA), *Enterococcus* sp. (ETC), *E. cloacae* (ETB)

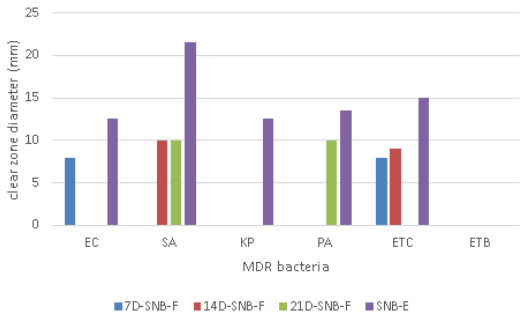

Figure 2. Inhibitory zone produced by actinomycetes SAE4034 filtrate and crude extract cultured in SNB medium during 7, 14 and 21 days

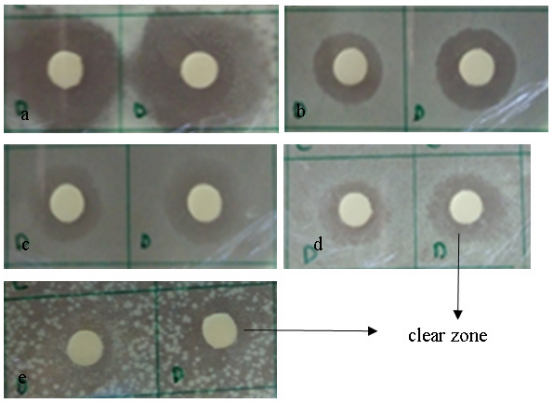

Figure 3. In orderly from left to right: clear zone produced by actinomycetes SAE40.3.4 isolate (notation D) against *S. aureus* (a), *K. pneumonia* (b), *E. coli* (c), *P. aeroginosa* (d), and *Enterococcus sp. (e)*

The capability of actinomycetes SAE40.3.4 isolate in producing antibacterial compounds was shown in the TLC test that produced 4 spots, each with a value of Rf 0.36; 0.45; 0.54; and 0.6. The bioactive compound was produced by biomass during the incubation period of 7-21 days which increases on day 14 and decreases at day 21 of incubation period. The culture changed pH during the incubation period, from 4.92 at the beginning to 6.3 at the end of the incubation period (Table 1 and Figure 4).

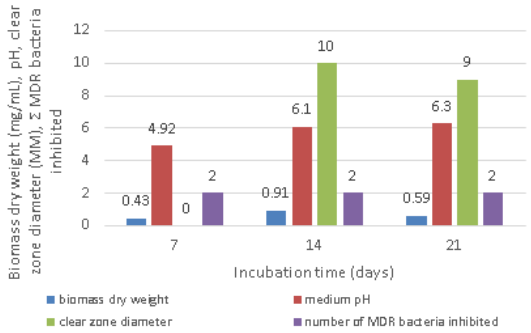

Figure 4. Biomass dry weight, culture pH, clear zone produced against *S. aureus*, and number of MDR bacteria inhibited by actinomycetes SAE4034 isolate during incubation periods of 7, 14, and 21 days

Morphology and biochemical observation of actinomycetes SAE4034 isolate

Isolate SAE4034 showed actinomyce-

Table 1. Biomass dry weight, culture pH, clear zone produced against *S. aureus*, and number of MDR bacteria inhibited by actinomycetes SAE4034 isolate during incubation periods of 7, 14, and 21 days

Incubation time (days)	Biomass dry weight (gram/100 mL)	Medium pH	CZ produced by filtrate against *S. aureus* (mm)	Number of MDR bacteria inhibited
7	43	4.92	0	2 (EC & ETC)
14	91	6.1	10	2 (SA & ETC)
21	58.5	6.3	9	2 (SA & `PA)

tes characteristics, such as slow growth, exhibited mycelium structures, and aerial mycelium supported the chain of spores. This isolate was obtained from the mud of *Rhizophora apiculata* rhizosphere at location E40 of Segara Anakan lagoon area. The environmental condition of the sampling site has an ambient temperature of 25-28˚C, soil pH of 6-7, and salinity of 41 ppm.

Isolate SAE4034 has aerial mycelium on the colony surface, substrate mycelium under colony and no pigment diffused into the medium. The colony has cream color and the surface showed powdery type colony. The aerial mycelium looks spiral form, fragmented into coccoid, consisted of long-chain spores (Figure 5 and 6). Based on the colony and mycelium characters, isolate actinomycetes SAE4034 was predicted as *Streptomyces*.

