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Enzyme production, antibacterial and antifungal activities of actinobacteria isolated from *Ficus deltoidea* rhizosphere

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Abstract. *Janatiningrum I, Lestari Y. 2022. Enzyme production, antibacterial and antifungal activities of actinobacteria isolated from* Ficus deltoidea *rhizosphere. Biodiversitas 23: 1950-1957.* Actinobacteria have long been known to have the ability to produce bioactive compounds and secondary metabolites. Actinobacteria from the rhizosphere of *Ficus deltoidea* Jack were expected to produce beneficial secondary metabolite compounds with antibacterial, antifungal activity, and enzymes. A total of 34 different actinobacteria were isolated from *F. deltoidea* rhizosphere soil. They were assessed for their antimicrobial activity against four fungi, four bacteria, and enzymatic activity. The enzyme activity test showed that almost all isolates had amylase, protease, and cellulase activities. The results of antibacterial indicated that 61.8% of all isolates are active against bacterial tested, i.e., *Bacillus subtilis* ATTC 3061 (10), *Staphylococcus aureus* ATCC 6538 (13), and *Escherichia coli* ATCC 8739 (3). The antifungal test showed that 64.8% of the isolates had antifungal activity against four fungal tested, i.e., *Fusarium oxysporum* (13), *Candida albicans* (7), *Colletotrichum capsici* (17). Based on the enzymes production, antibacterial activity, and antifungal activity, RTB 1 and RTB 34 isolates were selected to be characterized by morphological and 16S rRNA gene identification. The morphological characterization and molecular identification showed that RTB 1 and RTB 34 belong to the *Streptomyces* genus. This study indicated that the selected actinomycetes could be antimicrobial and enzyme sources.

Keywords: Actinobacteria, antimicrobial activity, extracellular enzymes, *Ficus deltoidea*, rhizosphere

INTRODUCTION

Actinobacteria are filamentous and Gram-positive bacteria that could produce various bioactive compounds (Amrita et al. 2012). The bioactive compounds produced by microbes are around 23.000, of which actinobacteria produce 10.000, thus representing 45% of all bioactive microbial metabolites discovered (Brzezinska et al. 2014)). Antimicrobials are the most bioactive compounds produced by actinobacteria. Most of the antibiotics in use today are derivatives of natural products of actinobacteria (Hutchings et al. 2019). In addition to antimicrobial, actinobacteria are also known to produce secondary metabolite compounds such as enzymes. They are considered valuable as they produce various antibiotics, other therapeutically beneficial compounds, and enzymatic activity with diverse biological activities (Prado et al. 2013).

Actinobacteria are distributed in all ecological habitats such as soil, freshwater, backwater, lake, compost, sewage, and marine environment (Van der Meij et al. 2017). Actinobacteria are abundant in soil and are responsible for bioactive and secondary metabolites compounds. The soil actinobacteria have been important sources of various bioactive compounds. New microbial habitats need to be examined to search for novel bioactive compounds (Yan et al. 2021). Plant rhizosphere soil represents a unique biological niche with diverse microflora. It comprises bacteria, fungi, protozoa, and algae (Mendes et al. 2014), and they obtain 30-60% root exudates from plants necessary for microbial growth (Van der Meij 2018). Roots exudates contain various compounds such as salicylic acid, a phytohormone. The chemical compounds in root exudates were positively correlated with the composition and quantity of microbes. Therefore, the diversity of actinobacteria rhizosphere depends on the plant species (Badri et al. 2013).

Ficus deltoidea Jack is a medicinal plant from Indonesia that is useful for treating various diseases (Sari and Kusharyoto 2016; Ashraf et al. 2021). The bioactive compounds produced by the plant cannot be separated from their interaction with microbes that live on its tissue or are called endophytes (Janatiningrum et al. 2020). The previous report showed a high diversity of actinobacteria from *F. deltoidea*, especially in root and stem (Janatiningrum et al. 2018). The diversity of actinobacteria is affected by the environment around the roots or the rhizosphere (Mahyarudin et al. 2015). The rhizosphere is the soil around plant roots affected by root secretions. Therefore, the rhizosphere ecosystem is rich in nutrients that can support microbial growth. From this ecosystem, it is expected that actinobacteria can produce bioactive compounds. However, reports on actinobacteria rhizosphere of *F. deltoidea* have not been reported previously. This study aims to isolate and identify actinobacteria from the rhizosphere of *F. deltoidea* and to determine their antimicrobial activity and enzymatic production.

