ISOLATION AND SCREENING OF SOIL CHITINOLYTIC ACTINOBACTERIA AS THE ANTI-FUNGAL PRODUCER OF PLANT PATHOGENS

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Abstract: Chitinolytic actinobacteria are currently more widely used because of their ability as the biological control agents to the pathogenic fungi, especially in horticultural and plantation crops. This research was conducted to obtain isolates of chitinolytic soil actinobacteria from the rhizosphere of the rubber plant (*Hevea brasiliensis*) area in IPB University. Antifungal activities from these actinobacteria hopely able to inhibit the growth of plant pathogenic fungi in Vitro on chitin agar media. *Fusarium oxysporum* and *Sclerotium rolfsii* are used in the inhibition test of chitinolytic actinobacteria. The results successfully obtained 16 isolates of actinobacteria were grew on *Humic Acid Vitamin* (HV) agar. It showed that six of 16 actinobacteria isolates were able to produce inhibition zones to the growth of hyphae of pathogenic fungi on potato dextrose agar (PDA). KK-15 and KK-07 isolates were able to produce the largest inhibition percentages in *F. oxysporum* and *S. rolfsii*. Based on the chitinolytic index (CI) values, KK-15 and KK-07 isolates produced CI values of 1.25 and 1.5, respectively. The morphological characteristics and Gram staining of both KK-15 and KK-07 isolates are closely relative with *Streptomyces* sp.
Keywords: Chitinolyitic actinobacteria; chitinolytic index; pathogenic fungi

Abstrak: Bakteri kitinolitik saat ini banyak diteliti karena kemampuannya sebagai agens pengendali hayati jamur patogen khususnya pada tanaman hortikultura dan perkebunan. Penelitian ini dilakukan untuk mendapatkan isolat-isolat bakteri kitinolitik asal tanah dari bagian perakaran tanaman karet (*Hevea brasiliensis*) di daerah perkebunan karet Institut Pertanian Bogor (IPB). Aktivitas antifungi dari bakteri ini diharapkan mampu menghambat pertumbuhan jamur patogen tanaman secara in Vitro pada media agar-agar kitin. *Fusarium oxysporum* dan *Sclerotium rolfsii* digunakan dalam uji hambat aktinobakteri kitinolitik. Hasil penelitian memperoleh 16 isolat aktinobakteri berhasil diisolasi dengan menggunakan media agar-agar *Humic Acid*

Vitamin (HV), dan dari 16 isolat tersebut, enam isolat mampu menghasilkan zona hambat terhadap pertumbuhan hifa dari kedua jamur patogen pada media *Potato Dextrose Agar* (PDA) secara *in Vitro*. Isolat KK-15 dan KK-07 mampu menghasilkan diameter hambatan terbesar pada jamur *F. oxysporum* dan *S. rolfsii*. Berdasarkan nilai index kitinolitik (IK), isolat KK-15 dan KK-07 menghasilkan nilai IK sebesar 1,25 dan 1,5 secara berurutan. Karakteristik morfologi dan pewarnaan Gram dari kedua isolat yaitu KK-15 dan KK-07 memiliki kedekatan dengan kelompok *Streptomyces* spp.
Kata kunci: Aktinobakteri kitinolitik; indeks kitinolitik; jamur patogen

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Introduction

Actinobacteria is a group of Gram-positive bacteria that are widespread and have special characteristics that the G-C content is relatively high compared to other bacteria (Harvey, Brzezinski, Beaulieu, 2018; Lewin et al., 2016). These groups of organisms spread in the soil and the water as both free-living saprophytes, and some can live in symbiosis in plant tissues to form the endophytes (Matsumoto and Takahashi, 2017). Actinobacteria have the main role in the process of the carbon cycle, especially in the weathering and dissolving of plant and fungal cell walls, and also in insect cuticles and crustacean shells (Chater, 2016).

