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ACTINOMYCETES FROM THE SOIL OF CHILLI PLANTATION IN YOGYAKARTA SHOWING AN ANTAGONISM TO *Fusarium oxysporum* FU3

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Abstract. *Application of biocontrol agent is an environmentally-friendly method to overcome the Fusarium wilt in chilli due to Fusarium oxysporum. This research aimed to isolate actinomycetes antagonist to F. oxysporum FU3 as biocontrol agent from the soil of chilli plantation in Kulon Progo, Yogyakarta. The actinomycetes were isolated using SCA medium and followed by screening for anti-F. oxysporum FU3 on PDA. The filtrate from isolates which showing the highest inhibition zone was produced and tested against strain FU3. The best isolate was then characterized. There are four isolates of actinomycetes showing antagonistic activity against F. oxysporum (AK4, AK5, AK7, and AK9). The highest antagonistic activity was shown by AK5 filtrate with inhibitions on PDA and PDB are 41.71 ± 3.5 % and 81.77 ± 5.1 %, respectively. Further observation showed that filtrate of AK5 caused abnormality and lysis on FU3 hyphae. From the results, we concluded that AK5 is a potential biocontrol agent against F. oxysporum. Further studies to optimize the production and formulize the bio-fungicide are suggested.*

Keywords: *biocontrol, bio-fungicide, fusarium wilt, antagonist, isolation, screening*

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INTRODUCTION

Chilli is an economically valuable plant. According to the data from the Ministry of Agriculture (2016), Indonesia consumed 400.91 thousand tons of red chilli in 2016 and is expected to increase in 2020 to 432.82 thousand tons. One obstacle to increasing chilli production is overcoming fusarium wilt. The disease can cause up to 50% of crop failures (Mona et al., 2012).

Control of *Fusarium* sp. by local farmers generally still use high-dose fungicides which are not environmentally friendly because they can kill mycorrhizae and endanger consumers (Sari et al., 2014). One method of preventing diseases in plants is by giving antifungal compounds. Antifungal compounds can be isolated from bacteria. The ability of bacteria to produce antifungal compounds indicates that the bacteria are potential as biocontrol.

Actinomycetes have been widely studied as antifungal bacteria. Isolation of actinomycetes from various samples has been carried out. Vurukonda et al. (2018) isolated *Streptomyces* sp. from plant rhizosphere and antagonist *Fusarium* spp., *Rhizoctonia solani* and *Phytophthora* spp. Ohike et al. (2018)

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also succeeded in isolating *Streptomyces* sp. AR10 from the soil around the ant nest. The strain can inhibit the growth of *Fusarium solani* properly.

Actinomycetes in the environment play a role in the carbon cycle and the substitution of organic matter. They also act as endophytes in plants and able to stimulate the secretion of secondary metabolites of antibacterial and antifungal compounds (Anandan et al., 2016; Vurukonda et al., 2018). It is assumed that healthy plants interact with actinomycetes antagonist to pathogenic fungi by secreting antifungal. This study aimed to isolate actinomycetes from soil of chilli (*Capsicum* sp.) plantation in Bugel, Kulon Progo, Yogyakarta. Actinomycetes obtained were tested for antagonistic activity against *Fusarium oxysporum* FU3.

MATERIALS AND METHODS

Materials

Soil samples were taken from chilli plantation in Bugel Village. Starch Casein Agar (SCA) [Merck] was used as isolation media. *Fusarium oxysporum* FU3 is a collection of Plant Systematics Laboratory, Biology, UGM and had been isolated from chilli (*Capsicum annuum* L.). Potato Dextrose Agar (PDA) [Merck] and Nutrient Broth (NB) [Himedia] were used to maintain the microorganisms. Potato Dextrose Broth (PDB) [Himedia] and PDA were used in antagonism activity. Other materials used were distilled water [CV. General Labora], NaCl 85%, alcohol 70% [CV. General Labora], Nystatin [PT. Metiska Farma], gram staining, and hydrogen peroxide (H2O2) 3%.

