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Credit Author Statement

Achmad Arifiyanto: Conceptualization, Writing-Original draft preparation, Software. **Tini Surtiningsih**: Data curation and Visualization. **Ni'matuzahroh**: Methodology and Investigation. ***Fatimah***: Supervision and Validation **Dyah Agustina**: Project administration. **Nur Hidayatul Alami:** Resources.

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Antimicrobial Activity of Biosurfactants Produced by Actinomycetes Isolated from Rhizosphere of Sidoarjo Mud Region

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Abstract

A unique location with a unique environment attracted many researchers to explore bacteria as an isolate collection especially for actinomycetes group because of the potential of their antimicrobial metabolites. Information on antimicrobial activity by Streptomyces from the Sidoarjo mud area is limited. Biosurfactant was also one of the metabolites produced by the group of actinomycetes. Biosurfactant screening was carried out by growing isolates in a series of tests in the hemolytic, tributyrin agar, surface tension, and emulsification activity. They fermented on glycerol, n-hexane and molasses substrates for 7 days. The antimicrobial activity of organic extract evaluated using a paper disc diffusion method against E. coli ATCC 25922 and S. aureus ATCC 6538P. The results of biosurfactant screening tests showed differences in surface tension and varying emulsification activities in the isolates obtained. Supernatants produced by biosurfactants have been shown to be able to show antimicrobial activity as indicated by the formation of inhibitory zones associated with pathogenic growth. AF1 as a selected isolates identified as *Streptomyces* sp. MN394821 using 16S rDNA gene analysis. It has 100% similarity with accession-coded strain KY236015.1 and 98% with accession coded Streptomyces rochei AB1 strain GU434672.1.

Keywords: Antibiotic; Active compound; Biosurfactant; Streptomyes

1. Introduction

Microbes are found to live freely or stick to the surface of soil particles, but most soil bacteria interact with plant roots known as the rhizosphere. The main reason was the increasing rate of availability of dissolved organic compounds derived from exudation of plant roots [1]. The location around Sidoarjo mudflow was once a fertile agricultural land, the environmental conditions at that location are also interesting to be investigated further about the potential of microorganisms in these habitats. A number of studies using the microflora of these habitats have been widely reported. Poernomo [2] interested in proteolytic enzymes, while Habibie [3] examined the potential of xylanase, Pracahyo [4]

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identified potential bacteria to inhibit pathogenic molds in rice, and overall informs the thermophilic ability of bacteria isolated, as the research focus by Dagdag [5].

Actinomycetes or Order Actinomycetales are members of the Actinobacteria group. Actinomycetes belong to the group of Gram positive bacteria, with filaments characteristic. He plays an important role in various industries because of his ability produced a number of metabolite compounds that vary in structure and function. Many metabolites by actinomycetes are reported to have antagonistic abilities against bacteria, yeast, and mold. For this reason, many actinomycetes are developed and used as medicinal ingredients in the prevention of various diseases. The search for actinomycetes metabolites develops from exploration of certain regions, unique environments, variations in isolation methods, innovation to manipulation of genetic material [6]. Most microorganisms, including actinomycetes, are known to produce active metabolites which can reduce surface tension. This compound itself is better known as surfactant. A number of biosurfactant groups are known to have antimicrobial activity, including glycolipids, lipopeptides & lipoproteins [7]. On the other hand, actinomycetes bacteria are spread over a wide habitat. It can be found in various types of soil samples, fresh and marine waters, mangrove ecosystems [8], thermofilic and alkaliphilic [9], acidophilic [10] and halophilic [11] or even though at Antartic region [12]. Therefore it is also called the extremophilic group. Different ecological environments, with the surrounding physical, chemical and biological conditions often play a role in the discovery of new types of bacteria and metabolites.

The actinomycetes study in Indonesia itself is being carried out. Susilowati [13] collected actinomycetes in various agricultural and plantation areas on the islands of Java, Sumatra, Sulawesi, and NTT by focusing isolation in the soil's rhizosphere. Nurkanto [14] explored land actinomycetes in Ternate. Meanwhile there is no information about actinomycetes isolated from Sidoarjo mud.