MATERIALS AND METHODS

Procedures

Samples collection

Sample of rhizosphere soil of *F. deltoidea* was obtained from the Collection of Medicinal Plants Garden of Biopharmaca Research Center, Bogor Agricultural University, Bogor, West Java, Indonesia (6°35'48.2" S106°54'00.7" E).

Isolation of actinobacteria

The soil sample was dried at 60°C for 2 hours. One gram of soil samples was separately suspended in 9 mL of physiological saline (0.85% of NaCl). The soil samples were serially diluted up to 10^4 dilutions with physiological saline. One hundred µL of the diluted sample (10^2-10^4 dilution) to be plated on Humic Vitamin Agar (HVA) medium by spread plate technique. The HVA medium contains 50 ppm of griseofulvin and 30 ppm of nalidixic acid (Hayakawa et al. 1988). Then, samples were incubated at room temperature (25-28°C for 3-7 days. The actinobacteria colonies growing from agar medium were purified on International Streptomyces Project (ISP) 2 medium.

The enzymatic activities of actinobacteria isolates (Retnowati et al. 2019)

Amylase production

Actinobacteria isolates were evaluated for their amylase activity using Yeast Starch Agar medium containing 10% starch. Isolates were incubated at room temperature (25-28°C) for 3 days. After 3 days, the sample was dropped with iodine solution. The plates were observed for halo zone formation around the actinobacteria colonies. The halo zone indicates the presence of amylase.

Protease activity

Actinobacteria isolates were screened for their protease activity using skim milk agar medium containing 10% skim milk. Isolates were incubated for 3 days at room temperature (25-28°C). The hallo zones around actinobacterial colonies indicate the presence of protease.

Cellulase production

Actinobacteria isolates were cultured on carboxymethyl cellulose (CMC) agar medium containing 10% CMC. Isolates were incubated for 3 days at room temperature (25-28°C). After incubation, the sample was dripped with iodine solution. The hallo zone around the actinobacteria colony indicates the presence of cellulase.

Antibacterial activity

Antibacterial activity of actinobacteria was tested against two Gram-negative bacteria (*Escherichia coli* ATCC 8739 and *Salmonella typhimurium* ATCC14028) and two Gram-positive bacteria (*Bacillus cereus* ATTC 13061 and *Streptococcus aureus* ATCC 6538) by the agar plug method (Kumaran et al. 2020). First, the isolates were inoculated on yeast malt extract agar medium (YMA) and incubated at room temperature (25-27°C) for 7 days. Then, three discs (9 mm in diameter) isolates were cut and placed on Nutrien Agar (NA) inoculated with pathogenic bacteria and then incubated at 37°C for 24 hours. The growth inhibition zone of bacteria indicates the antibacterial activity produced by the actinobacteria.

Antifungal activity

Antifungal activity was tested against four fungi, *Sclerotium roflsii*, *Fusarium oxysporum*, *Colletotrichum capsici*, and *Candida albicans* (Culture collection of microbiology laboratory IPB). Antagonist test was observed to determine the Percentage Inhibition of Radial Growth (PIRG) of fungi (Fadhilah et al. 2021) by dual culture method using Potato Dextrose Agar (PDA) medium. First, Actinobacteria isolates were streaked onto the PDA plate 3 cm from the edge of the Petri dish. Then the isolates were incubated for 4 days. After incubation, an agar disc cut of 5-days olf of pathogenic fungi with a diameter of 5 mm was placed in the middle of the same plate. The control plate contained only pathogenic fungi placed in the same way without actinobacteria isolates on a fresh Petri dish. The plates were incubated at room temperature (25-28°C) for seven days.