Actinobacteria secrete extracellular hydrolytic enzymes that are involved in the degradation of cellulose compounds (Wang et al., 2016), keratin (Mukhtar 2017), and chitin compounds (Beier et al., 2013). Chitin is a polysaccharide (polymer) built by N-acetylglucosamine monomer units arranged linearly with β (1-4) glycoside bonds, and the monomer structure of chitin is linked with β (1-4) glycoside bonds. Chitin spreads in nature, such as in insect cuticles, shrimp shells, nematodes, and cell walls of most fungi (Veliz, Martinez, and Hirsch, 2017). This compound can be degraded into a simple monomer of N-acetyl glucosamine by the chitinase enzyme produced by chitinolytic microorganisms, which are currently used as biocontrol agents that are effective against fungal and insect attacks.

Chitinolytic microorganisms are currently being researched, especially their ability to control plant disease, especially those caused by pathogenic fungi (Nagpure, Choudhary, and Gupta, 2014). Fungi generally have cell walls containing chitin compounds. The presence of chitinolytic microorganisms in the soil, especially in plant rhizoplanes and filoplanes, can protect plants from fungal infections. Chitin contained in the cell walls of pathogenic fungi can be degraded

or lysed by chitinolytic microorganisms, thereby reducing the occurrence of disease infections.

Microorganisms with chitinolytic abilities are believed to be able to play a role in controlling the attack of pathogenic fungi by making chitin a source of carbon and nitrogen (Jholapara, Mehta, Bhagwat, and Sawant, 2013). Several groups of bacteria and fungi with chitinolytic abilities are used in controlling plant pathogens such as *Bacillus* (Wibowo, Mubarik, Rusmana, Thenawidjaya, 2017, Wibowo et al., 2020), *Pseudomonas* (Saranya et al., 2013), Trichoderma (Agrawal et al., 2012, Toshy et al., 2012) and *Streptomyces* (Arias et al., 2016). Pattanapipitpaisal & Songklanakarin (2012) stated that *S. hygroscopicus* in vitro was antagonistic to *Colletotrichum gloeosporioides* and *Sclerotium rolfsii* and inhibited the growth of pathogenic fungi with the activity of hydrolytic enzymes such as chitinase and glucanase.

However, the information about chitinolytic actinobacteria, especially in the rhizosphere and soil in Indonesia, is still very lack and limited, both in terms of their types and uses. The role of chitinolytic actinomycetes provides many benefits to humans and the environment, including controlling pests and plant diseases, because they have the chitinase to degrade pathogenic fungi so that it is thought to be able to control the growth of *Fusarium oxysporum* and *Sclerotium rolfsii*, which can cause the dumping-off disease in various cultivated crops, these two fungi are economically important soil-borned pathogens. So, research is needed to study the diversity of actinobacteria in the soil and their productivities to produce anti-fungal compounds. Then measure the chitinase activity of chitinolytic actinobacteria isolates in vitro and measure the antagonistic ability (zone of inhibition) of chitinolytic actinobacteria isolates from rubber plantation soil of IPB University as a biological control against *Fusarium oxysporum* and *Sclerotium rolfsii* infections in vitro.

Methods

Soil Sampling

Soil samples were collected from the roots of rubber plants (*Hevea brasiliensis*) in the rubber plantation area of IPB University. 250 grams of soil taken from the roots attached to the edge of the excavated soil to a depth of an average of 15-30 cm. Samples were put into sterile plastic containers. Then the soil sample was heated at a temperature of 60-70 °C for 1 hour, then wind-dried in a sterile container.

Soil Actinobacteria Isolation

A total of 1 gram of soil sample was measured, then mixed in 10 ml of sterile distilled water. Then the sample was homogenized for 3 minutes. Serial dilution was carried out by diluting 10^{-3}, 10^{-4}, 10^{-5} and taking 0.1 ml of the sample

solution with a volume pipette, then spread it on HV agar media using a spreading rod. The samples were incubated at room temperature for 7 days. The growing actinobacteria colonies were then observed and purified on ISP 2 media.

Morphological Characterization

Colonies of actinobacteria isolates were characterized morphologically based on the substrate mycelium colour, aerial mycelium colour, dissolved pigment colour, colony edges, colony surface shape, gram staining using Bergey's manual of systematic bacteriology. The morphological characterization of the hyphae/mycelium was observed visually under a light microscope with a magnification of 10 x 40.