2. Materials and Methods

2.1. Sampling and isolation of actinomycetes

Soil samples taken using soil borers at a depth of 30 cm through line transect method around six burst embankments (112.70487,-7.52393) (112.70325,-7.52912) (112.70326,-7.53282) (112.70355,-7.53272) (112.70734,-7.51744) (112.71107,-7.51237). Isolation of actinomycetes was carried out using the dry-heating method. The soil dried in the oven for approximately 8 days at 70°C. Soil samples crushed and filtered with a 3-5 mm sieve. Then 1 g of soil was taken, suspended in 9 mL of sterile water, shaken in 2 minutes, and left for 1 minute. They diluted fully for 10-8 dilutions. The last 3 series of dilutions are 10-6, 10-7, and 10-8 also the first dilution is taken as much as 0.1 mL and spread on a Petri dish containing Starch Casein Agar or SCA (Himedia®) medium which is added with griseofulvin and chloramphenicol 0.05 ppm. The surface of the medium is flattened using a spatula. The Petri dish was then incubated for 1-3 weeks at 70°C.

Isolates were observed microscopically and accompanied by Gram staining to determine whether or not the actinomycetes group was present. Storage of isolates was carried out on sloping SCA media and as stock culture and work culture. Long-term storage of o them stored using liquid drying method with cryoprotective media whose composition consisted of ribitol or adenitol 1.5 g; phosphate buffer pH 7.2 100 mL, and monosodium glutamate 3 g.

2.2. Hemolytic test

Isolate is grown on Blood agar medium. A total of 1.5 mL of fresh sterile lamb blood was pipetted into an Erlenmeyer flask containing 100 ml of mineral-agar media and then homogenized. The blood medium poured and kept sterile until it got frozen and solid. Biosurfactant potential bacterial strains were scratched on fresh blood media. It was incubated for 1-2 days at 37°C. It was checked for the zone of hemolysis and color changes formed from the activity of the test bacteria

2.3. Lipase test

Lipase activity was measured by growing bacterial isolates on agar tributyrin medium. This medium is made by adding 10 mL of tributyrin (Himedia®) to 990 ml of agar solution (5 g of peptone, 3 g of yeast extract, and 15 g of agar) and then heated and sterilized in an autoclave at 121°C. This medium should not be poured on disposable Petri dishes or other tools made of plastic. It affected the objectivity in assessing the ability of bacteria in lipase activity tests, because plastic included as a hydrocarbon group that was sensitive to the lipase enzyme.

2.4. Surface tension test

Surface tension measurement conducted using the Kruss K100 tensiometer (Kruss GmbH, Hamburg, Germany) using the Du Nouy ring method. Measurements were made using crude surfactant previously obtained. It tested at room temperature (3x replication used to improve measurement accuracy and take an average). The formula below referred to [15],

$$\gamma 2 = \gamma 1 \frac{n1\rho 1}{n2\rho 2} \quad (1)$$

γ 1 = water surface tension, 72 dyne / cm, γ 2 = biosurfactant surface tension, ρ 1 = water density, ρ 2 = biosurfactant density, n1 = number of drops of water, and n2 = number of drops of bsiosurfactant solution.

[1] Chakraborty *et al.*, 2015.

2.5. Index of Emulsification

The ability of biosurfactant emulsifies liquid of hydrocarbons determined using kerosene. Bacterial cultures were inoculated on a liquid production medium and incubated for 24 hours. Bacterial cell-free supernatant had obtained through centrifugation at 20.000 G for 10 minutes and homogenized with hydrocarbons on the vortex machine at high speed for 2 minutes. Index of emulsification counted from height of fluid layer emulsion divided by the entire system height multiplied by 100. The stability measured after one hour. The emulsification activity of biosurfactant was determined by measuring the emulsification index (E24 or EI). It can be calculated using the following formula:

$$\mathrm{EI} = \frac{Total\ height\ of\ the\ emulsion\ layer}{Total\ height\ of\ the\ system} \text{ X } 100 \quad (2)$$

[2] Chakraborty *et al.*, 2015.