Isolation of Actinomycetes

The soil sample on the surface of the chilli root was collected. A 10 g of soil sample was dissolved in 100 ml of sterile distilled water with a series of dilutions 10^{-1} to 10^{-7}. The soil suspension was homogenized by the vortex. 0.1 mL of the suspension was spread into the medium of SCA containing *Nystatin* (1%). Isolates were incubated for seven days at 28°C. The suspension with the results of bacterial growth with good separation was treated three times. Bacterial isolates that grew with different morphological characters were streaked on NA medium until single colonies were obtained. Pure isolates were kept on NA as stock (Zhao et al., 2014; Gopinath et al., 2018).

Screening Actinomycetes Antagonist *F. oxysporum* FU3

A 5-mm agar plug cut out from 5 days agar culture of *F. oxysporum* FU3 was inoculated at the centre of the PDA plate. Four different isolates were streaked at different points, 2.5 cm from the centre. The negative control plate only contained *F. oxysporum* FU3. Isolates that able to inhibit the growth of *F. oxysporum* FU3 were assayed further.

Antagonism Assay of Potential Isolate Against *F. oxysporum* FU3

Potential isolates were streak at different points 2.5 cm from the centre. The negative control plate only consisted of *F. oxysporum* FU3. The plates were incubated at 28°C for seven days. All treatments were triplicated. Inhibition ratios were calculated using the following formula (1):

$$\text{Inhibitory} = \frac{\text{fungal diameter in the control plate} - \text{fungal diameter in the assay plate}}{\text{pathogenic fungi diameter in the control}} x\ 100\% \quad (1)$$

(Zhao et al. 2014)

Antagonism Assay by Filtrate Against *F. oxysporum* FU3

The filtrate was produced by inoculating one loopful of the best potential isolate into 90 ml of SCB and incubated in shaking culture (28ºC, 150 rpm) for five days. The filtrate was collected by taking 10 mL of shake fermentation and centrifuging at 15000 rpm, 10 minutes, 4°C, which then the supernatant was collected.

Antagonism assay in PDA was done by inoculating 0.2 ml of filtrate into the wells on the medium plates that have been inoculated by *F. oxysporum* FU3 at the centre. All assays were made in 3 replications. The plates were incubated and the inhibition percentages were measured as the previous method.

For antagonism test in PDB, Erlenmeyer flasks containing 20 mL of the media with various concentrations of filtrate from the potential isolate (0 (as control), 1, 2.5 and 5 %vol) were inoculated by *F. oxysporum* FU3 fungal cake (Ø5 mm). The mixtures were incubated in shaking incubator at 28°C and 150 rpm for two days. Fungal biomass was filtered on filter paper, and the dry weight was determined. All assays were done in triplicate. The inhibitory percentage was calculated using the following formula (2):

$$\text{Inhibitory} = \frac{\text{fungal diameter in the control plate} - \text{fungal diameter in the assay plate}}{\text{pathogenic fungi diameter in the control}} x\ 100\% \quad (2)$$

(Wang et al. 2015)

Observation of Hyphae and Spores

Hyphae and spores of *F. oxysporum* FU3 were sampled and observed under a light microscope with the addition of 5 ml 0.01% Tween 80 and 85% NaCl (Zhao et al., 2014).

Characterization of Potential Isolate

Morphological characterization of the potential isolate was based on observation cell shape, colour shape, and margin of the colony. Biochemical characters were based on gram staining, catalase test and oxidase test.

Statistical Analyses

Quantitative data were analysed statistically using Duncan Test.

RESULTS AND DISCUSSION

Isolation and Screening Actinomycetes

This study has isolated antagonist *F. oxysporum* FU3 from the soil. The soil was taken from the root rhizosphere of chilli plantation in Bugel village farmer group's, Kulon Progo, Yogyakarta. The character of the soil was sandy soil and grayish. There were nine actinomycetes obtained (AK2, AK4, AK5, AK6, AK7, AK8, AK9, AK10, AK11) (Figure 1). Actinomycetes are gram-positive bacteria that can produce mycelium. Actinomycetes colonies are very compact; the surface is dry and covered by air mycelium. Actinomycetes colony pigmentation is influenced by the environment and nutrients. The colour of mycelium can be affected by pigmentation. The pigments are blue, purple, red, rose, yellow, green, brown and black (Anandan et al., 2016).