Chitinolitic Activity Test of Actinobacteria

Chitin media were made with the composition: K_2HPO_4 0.14gr, KH_2PO_4 0.06 gr, $MgSO_4$. $7H_2O$ 0.1 gr, $FeSO_4.7H_2O$ 0.02 gr, $MnCl_2$ 0.001 gr, 2 % Colloidal Chitin, 2 % Agar. These components are mixed and added with aquadest until the volume reached 200 ml. After that, it was homogenized with a magnetic stirrer and heated until the media became clear, then sterilized by the autoclave. Chitin medium was poured into a Petri dish and allowed to be solid for 5-10 minutes. Then using a cork borer, the actinobacteria isolates were placed on the medium for chitin agar. The cultures were incubated at 30 °C for 24-72 hours. Chitinolytic activities index was determined by measure the halo zone / clear zone formed around the colony.

In Vitro Antagonism Test of Chitinolytic Actinobacteria

Chitinolytic actinobacteria were reinoculated into the chitin colloidal medium for 72 hours at 32 ^oC. The ability of chitinolytic actinobacteria to inhibit fungal growth was tested by in vitro antagonism test in Petri dishes. The cultures of *Fusarium oxysporum* and *Sclerotium rolfsii* were grown on chitin colloidal medium, respectively, at a distance of 3.5 cm from the actinobacterial growth disc. The cultures were incubated for 72 hours at room temperature. Furthermore, using a cork borer, chitinolytic actinobacteria isolates were inoculated on chitin media with calculated the average of colony diameters of 0.8 cm in the submedian section of chitin. The culture was inoculated at 30 ^oC for 5-10 days. Inhibition activity was determined based on the zone of inhibition that was formed around the colony. The observations were started from day 5 to day 9. The radius of the bacterial inhibition zone was measured using a calliper.

Result and Discussion

Morphological Characterization of Actinobacterial Isolates

Sixteen actinobacterial isolates were isolated from rubber plantation soil at IPB University. Pure colonies coded as KK-1 to KK-16. Sixteen isolates were grown on Humid Acid Vitamin (HV) agar media and then reinoculated and purified on ISP-2 media. Morphological characterization of actinobacteria colonies was determined from their pigmentation, hyphal filaments, colony margins, mycelium production, aerial hyphae, and substrate hyphae (Table 1).

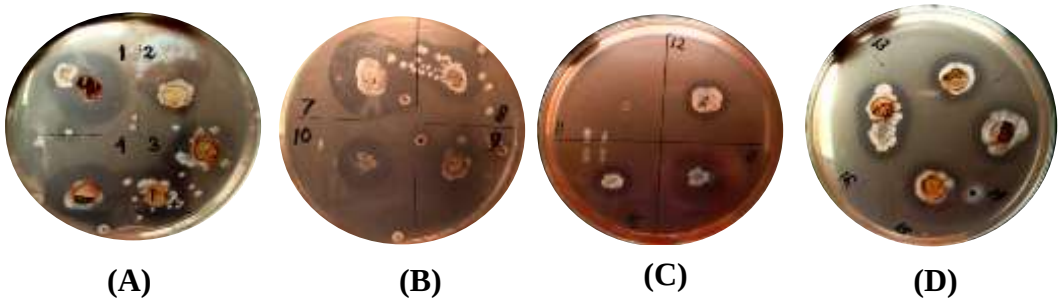
Table 1. Morphological characters of actinobacteria isolates

Isolate Code	Growth of ISP-2	Colour of Aerial Mycelium	Colour of Substrate Mycelia	Fungal Pigments	Margin	Elevation
KK-01	+	Grey	White	-	filament	Flat
KK-02	+	Dark grey	Light green	-	Filament	Flat
KK-03	+	Grey	Dark green	-	Filament	Convex
KK-04	+	Grey-White	White	-	Filament	Flat
KK-05	+	Grey-White	Dark red	Red	Filament	Flat
KK-06	+	Grey-White	Rosy/pink	Red	Filament	Flat
KK-07	+	Grey- Brown	Grey	-	Filament	Flat
KK-08	+	Grey- Brown	Light green	-	Filament	Flat
KK-09	+	pale red	White	-	Filament	Irregular
KK-10	+	Dark brown	Grey	-	Filament	Flat-apex
KK-11	+	Grey-White	White	-	Filament	Flat apex
KK-12	+	White	White	-	Filament	Irregular
KK-13	+	White	White	-	Filament	Flat
KK-14	+	Grey	White	-	Filament	Convex
KK-15	+	Grey	White	-	Filament	Convex
KK-16	+	Grey	White	-	Filament	Convex