2.6. Antimicrobial activity test

Antimicrobial activity tested using agar disc diffusion test at Mueller-Hinton agar (MHA; Himedia®). *E. Coli* ATTCC (-), and *Staphylococcus aureus* ATTCC (+) cultured overnight in Mueller-Hinton broth at 37° C. Optical Density broth cultures were adjusted to 0.1 or equivalent to 108 CFU mL-1 inoculum (according to McFarland turbidity standards). About 500 μL inoculated on Petri dish then the MHA medium (10mL) was poured on the surface and spread uniformly. It air dried for 10 minutes under laminar sterile air flow. Biosurfactant extracted using chloroform: methanol (3:1/v:v). Sterile Whatman disc filter (5 mm in diameter) dripped with 10 μL of a different solution of biosurfactant in methanol aseptically on the surface of the MHA agar. A disc dipped by methanol used as a negative control. The dish is incubated in 37°C for 24 hours. The inhibition zone (mm) around the well and discs measured triplicate reegarding the antibiotic zone scale (Himedia®).

2.7. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy (8201PC Shimadzu, Japan) was carried out using crude biosurfactant (extracted using chloroform: methanol (3:1/v:v)). The FTIR spectrum, with a resolution of 4 cm^{-1}, was collected from 500 to 4000 cm^{-1}. The basic functional groups of the biosurfactant were analyzed

2.8. Isolate identification

Suspected isolates of actinomycetes were identified by morphological, physiological, biochemical [16] and molecular approaches. Molecular identification of bacteria suspected of actinomycetes tested using primers 27F-5'AGAGTTTGATCCTGGCTCAG-3 'and 149R-5'GGTTACCTTGTTACGACTT3'. PCR was carried out by mixing 2.5 μL 10X PCR buffer, 0.5 μL mixtures of dNTP, 1.0 μL of each PCR primer, 0.5 μL of Taq DNA polymerase, and 1.0 μL of each template DNA. Initial denaturation at 94°C for 25 seconds, followed by denaturation at 94°C for 10 seconds for 35 cycles, annealing at 46°C for 30 seconds, elongation at 72°C for 1.5 minutes, then final extension at

72°C for 10 minute. The PCR results were then further sequenced and the results were compared with other bacterial sequences at Genbank.

3. Results and Discussion

3.1. Biosurfactant screening

Biosurfactant is a variety of groups of active compounds produced by various types of microorganisms. Has a polar and non-polar group that is able to separate water from oil or air surface with water so that it can reduce interface tension or surface tension. Because of this ability, biosurfactants are widely offered as a solution to overcome waste oil pollution [17]. A number of biosurfactants are known as antibacterial, antifungal and antiviral agents. Actinomycetes are a large group of actinobacteria members known for their ability in antibiotic activity and many are reported to be able to produce biosurfactants.

3.1.1. Hemolytic activity

Hemolytic reaction on the Blood agar plate was observed by lifting Petri to the light source, the results of hemolytic activity can be seen in table 1. There are 3 types of hemolysis namely beta hemolysis (β), alpha hemolysis (α), and gamma hemolysis (γ). Beta hemolysis (β) or commonly called total hemolysis is defined as the lysis of all red blood cells. A clear zone, close to the color of the transparency of the base media and surround the colony. Many species of bacteria produce toxins or poisons that can destroy red blood cells. Some species display various levels of beta hemolysis. The hemolysis alpha (α) also called partial hemolysis is a decrease in red blood cell hemoglobin for methemoglobin in the medium around the colonies. This causes changes in green or brown in the medium. Color can be likened to "bruising" cells. Microscopic examination of alpha-hemolyzed red blood cells shows that the cell membrane is intact, so the cell is not really lysis. Gamma hemolysis (γ) is also called non hemolysis. Gamma shows a lack of hemolysis, because there is no reaction in the surrounding medium.