Screening Actinomycetes antagonist *F. oxysporum* FU3

Purified actinomycetes were tested antagonism against *F. oxysporum* FU3 for screening. Screening aimed to obtain potential isolates that have antifungal compounds. In screening, *F. oxysporum* FU3 and four isolates of actinomycetes were grown on single Petri dish (Figure 2).

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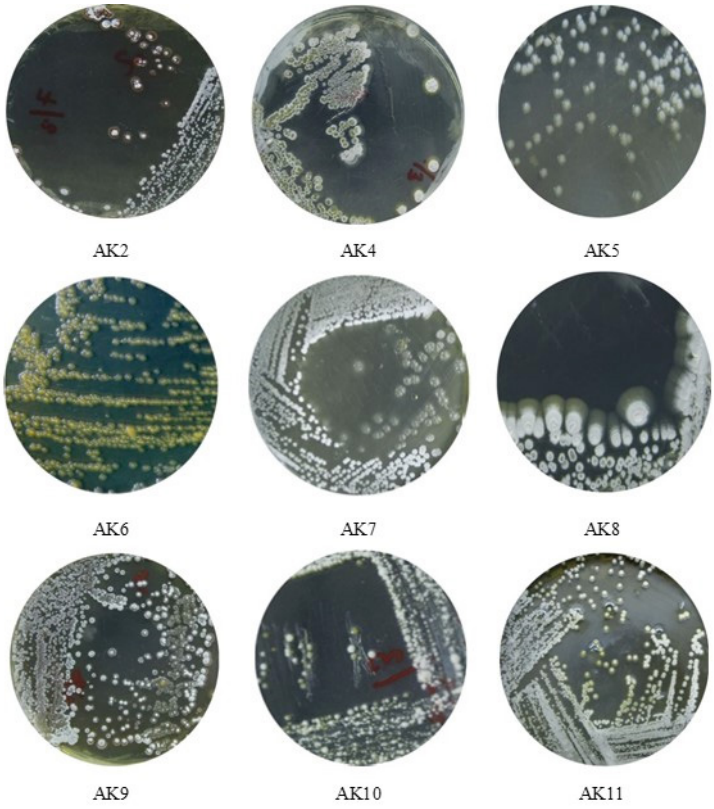

Figure 1. Actinomycetes isolates from the soil of chili plantation.

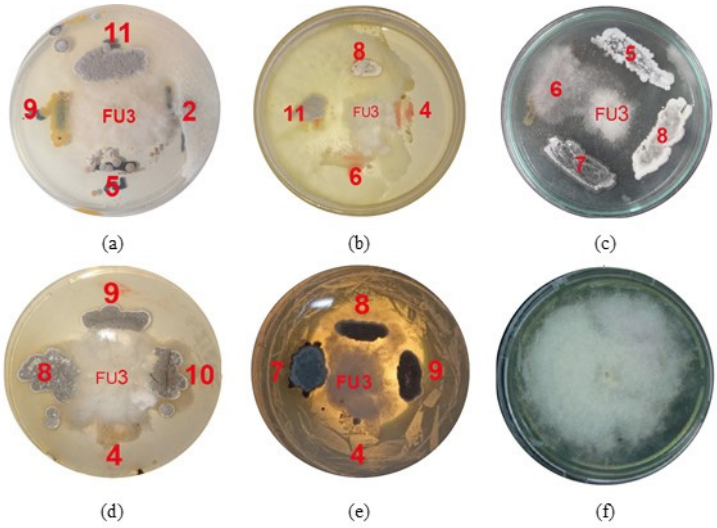

Figure 2. Screening of actinomycetes antagonist *F. oxysporum* FU3. (a) Isolates AK11, AK2, AK5, AK9; (b) Isolates AK4, AK8, AK6, AK11; (c) Iisolates AK5, AK8, AK7, AK6; (d) Isolates AK9, AK10, AK4, AK8; (e) Isolates AK8, AK9, AK4, AK7; (f) Control of *F. oxysporum* FU3.