According to Zinhua dan Liping (2011), HV media is a medium that is very poor in nutrients. Therefore it is effective in isolating actinobacteria from the soil. The growing actinobacteria colonies are white on this HV agar medium. Actinobacterial colonies were then repurified on ISP-2 media to observe the colony morphology. The diversity of colony morphology can be categorized from the aerial mycelium colour, substrate mycelium colour, pigmentation, and colony surface. In ISP-2, medium isolates were able to produce aerial mycelia and were able to grow well and sporulate after 7-14 days incubation time. Actinobacteria colonies that were successfully purified on ISP-2 media had high morphological diversity, seen from their aerial mycelium, which consisted of various colours, ranging from white, grey, grey-white, pale red, and dark brown (Table 1). Actinobacteria colonies that have been isolated able to produce pigments that dissolve into the media that produce red pigments. Actinobacteria can be distinguished easily from other bacteria by seeing their colony looks stiff, in contrast to other bacteria whose colonies are soft when they grow on agar.

Chitinase Activity Assay in The Chitin Agar

The chitinase activity assay results of sixteen actinobacteria isolates on chitin agar showed that the sixteen isolates had various chitinase enzyme activities after incubation at room temperature, and observations were made on the fifth day after treatment. The mechanism of chitinase activity was observed by the formation of a clear zone around the isolated colony as a degradation zone of chitin by chitinolytic activity. Observations were made with the addition of the Congo Red dye to clarify the clear zone on the chitin medium. (Figure 1).



(A) **(B)** **(C)** **(D)**

Figure 1. The chitinase enzyme activity test of sixteen isolates of chitinolytic actinobacteria (A), KK-01, KK-02, KK-03, KK-04 (B) KK-07, KK-08, KK-09, KK-10 (C) KK-13, KK14, KK-16 (D) KK-11, KK-12

The presence of chitinase activity is indicated by the formation of a clear zone around the bacterial colony on the chitin agar medium. The clear zone is formed due to the hydrolysing of the ß-1,4 homopolymer N-acetylglucosamine bonds in chitin by chitinase to become N-acetylglucosamine monomers (Veliz, Martinez, and Hirsch, 2017). The differences in the chitinolytic index of the isolates were due to differences in the activity of the chitinase enzyme from each of these isolates. The clear zone began to appear on the first day, and the zone size increased until the fifth day of observation (Wibowo, Mubarik, Rusmana, Thenawidjaya, 2017).

Screening of actinobacteria was carried out to obtain the potential isolates capable of inhibiting the growth of pathogenic fungal mycelium *Fusarium oxysforum* and *Sclerotium rolfsii* in vitro. The results of measuring the chitinolytic index and antagonistic testing against the fungal pathogens from sixteen actinobacteria isolates can be showed in Table 2.

Table 2. Chitinolytic index of actinobacteria isolates and the antagonistic test against the fungal pathogens (Observations were conducted at the fifth days after incubation)

Isolate Code	Chitinolytic Index (CI)	Potential inhibition	
		Fusarium oxysporum	*Sclerotium rolfsii*
KK-01	1.3	-	-
KK-02	0.4	-	-
KK-03	1.0	-	-
KK-04	1.2	-	-

Isolate Code	Chitinolytic Index (CI)	Potential inhibition	
		Fusarium oxysporum	*Sclerotium rolfsii*
KK-05	1.3	+	+
KK-06	1.0	+	+
KK-07	1.5	+	+
KK-08	1.4	+	+
KK-09	1.1	-	-
KK-10	1.1	-	-
KK-11	0.25	-	-
KK-12	0.5	-	-
KK-13	1.2	-	-
KK-14	1.3	-	-
KK-15	1.25	+	+
KK-16	1.2	+	+