Table 1: Hydrolysis results for actinomycetes grown in blood agar medium

No	Isolate	Hydrolysis results	
1	AA1	+	α
2	AA2	-	γ
3	AB1	+	α
4	AB8	+	α
5	AF1	+	β
6	AF2	+	β
7	AF3	+	α
8	AF4	+	α
9	AF5	-	γ
10	AF6	+	α
11	AG1	-	γ
12	AG2	+	α

From the results of the study as presented in Table 1, there are 4 isolates that are negative for hemolytic activity, namely AA2, AF3, AF5, and AG1. While 8 other isolates were categorized positively hydrolyzing the blood agar medium namely AA1, AB1, AB8, AF1, AF2, AF4, AF6, and AG2. There are only two isolates namely AF1 and AF2 which show the results of β hemolysis. Suthindhiran [18] suspected that the ability of actinomycetes in hemolytic activity is likely to be used as an anticancer agent because of its cytotoxic properties in human and mouse blood cells.

3.1.2. Lipolytic activity

The tributyrin medium is widely used to test the lipase enzyme, which is exoenzyme, which is able to hydrolyze the medium containing tributyrin oil. Lipase plays a role in breaking the lipid complex into simple fragments. Tributyrin oil is a type of lipid group called triglycerides. Triglycerides are composed of glycerol and fatty acids. The tributyrin oil suspension will make the medium become opaque or look thick. Lipase enzymes produced by microorganisms will

break down tributyrins, so that a halo zone (clear) formed around the area of microbial growth. Microbes including actinomycetes will break the bonds of glycerol and fatty acids so that they can be converted into final products that will be used to produce energy and other metabolic processes. According Fig.1 AF3 and AF1 was top pruducer of lipase.

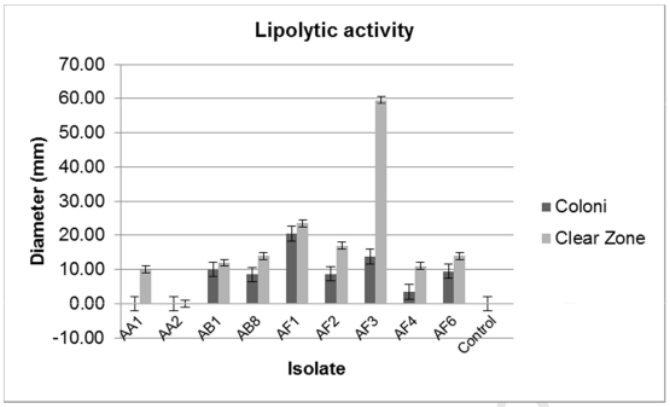

Fig. 1. Comparison of colony growth and formation of clear zones in actinomycetes isolates through lipolytic test

3.1 3. Surface tension test and Emulsification index

The resulting free cell supernatant is measured by its surface tension value using a Du Nouy tensiometer at room temperature. The results of surface tension measurements and a decrease in the value of the surface tension to the control can be seen in Fig 2. The value of the surface tension measured at day 0 (control) and day 7). The decrease in surface tension is caused by the presence of biosurfactants in the supernatant produced by bacterial isolates during the growth process.

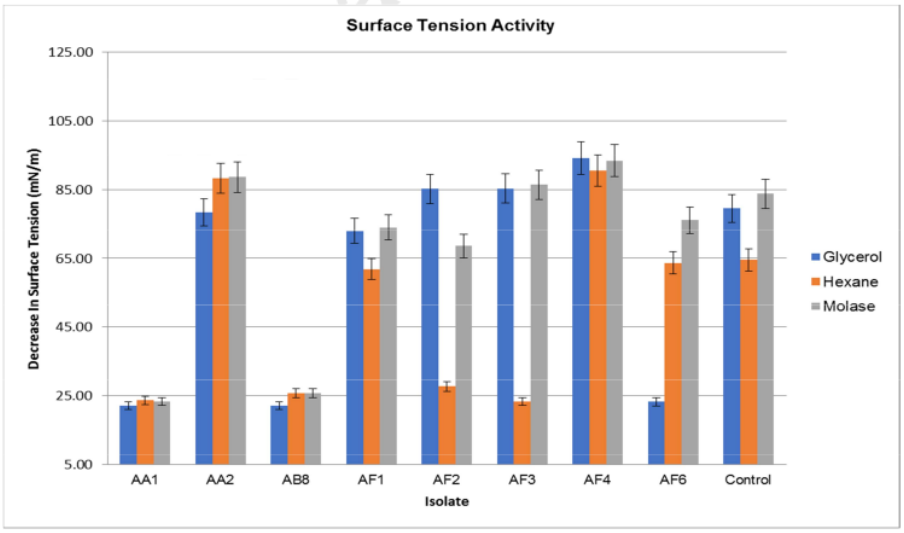

Fig. 2. Surface tension on actinomycetes isolates growing on glycerol, n-hexane and molase substrate media