Table l. Screening of actinomycetes against *F. oxysporum* FU3 based on inhibition distance.

No.	Isolate	Inhibition Distance (mm)
1	AK2	-
2	AK4	1.7±0.4
3	AK5	6.2±0.58
4	AK6	-
5	AK7	3.4±2.4
6	AK8	2.7±4.6
7	AK9	1.9±0.45
8	AK10	-
9	AK11	-

There were four isolates of actinomycetes isolates that showed antagonistic activity against *F. oxysporum*. These isolates were AK4, AK5, AK7 and AK9. The inhibitory distance measured by AK5, AK7 and AK9 were 1.7 ± 0.4 (mm), 6.2 ± 0.58 (mm), 3.4 ± 2.4 (mm) and 1.9 ± 0.45 (mm) respectively. AK8 showed inconsistent inhibition that results in a large standard deviation. AK8 in Figure 2(c) showed good inhibition, while in Figure 2(b) showed no inhibition and overgrowth of *F. oxysporum* FU3. Therefore, AK8 isolates were not included as potential isolates in this study. The potential isolates were tested against *F. oxysporum* FU3 to determine the percentage of inhibition. The test consists of testing isolates and filtrates to inhibit fusarium growth.

AK5 showed higher inhibition than AK4, AK7 and AK9 (Figure 3, Table 2). The inhibitions by AK4, AK5, AK7 and AK9 were 0.65 ± 5.1 %, 47.8 ± 2.96 %, 24.79 ± 15.9 % and 27.19 ± 2.9 %, respectively. AK5 was a potential isolate; therefore, the filtrate was produced and tested.

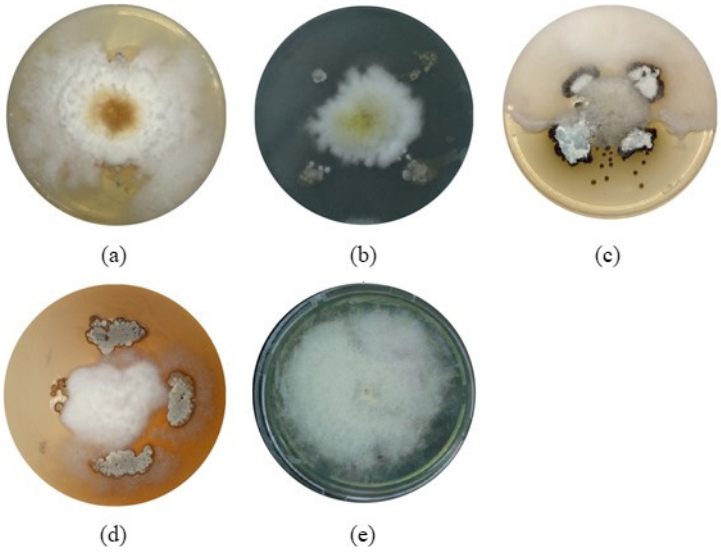

Figure 3. Inhibition of potential isolate against *F. oxysporum* FU3 in PDA. (a) AK4; (b) AK5; (c) AK7; (d) AK9 and (e) Control.

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Table 2. Inhibition by potential isolates against *F. oxysporum* FU3.