Based on the observation on the fifth day (table 2), the chitinolytic index is different among the chitinolytic isolates. The KK-07 isolate showed the highest chitinolytic index in chitin media, it was about 1.5. Meanwhile, the lowest chitinolytic index was shown at KK-11 and KK-02 isolates, only showing the chitinase activity index of 0.4 and 0.25, respectively. The presence of chitinase activity is indicated by the formation of a clear zone around the bacterial colony on the chitin agar medium. The clear zone is formed due to the breaking of the ß-1,4 homopolymer N-acetylglucosamine bond in chitin by chitinase to become N-acetylglucosamine monomers. The difference in the chitinolytic index of the isolates was due to differences in the activity of the chitinase enzyme from each of these isolates. Kuddus dan Ahmad (2013) stated that the amount of clear zone produced depends on the amount of N-acetylglucosamine monomer produced from the chitin hydrolysis process by breaking the ß-1,4 bond of the N-acetylglucosamine homopolymer. In addition, the resulting clear zone depends on the type of chitinase enzyme produced by various organisms.

Chitin as a substrate will also induce chitinase enzyme activity, the enzyme regulated through genetic control, which involves the induction of enzyme synthesis at the genetic level. For enzyme synthesis, an inducer is needed, which is a substrate or compound that is related to the substrate of the reaction catalyzed by the enzyme (Muharni, 2009).

The actinobacterial antagonist test against *F. oxysforum* and *S. rolfsii* showed that from 16 isolates of chitinolytic actinobacteria, only 6 isolates had the potential to inhibit the growth of the two tested pathogenic fungi. The six isolates were KK-05, KK-06, KK-07, KK-08, KK-15, and KK-16. The six isolates will later be used for further treatments. Chitinolytic microorganisms have strong antagonistic activity against pathogenic fungi with their hyperparasitic mechanism, and they are effective in inhibiting the growth of plant pathogenic fungi by degrading their cell walls.

Microscopic Observation of Potential Actinobacteria Isolates

Microscopic observations were carried out on six potential actinobacteria isolates by using a Gram stain. The results of Gram staining showed that the six isolates were included in Gram-positive bacteria and had the same characters as the *Streptomyces* group with a spiral chain shape and had the septic hyphae with varying hyphae colours, such as grey, brownish, and reddish-brown (Table 3) and Figure 2.

Table 3. Microscopic observations of six chitinolytic actinobacteria isolates.

Isolate Code	Genus	Microscopic Observation			
		Gram-staining	Hyphae		
			Form	Septate	Colour
KK-05	*Streptomyces*	Positive	Spiral chain	Septate	Grey
KK-06	*Streptomyces*	Positive	Spiral chain	Septate	Brown-reddish
KK-07	*Streptomyces*	Positive	Spiral chain	Septate	Grey
KK-08	*Streptomyces*	Positive	Spiral chain	Septate	Grey-Brown
KK-15	*Streptomyces*	Positive	Spiral chain	Septate	Grey
KK-16	*Streptomyces*	Positive	Spiral chain	Septate	Grey

Overall, the potential chitinolytic actinobacteria belong to the genus *Streptomyces* spp. and has the greatest number of all isolates. This is following what was reported by previous researchers that the genus *Streptomyces* spp. are the most dominant genus of all Actinomycetes sources (Chandrasekar et al., 2012). Research by Nurjasmi and Suryani (2013) obtained nearly 80 % of its isolates, including the *Streptomyces* spp. In inhibiting the fungal colonies of *F. oxysforum* and *S. rolfsii*. Ali (2009) found that 63.8 % of actinobacteria belong to the *Streptomyces* spp. which can inhibit the growth of *Fusarium* spp. Pattanapipitpaisal & Kamlandharn (2012) found that almost all isolates isolated from the rhizosphere of chilli plants were *Streptomyces* spp. and able to inhibit the growth of *Sclerotium rolfsii*. Rozas, Gullon, and Mellado (2015) found *Streptomyces* as the most dominant genus of Actinomycetes isolated from compost. This is possible because *Streptomyces* can grow fast and has a diversity of bioactive compounds so that it can adapt and compete in its environment or habitat.