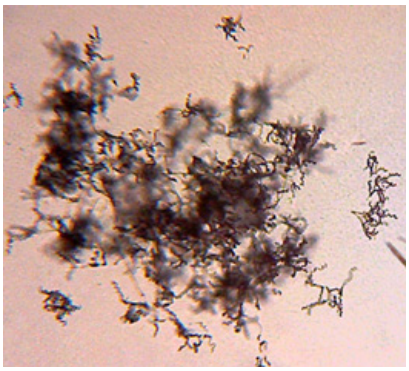

Figure 5. The morphology of aerial mycelium was observed with a 400x magnification light microscope

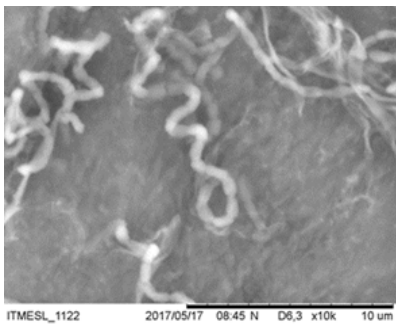

Figure 6. The morphology of aerial mycelium was observed with an SEM of 10,000x magnification

Table 2. The observed physiological characteristics

Characteristics	Results of Observation
Colony shape	circular
Colony diameter	2-3 mm
Colony surface	powdery
Aerial mycelium	Form spiral, fragmented into coccoid
Substrate mycelium	positive
Diffusion pigment	negative
Spore chain	Long chain spores
Fructose	+
Sucrose	+
Raffinose	+
Rhamnose	-
Inositol	-
Mannitol	-
Arabinose	+
Xylose	+
Amylolytic	-
Catalase test	+
Oxidase test	+
Growth in pH 4	+
Growth in pH 8,5	+++
Growth in temperature 50°C	+
Growth in temperature 37°C	++
Growth in room temperature	+++
Growth in temperature 4°C	+
Growth in salinity 3%	++
Growth in salinity 5%	+

The results of physiological and biochemical assays showed that isolate SAE4034 was able to use sugars in the form of fructose, sucrose,

raffinose, arabinose, xylose and negative test in rhamnose, inositol, mannitol sugars. The isolate also exhibited positively result in amylolytic, oxidase and catalase tests.

Amplification and sequencing of 16S rRNA gene

The result of amplification test by using a universal 16S primer and sequencing of 16S gene showed a band with nucleotide base sequence was most closely related to *Streptomyces* sp. N56 with similarity index of 95% (access number HQ132789.1) as shown by Figure 7.

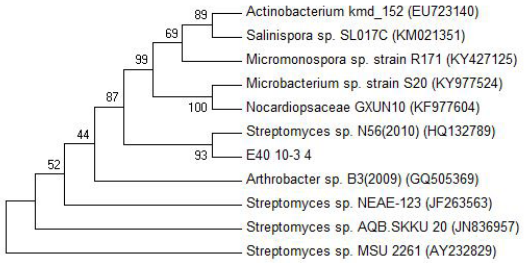

Figure 7. Phylogeny tree of *Streptomyces* sp. SAE4034 isolate and its position between other actinomycetes

At the same volume, the crude extract contains more concentrated antibacterial compounds, while the filtrate is more dilute. This led the crude extract results in higher inhibition than the culture filtrate. Bioactive compounds produced by actinomycetes biomass were affected by the incubation period. The exponential phase growth of SAE4034 isolate was at 7-10 days of incubation period. Incubation period of 14 days produced a higher biomass than other incubation period. Incubation period of 14 days could be the best phase for producing bioactive compounds, as well as five actinomycetes isolated from mine soil samples collected from Salem, Tamilnadu, the maximum antibacterial activity toward multi drugs resistant bacterial wound isolates was also observed on 14th-day incubation (Ashokkumar *et al.*, 2012). At the end of the incubation time (21 days), the biomass weight and inhibitory activity was decreased, since the antibacterial compound was synthesized at the end exponential growth phase, however, it may accumulate until the end of the incubation period. Production of tetracycline was optimum during 4-7 days, the longer the incubation time the the decrease of antibiotic produced was occurred (Mahesh & Nath, 2013). The growth of *Streptomyces kananmyceticus* M27 was not directly related to antibiotic synthesis. The pH of the medium might be an important factor for antibiotic formation, as media giving high antibiotic yields showed an alkaline pH (Pandey *et al.*, 2005).