Isolate	Ø Growth on the day to day - (mm)						I (%)
	2	3	4	5	6	7	
AK4	20.28±0.63	37.33±1.3	47.44±2.3	55.89±3.7	62.89±2.6	67.89±3.5	0.64±5.1
AK5	17.2±1.74	24.22±1.2	29.67±4.14	33.44±1.73	35.22±1.87	35.67±2*	47.8±2.96
AK7	12.5±0.6	20.17±1.17	29.97±7.16	38.5±8.17	44.94±9.19	51.39±10.9*	24.79±15.9
AK9	12.92±0.82	22.08±0.11	31.67±1.4	37.92±2.47	44.17±3.3	49.75±2*	27.19±2.9
Control	27.77±1.5	38±0.5	48±1	57.77±1.5	72.77±1.1	78.33±1.5	-

*) significant at P<0.05

The inhibition of AK5 filtrate was 41.71 ± 3.5 %. this is lower than inhibition by the isolate directly (Table 3). The filtrate inhibition assay was then continued on PDB by adding a fungus cake (Ø 5 mm) of *F. oxysporum* FU3 and AK5 filtrate to the medium. The filtrate concentration was varied to 1, 2.5 and 5 %vol.

The inhibition of AK5 filtrate concentration of 1, 2.5 and 5 %vol were 61.56 ± 3.1 %, 69.31 ± 3.6 % and 81.77 ± 5.1 %, respectively (Table 4). The one-way ANOVA test at 95% of confidence showed the concentration had a significant effect on the dry weight of FU3. The 5 %vol concentration gave the best inhibition with a significant difference with other concentrations. The concentrations between 2.5 and 1 %vol give a no different effect.

The hyphae *F. oxysporum* FU3 has abnormalities. Figure 5a-b showed lysis hyphae. Figure 5 c-d showed normal microconidia and macroconidia. Hyphae abnormalities indicate the antifungal released by AK5 can affect the hyphae cell wall.

Table 3. Inhibition by filtrate of AK5 against *F. oxysporum* FU3 on PDA.

Isolate	Ø Growth on the day to day - (mm)						I (%)
	2	3	4	5	6	7	
AK5	19.17±1.5	23.83±2.9	28±2.7	34.57±3	38.3±4	39.83±5.8*	41.71±3.5
Control	27.77±1.5	38±0.5	48±1	57.77±1.5	72.77±1.1	78.33±1.5	

*) significant at P<0.05

Table 4. Inhibition by filtrate of AK5 against *F. oxysporum* FU3 in PDB.

Concentration (%vol)	AK5	
	Dry weight (mg)	Inhibition (%)
1	2.13±0.17	61.56±3.1*
2.5	1.7±0.2	69.31±3.6*
5	1.01±0.28	81.77±5.1*
Control	5.54±0.1	-

*) significant at P<0.05

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Figure 4. Inhibition of AK5 filtrate against *F. oxysporum* FU3 in PDA. (a) AK5; (b) Control.

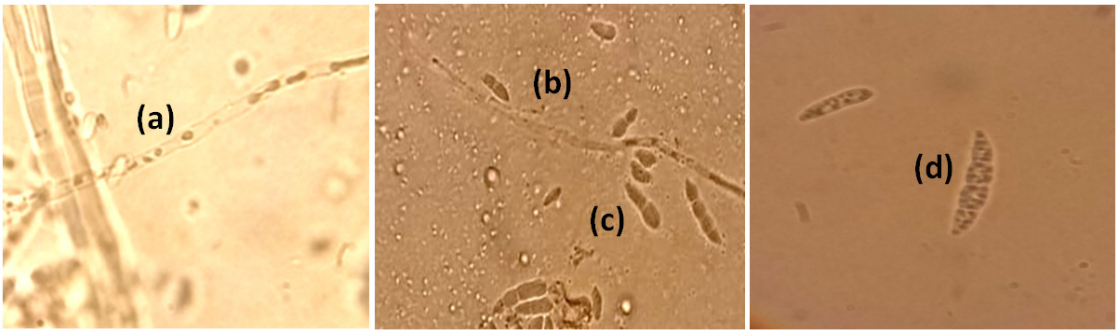

Figure 5. Observation of *F. oxysporum* FU3 after treatment of the antagonism test under a light microscope (1000x). (a-b) Hyphae lysis; (c) Normal microconidia; and (d) Normal macroconidia.