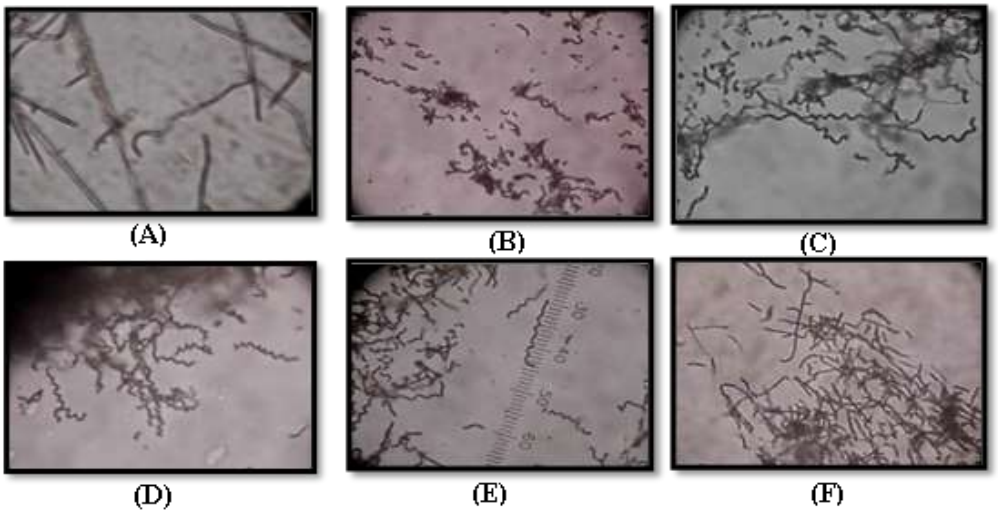


Figure 2. Observation of hyphae of chitinolytic actinobacteria isolates with light microscopy (magnification 10x40) (A) isolate KK-05, (B) isolate KK-06 (C) isolate KK-07, (D) isolate KK-08, (E) isolate KK-15, (F) isolate KK-16

In Vitro Antagonism Ability of Chitinolytic Actinobacteria against *F. oxysporum* and *S. rolfsii*

Six isolates of actinobacteria with the largest clear zone were tested for their chitinolytic activity against two pathogenic fungi, *F. oxysporum* and *S. rolfsii*. The results of the antagonism test of chitinolytic actinobacteria isolates against *F. oxysporum* showed that all of the actinobacteria isolates were able to inhibit the growth of the *F. oxysporum*, and five of the six isolates showed inhibition of *S. rolfsii* with different abilities. The inhibitory mechanism that occurs in the antagonism test can be observed by the formation of a clear zone as a growth inhibition zone for chitinolytic actinobacteria isolates (Figure 4.).

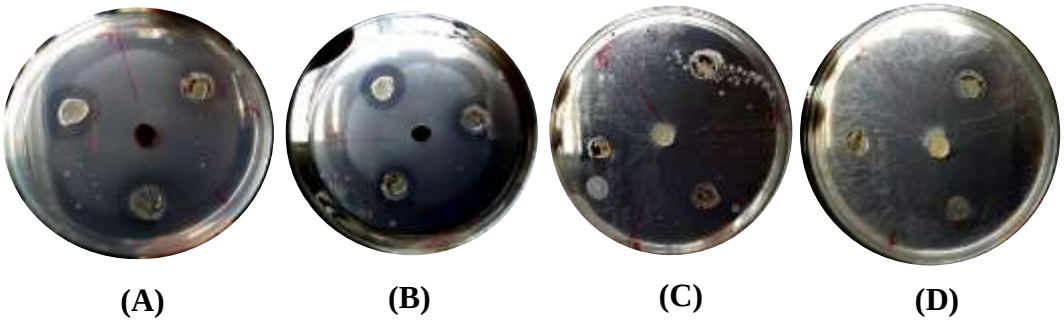


Figure 4. Antagonism abilities of chitinolytic actinobacteria against *F. oxysforum* (A) Isolates of KK-05, KK-06, and KK-07 (B) Isolates of KK-08, KK-15, and KK-16. The antagonism abilities of chitinolytic actinobacteria against *S. rolfsii* (C) Isolates of KK-06 and KK-07, (D) Isolates of KK-08, KK-15, and KK-16.

The inhibition zone starting to be observed on the fifth day, and the size of the inhibition zone continues to increase until the ninth day of observation. The

results of the antagonism test for the six chitinolytic isolates can be shown in Tables 4 and 5 below.