Based on the results of surface tension measurements (Fig. 2), it was found that most of the isolates were able to reduce surface tension except AF2, AF3 and AF4 isolates on glycerol fermentation medium, AA2 and AF4 isolates on n-hexane medium and AA2, AF3, and AF4 isolates on molasses medium. Three isolates that were characterized as actinomycetes had surface tension values ranging from Δ 56.22 mN / m for AF6, Δ 57.27 mN / m for AB8, and Δ 57.33 mN / m for AA1.

AF3 isolates ranked first in reducing surface stress on fermentation using n-hexane substrate (Fig. 2) with a value of Δ 41.28 mN / m followed by AA1 with a reduced surface tension of Δ 41.28 mN / m. On molasses substrate (Fig 2) AA1 isolate reduced the surface tension by Δ 60.39 mN / m, at once being the highest among other isolates.

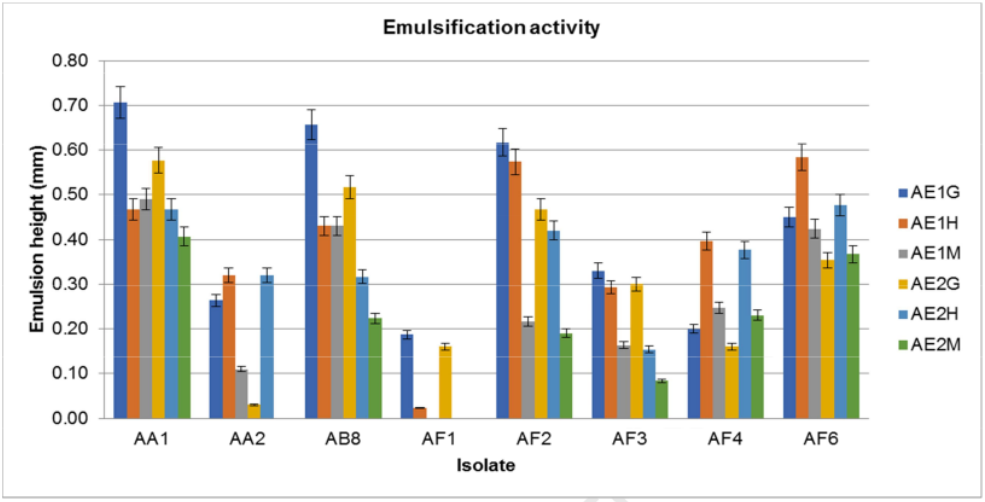

Fig. 3. Emulsification activity conducted in the glycerol, n-hexane and molasses fermentation medium. AE1G: Emulsification activity of 1st hour at glycerol media, AE2G: Emulsification activity after a day at glycerol media, AE1H: Emulsification activity of 1st hour at n-hexane media, AE2H: Emulsification activity after a day at n-hexane media, AE1M: Emulsification activity of 1st hour at molase media, AE2M: Emulsification activity after a day at molase media.

The emulsification activity (AE) of biosurfactants determined by measuring the emulsification index produced (Fig. 3). The presence of emulsification activity in the media can be an indicator that biosurfactant compounds have been produced by microorganisms. AE data indicate that there was an emulsion forming which appeared to be a gas bubble enveloping the fermentation medium. Bacteria use an emulsion as a mechanism of interaction with the substrate and it is one of the biosurfactant characters produced by microorganisms. Bacteria expand surface contact with the substrate by producing biosurfactants, as well as facilitating substrate transport into the cell. AE was observed in the first hour of contact of the supernatant and hydrocarbons namely kerosene and it subsequently observed again after 24 hours.