The antagonism test showed that AK5 is able to produce bio fungicide with good and consistent inhibition. The best inhibition by AK5 filtrate was seen at 5 %vol concentration; this is assumed to be influenced by concentration. The higher the concentration added, the higher the inhibitions. However, the inhibitory ability in liquid media cannot be compared with the inhibition on solid media. Koley & Mahapatra (2015) stated that there are dissolved components in the media which can increase dry weight, such as carbon, nitrogen, phosphate, potassium, magnesium and sulfur. These components are contained in both liquid and agar media but can have different influences on the growth rate in liquid or agar media. Therefore, the quantity of growth in the agar and liquid media does not always correlate between the diameter of the colony and the dry weight. Based on the AK5 antagonism test on PDA, the filtrate showed a lower inhibition compared with the isolate. We assumed that the difference was caused by the dependency of filtrate absorption to the agar on the amount inoculated. Therefore, distribution of the filtrate was limit. On the other hand, the growth of colonies and quorum sensing of the isolate resulted in continuous secretion of the bio-fungicide and caused its wide distribution.

Quorum sensing is a signal transduction system that depends on cell density and controls various physiological behaviours in bacteria. Quorum sensing acts as a communication medium between bacterial cells that are not limited by the species. The communication occurs when bacterial cells produce and secrete molecular signals.

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Signals spread to nearby cells and will be recognized by specific receptors after exceeding the threshold concentration. After the molecular signal is recognized, physiologically, the bacterial cell will regulate the gene and induce a larger molecular signal. The mechanism has positive feedback which causes the population to work synchronously (Kavitha et al., 2014).

The inhibition on PDA of AK5 isolate was higher than its filtrate. It assumed that the intensity of AK5 antifungal secretions is influenced by quorum sensing between AK5 cells. In gram-positive bacteria, such as actinomycetes, quorum sensing mechanism involves small peptides as inducers which will bind to specific receptors. The receptor conformation changes and produces phosphorylation of proteins in the cytoplasm. It affects the transcription process because phosphorus is one of the transcription factors. Therefore, it can induce changes in gene expression patterns. Quorum sensing occurs naturally in biofilm formation, virulence, bioluminescence, symbiosis, conjugative plasmid transfer, competence, motility, sporulation, peptide synthesis and antimicrobials (Kavitha et al., 2014).

Wang et al. (2015) compared the inhibition of actinomycetes (Bn035) against *F. oxysporum* in liquid and solid media. In the study, the author used 0.2 mL crude chitinase enzyme produced by Bn035. The filtrate was collected from supernatant of flask fermentation. The inhibition of Bn035 on PDA was 54.70 ± 2.00 % while in PDB was 35.13 ± 1.86 %. Based on their inhibition, Bn035 on PDA has a higher inhibition than AK5. However, AK5 showed better inhibition in PDB. The differences in inhibition are influenced by many factors, such as the ability of secretion of enzyme and active compounds, purity of enzymes, the strain of *F. oxysporum* used, and concentration of the filtrate.

Several antifungals and enzymes can be secreted by actinomycetes. Some of the enzymes are chitinase, cellulase, ligase, amylase and β-1-3-glucanase (Li et al., 2016). The actinomycetes can secrete more than one antifungal. The production of antifungal in *Streptomycetes* begins in response to nutritional stress conditions. Substrate mycelium releases small molecules as signals for aerial initiation of hyphal growth as well as for the production of antibiotics and enzymes in response to competition (Li et al., 2016). In this study, the media did not contain inducers, such as chitin which can induce chitinase enzymes to degrade cell walls of fungi. It assumed the ability of AK5 to lyse hypha cell walls was due to the presence of antifungal secretions instead of enzymes.