Table 4. The average value of in vitro antagonistic activity of chitinolytic actinobacteria isolates *against F. oxysporum* on a ninth day after incubation

Isolate Code	Inhibition zone		% Inhibition
	Mean ± Stdev (mm)	Category	
KK-05	12 ± 0.7	++	40 %
KK-06	12.3 ± 0.7	+	41 %
KK-07	12.5 ± 0.0	++	41.5 %
KK-08	12.2 ± 1.4	++	40.6 %
KK-15	14 ± 0.7	++	46 %
KK-16	9.5 ± 3.4	+	31.6 %

Table 5. The average value of in vitro antagonistic activity of chitinolytic actinobacteria isolates against *S. rolfsii* on a ninth day after incubation

Isolate Code	Inhibition zone		% Inhibition
	Mean ± Stdev (mm)	Category	
KK-05	-	-	-
KK-06	16 ± 0.0	++	53 %
KK-07	18.5 ± 0.7	++	61.6 %
KK-08	17 ± 3.5	++	56.7 %
KK-15	17.5 ± 0.7	++	58.3 %
KK-16	15 ± 0.0	++	50 %

It is seen that the six isolates of chitinolytic actinobacteria have almost the same ability to inhibit the growth of fungal mycelium *F. oxysporum* in vitro (Table 4). The mean inhibition zone of KK-15 chitinolytic actinobacteria isolates on the ninth day after testing had a higher percentage of 46 % with an inhibition zone radius of 14 mm compared to KK-05, KK-06, KK-07, KK-08, and KK-16 isolates. The results of this study are in line with research conducted by Rashad *et al*. (2017), which succeeded in isolating the bacterium *Streptomyces griseorubens* E44G from agricultural land from Saudi Arabia, which was able to inhibit the fungus *Fusarium oxysforum* with an inhibition zone diameter of 24 mm.

The results in Table 5 showed that on the ninth day of testing, the average inhibition zone of the KK-07 isolate produced the highest percentage of inhibition against *S. rolfsii* fungus, which was 61.6 % with an inhibition zone diameter of 18.5 mm compared to the KK-16, KK-06, KK-15, and KK-08 isolates, while KK-05 isolate did not show any inhibiting activity.

The inhibitory effect of these chitinolytic isolates against the fungus *F. Oxysporum* and *S. rolfsii* was influenced by the presence of chitin compounds in the test medium that the chitinase enzyme in the six isolates might be faster secreted. Several research results indicated that chitinolytic enzyme expression is

inducible. Generally, enzyme expression will increase rapidly if the growing medium contained chitin as the only carbon source, which can be in the form of pure chitin or its derivative, the cell wall, and mycelium of pathogenic fungi. Induction does not occur when the fungus is grown in a medium containing glucose and several other simple sugars (Corneliyawati, Massora, Khikmah & Arifin, 2018).

Chitinolytic microorganisms have strong antagonistic activity against pathogenic fungi with their hyperparasitic mechanisms and their antibiotics. So, they are effective in inhibiting the growth of plant pathogenic fungi by degrading their cell walls. The presence of chitin in the media causes the chitinase production of these bacterial isolates to be stimulated to degrade the fungal cell walls. When the chitin around the colony has broken down, the chitinase bacteria will colonize the fungal mycelium to break down the chitin in the fungal cell wall. According to Muharni (2009), chitinase is an enzyme that degrades chitin to N-acetylglucosamine. Chitin degradation can be carried out by chitinolytic organisms by involving the chitinase enzyme.

Actinobacteria, especially *Streptomyces* sp. group, have long been widely recognized as a group of microorganisms that produce secondary metabolites and important enzymes such as chitinase, mannose, and glucanase, which are widely used for agricultural applications, especially in biological control against the pathogenic fungal attacks and insect pests.

Conclusion

Sixteen actinobacteria isolates isolated from rubber plantation soil IPB University had chitinolytic activity. There were six chitinolytic isolates tested had the antagonistic ability (inhibition zone) against the pathogenic fungus of *F. oxysporum,* and five of them had antagonistic ability against *S. rolfsii*. The best isolates produced the largest percentage of inhibition zone in the two pathogenic fungi were KK-15 and KK-07 isolates. In the future, hopely these isolates can be applied as the potential candidates for biological control agents in plant pathogenic fungi.

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