3.2. Antimicrobial activity test

Media fermented untill 7th day was continued by testing the antimicrobial activity. The results of the antimicrobial activity test (figure 4) was show that AF1 isolates have the largest average inhibition zone diameter in inhibiting the growth of *S. aureus* ATCC 6538P which around 12 mm ± 3.75 mm especially in the molasses medium. AA1 isolates as isolates with the highest inhibitory zone in the molasses medium were 7.7 mm ± 2.15 mm. AB8 with inhibition zone of 6.65 mm ± 2.28 mm in the glycerol medium. Molasses medium contributes positively compared to other media in terms of producing antimicrobial biosurfactants. Isolate AA1, AA2, AB8, AF1, AF2 and AF6 produced biosurfactants that are more antagonistic to *S. aureus* ATCC 6538P when they had grown on molasses medium. AB8, AA1 and AF2 isolates are able to produce biosurfactants which inhibit the growth of pathogens both *S. aureus* ATCC 6538P and *E. coli* ATCC 25922 (see Fig. 4).

Streptomyces VITSDK1 spp., [18] are more antagonistic to Gram negative bacteria, with a inhibition zone of 10.3 mm in *Klebsiella pneumoniae*. *Streptomyces* VITSDK1 spp., inhibits *Staphylococcus aureus* by 5.3 mm. This shows that the antimicrobial activity obtained in this study was reach above it in terms of inhibition zones in Gram positive or negative Gram bacteria.

Antimicrobial mechanisms might be occur due to disruption of cell wall synthesis, damage to membrane permeability, inhibition of protein synthesis, DNA / RNA synthesis and metabolism. The ability to reduce surface tension in actinomycetes isolates was thought to play a role in damaging cell walls and permeability of pathogenic membranes. The integrity of the disturbed wall and membrane will inhibit various target cell metabolisms. Biosurfactants also acted

as fungicidal, bactericidal, insecticidal and anti-viral ingredients because of their anti-adhesive agents and enzyme inhibitors [19]. Rhamnolipid was one of the biosurfactant groups which has antibacterial activity [20], and anticapacity produced from *P. aeruginosa* AT10 [21]. Shoforolipids from *C. bombicola* yeast are broad-spectrum as antibacterial in Gram positive or negative [22]. Lipopeptide was also exploited and gave positive result against methicillin-resistant *Staphylococcus aureus* [23].

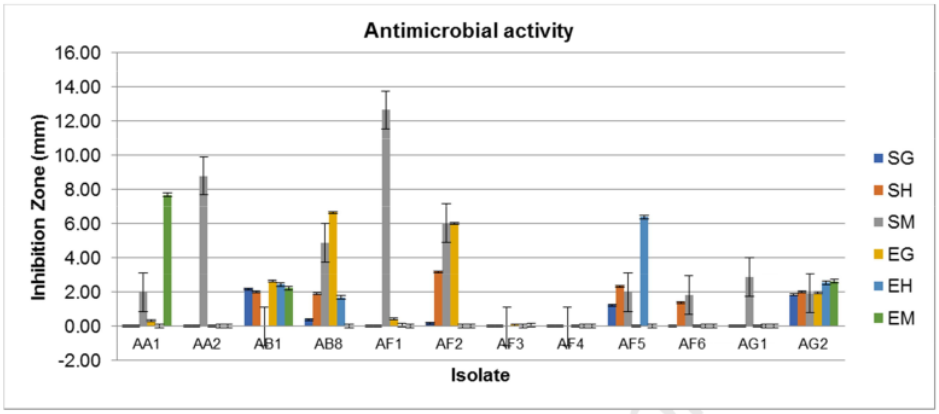

Fig. 4. The clear zone formation (mm) indicates the inhibition of bacterial growth due to exposure of the fermented supernatant after 7 days on the glycerol, n-hexane and molasses substrate media; SG: *S. aureus* ATCC 6538P as pathogen in medium glycerol, SH: *S. aureus* ATCC 6538P as pathogen in medium n-hexane, SM: *S. aureus* ATCC 6538P as pathogen in medium molase, EG: *E. coli* ATCC 25922 as pathogen in medium glycerol, EH: *E. coli* ATCC 25922 as pathogen in medium n-hexane, EM: *E. coli* ATCC 25922 as pathogen in medium molase.