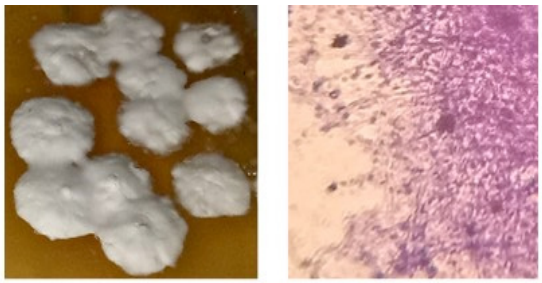
Based on its inhibitory mechanism, the antifungals can affect cell wall synthesis, plasma membrane integrity, nucleic acid synthesis, and ribosome function. Some of the antifungals secreted by actinomycetes are polyenes macrolides, ansamycin, triazole and anthracimycin (Anandan et al., 2016; Krzesniak et al., 2018). Polyene macrolides are common antifungals produced by soil actinomycetes such as *Streptomyces*. The other examples are Nystatin which isolated from *S. noursei*, Amphotericin B from *S. nodosus* and Natamycin from *S. natalensis* (Raja & Prabalarana, 2011; Kong et al., 2013). The inhibiting mechanism of the antifungals is by binding to ergosterol which an essential sterol of the fungus membrane, thus causing cellular leakage (Asif, 2017). A new type of ansamycin, Chaxamycins (A-C), was isolated from *Streptomyces* sp. inhibits the intrinsic activity of ATPase from heat shock 90 (Hsp) proteins. Triazole (azole) causes the depletion of ergosterol and the accumulation of 14-methylsterol in fungal cell membranes. Altered sterol composition affects membrane permeability and inhibits growth and dead cells (Thompson et

al. 2009). Anthracimycin was isolated from *Streptomyces* sp. CNH365 strain inhibits DNA and RNA synthesis (Krzesniak et al., 2018).

Based on its inhibition, antifungals are divided into fungicides and fungistatic. The concentrations of antifungal can be determined based on Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC). MIC is the minimum antifungal concentration that can inhibit fungal growth. MFC is defined as the minimum antifungal concentration that can kill fungi or not produce fungal growth (Dias et al., 2018). Microscopic observation of *F. oxysporum* FU3 aimed to determine the effect of antifungal compounds secreted by potential isolates. The apical hyphae of *F. oxysporum* FU3 has been lysed (Figure 5). The lysis in some parts shows AK5 secretion affecting the fungal cell wall. On the other hand, the microconidia and macroconidia morphology was normal. This indicates that the concentration released by AK5 on a laboratory scale was still fungistatic. However, when concentration increases, higher inhibition is observed (Table 4).

Characterization of AK5

The characters of AK5 based on observations were white air mycelium, creamy vegetative mycelium, colony form was curled, the margin was undulate, and the elevation was low convex, gram-positive and positive catalase (Figure 6(a), Table 5). *Streptomyces* spore types can be divided into several main types. Rectiflexibiles are straight and flexible spore chains. Retinaculiaperti is a spore chain with hooks, open loops or short, irregular spirals and has 1 to 4 turns. Spirales are a spiral spore chain (Li et al., 2016). Based on the shape of the chain (Figure 6(b)), AK5 is assumed to belong to the genus *Streptomyces* with the type of rectiflexibiles spore chain.



(a) (b)

Figure 6. Morphology of AK5. (a) Colony and (b) Hyphae (400x).

Table 5. Morphological and biochemical characters of AK5

Characters	AK5
Morphology	
Colour of air mycelium	White
Colour of vegetative mycelium	Cream
Pigmentation	Brownish
Shape	Curled
Margin	Undulate
Elevation	Low convex
Spore type	Rectiflexibiles
Biochemistry	
Gram	+
Catalase	+

The pigmentation of AK5 is brownish. Actinomycetes colony pigmentation is influenced by the environment and nutrients. The pigments are blue, purple, red, rose, yellow, green, brown, and black. Pigmentation can affect the colour of the media and mycelium. Pigments produced by actinomycetes such as *Streptomyces* sp. can be endopigmen (bound to specific cell structures) or exopigmen (excreted to the surrounding media). Sometimes the pigments produced show secretions from antibiotics (Anandan et al., 2016).

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