A higher observed inhibition activity came from SNB culture than the one from SCB medium. SCB medium was commonly used as a cultivation medium for actinomycetes that contain casein as the nitrogen source for supporting growth. As a growth medium, it supported growth rather than secondary metabolite synthesis unless isolate has a starvation condition. When nutrient become limited, it stimulates secondary metabolite synthesis and results in antibacterial activity. SNB medium contained inorganic nitrate salt that functionate as inorganic nitrogen source and cofactor (Mahesh & Nath, 2013) that supporting secondary metabolite synthesis. The maximum antibiotic biosynthesis by *Streptomyces* isolate J12 was obtained in medium supplemented with 10g/l starch as a sole carbon source and 2.5g/l potassium nitrate in addition to 0.3g/l casein as nitrogen sources at pH 7.2 after six days of incubation (Al-Zahrani, 2007). Starch was also the best carbon source for actinomycin-D production (Hamza *et al.*, 2013).

Inhibition activity was higher against Gram-positive bacteria *S. aureus* rather than to Gram-negative bacteria. This distinct response is due to the different sensitivity of Gram-positive and Gram-negative bacteria cell wall. Gram-negative cell wall has outer membrane structure composed by lipopolysaccharide beside thin peptidoglycan, while Gram-positive cell wall is only composed by thick peptidoglycan. This makes the cell wall impermeable to lipophilic solutes, so that the gram-positive bacteria should be more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier (Ashokkumar *et al.*, 2012).

The antibacterial compound produced by isolate SAE4034 was not known yet, but has Rf values of 0,36; 0,45; 0,54; and 0,6 in the TLC test. Possibly, these compounds affect the variation of inhibitory activities against MDR bacteria. Further research is required for elaborating chemical component to determine the compounds type contained in the extract, in addition to testing on various culture media to determine of higher antibacterial compounds synthesis.

Actinomycetes SAE4034 was isolated from *Rhizophora apiculata* rhizosphere mud, in location E40, a saline environment. It has a powdery colony surface with cream color and slow growth. Under SEM (10.000 times) investigation, the aerial mycelium looked forming a spiral struc-

ture, fragmented into coccoid, and smooth surface spore at the tip (figure 5a and 5b). Common aerial mycelium of *Streptomyces* may stable or fragmented into coccoid, short rod or elongated and based on spore chains it may be divided into rectiflexibiles, retinaculiperti and spirals. Refer to Holt *et al.* (2000), Miyadoh (1997), and Khanna *et al.* (2011) actinomycetes SAE4034 isolate was strongly predicted as *Streptomyces.*

Morphological identification is difficult enough to obtain results up to the species level, therefore 16S rRNA studies help to determine the phylogenetic relationship and make possible the recognition up to species level (Das *et al.*, 2006). Based on sequencing analysis of 16S rRNA gene, actinomycetes SAE4034 isolate has a 96% similarity to *Streptomyces* sp. N56, known inhibit plant pathogen, isolated from soil samples of wheat cropping system from Indo-Gangetic Plains of India with low salinity environment (Malviya *et al.*, 2010). In contrast to *Streptomyces* sp. N56, actinomycetes SAE4034 isolate was isolated from wet environment with higher salinity. This investigation showed an actinomycetes diversity and may contribute a novel actinomycetes list, then needs to be validated by DNA–DNA hybridization and (%) GC content for its identification up to species level. A microbe with a low similarity index (less than 97%) requires further study. Isolates with a high similarity index (greater than 97%) shows similarity at the genus level (Malviya *et al.*, 2011; Da Silva *et al.*, 2013). However, the sequence cannot be compared to the 16S sequence of *Streptomyces* sp. NEAE-102, because the sequence was not registered yet in gene bank and was still being proposed as novel species of *Streptomyces* (El-Naggar *et al.*, 2011). Since of the phenetic and phylogenetic characters of SAE4034 isolate were not similar as those of the existing actinomycetes isolates, it may be proposed as a new strain of *Streptomyces.* These informations usefully contribute in culture collection and new drug investigation for combating infection diseases caused by MDR bacteria.

CONCLUSION

Actinomycetes SAE4034 isolate was *Streptomyces* sp., with the ability to inhibit MDR bacteria. It produces four types of bioactive compounds and could be proposed as a new strain of *Streptomyces* as well as a new source of bioactive compounds.

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