3.3. Fourier Transform Infrared Spectroscopy (FTIR)

Result of IR spectral analysis shown different peak at Fig. 5, the sharp at 3410.15 cm^{-1} was expressed as O-H group. The appearance of this peak was confirmed that the sample contains phenol or alcohol group. Wave numbers ranging from 3600 cm^{-1} to 3100 cm^{-1} was observed. This corresponded to compound that contains amine (N-H) group. Sharp absorbance peaks are observed at 2924.09 cm^{-1}, 1157.29 cm^{-1}, and 1026.13 cm^{-1}.They were indicated aliphatic chains with ether and amines. The peak 1651.07 cm^{-1} was double bonds (alkenes). Spectrum 1419.61 cm^{-1} that is a bending vibration of C-H from CH_2 while 1373.32 cm^{-1} is contains nitro compounds. Amine group based on FTIR spectrum with positive lipolytic activity regarding halo zone at tributyrin media was allegedly the presence of a lipopeptide biosurfactant.

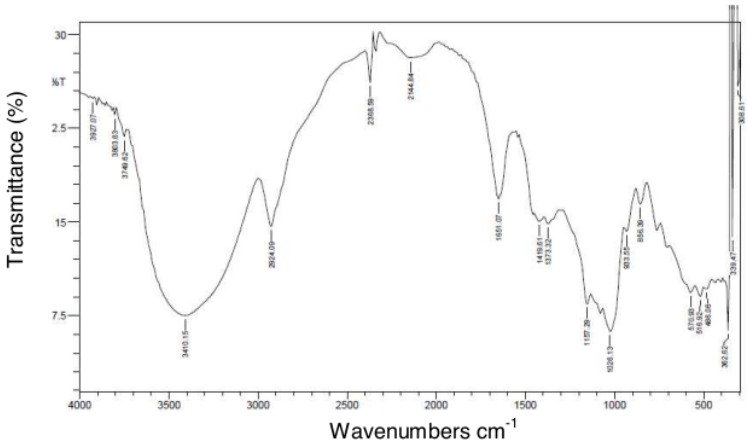

Fig. 5. FTIR spectrum of the biosurfactant.

3.4. Identification selected isolate

According to Fig. 4, AF1 was the highest isolate that has ability inhibit pathogen proven by clear zone formation. It sequenced using 16S rRNA and tabulation data from BLAST® results indicated that isolate AF1 belongs to the genus *Streptomyces* spp., and it deposited at GenBank under MN394821 accession number. The percentage of identity reached 100% similarity with the *Streptomyces* sp. strain of actinomycetes 16S ribosomal RNA with accession code KY236015.1. (Fig 6)

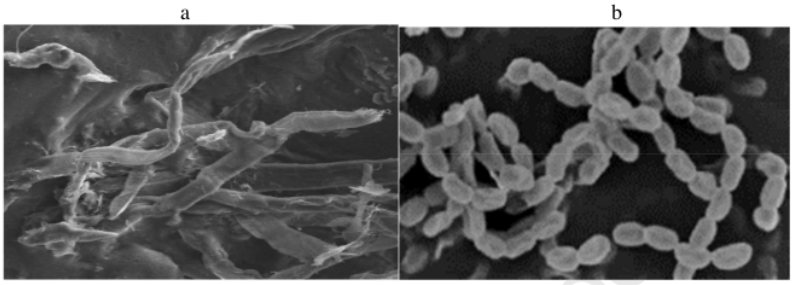

Fig.6. (a) *Streptomyces* sp. strain AF1 (left, Author collection) and (b) *Streptomyces rochei* strain AB1 (right, compared to [24])

4. Conclusions

Lapindo mud volcanoe in Sidoarjo proven as a unique environment that now *Streptomyces* sp., collected from there. *Streptomyces* sp. strain AF1 has a unique character. It's able to grow in high temperature around 70^{o} C and produce biosurfactant with antimicrobial activity. It potentially developed as candidate for enzymatic and antibiotic producer for further research.

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PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6

PAGE 7

PAGE 8

PAGE 9

PAGE 10

PAGE 11