Results were shown by measuring the radial growth of pathogenic fungi. PIRG was measured using the formulation below:

$$\%PIRG=(R1-R2)/R1 \times 100$$

Where:

PIRG: percentage inhibition of radial growth;

R1: radial growth of fungi in the absence of actinobacteria isolates (control);

R2: radial growth of fungi in the presence of actinobacteria isolates.

Screening of antifungal activity for *C. albicans* was assessed by the agar plug method. First, the actinobacteria isolates on ISP 2 medium were cut with a diameter of 5 mm. Then, it was placed on PDA media inoculated with *C. albicans*. Finally, the sample was incubated for 36 h at 36°C. Anticandida activity was indicated by a hallo zone formed around the actinobacterial colony (Kumaran et al. 2020).

Morphology identification of selected isolates

The morphology of actinobacteria isolates was characterized on three different media, i.e., Yeast Starch Agar (YSA), ISP 2, and ISP 4, based on mycelium substrate color, aerial mycelium color, and pigmentation using RAL color chart. In addition, microscopic observation was performed using a light microscope with a magnification of 4x10.

16S rRNA gene identification of culturable actinobacteria endophytes

Actinobacteria were identified with molecular analysis using the 16S rRNA gene. According to the protocol, the spores and mycelium of actinobacteria endophytes were collected in a 1.5 mL microtube and extracted using Presto Mini gDNA Bacteria Kit. The concentration and purity of the DNA genome were quantified using Nanodrop 2000 spectrophotometer (Thermo Scientific, USA).

Total genomic DNA was amplified using the Polymerase Chain Reaction (PCR) method with 16S specific primers for actinobacteria 16Sact1114R 5'-GAGTTGACCCCGGCRGT-3' (Martina et al. 2008) and 27F 5'-AGAGTTTGATCCTGGCTCAG-3' (Bruce et al. 1992). The PCR condition was pre-denaturation (94ºC, 5 minutes), denaturation (92ºC, 1 minutes), annealing (53ºC, 30 seconds), elongation (72ºC, 30 seconds), post elongation (72ºC, 3 minutes), followed by a 30 cycles amplification. The amplification results were visualized using gel electrophoresis with EtBr dyes on a UV transilluminator.

The amplified product was sent to the sequencing services company. The sequencing results are processed with Seqtrace software then identified using EzBioCloud web by entering primary data. Afterward, the phylogenetic tree was constructed using MEGA 7 software with the neighbor-joining method approach.

RESULTS AND DISCUSSION

The rhizosphere represents a unique biological niche. It supports an abundance of diverse microbes due to the high input of organic material from plant roots and root exudates (Schlatter et al. 2019). A total of 34 actinobacteria were successfully isolated from *F. deltoidea* rhizosphere soils. All isolates can produce enzyme amylase and cellulase (Table 1, Figure 1). However, two isolates were not produced protease (Table 1).

Previous studies reported that actinobacteria from soil possess a high number of enzymatically active actinobacteria. Isolated actinobacteria have been reported to have protease activity (Suthindhiran et al. 2013), amylase activity (Aguiar et al. 2022; Kafilzadeh and Dehdari 2015; Al-Agamy et al. 2021). In addition, cellulase activity in actinobacteria has been reported by Das et al. (2014). These results indicate that actinobacteria have the potential to produce a broad range of enzymes. The ability of actinobacteria may result from the natural microbial selection of microorganisms to survive in the competing environment (Arjit et al. 2012).

Antibacterial activity was indicated by the formation of hallo zones around the colony. Approximately 61.7% (21) of the isolates showed antibacterial activity, including broad and narrow spectrum. Twenty-one isolates (61.7%) were only active against Gram-positive bacteria, three isolates (8.8%) were active against Gram-negative bacteria, and three isolates (8.8%) were active against both Gram-negative and Gram-positive bacteria (Figure 2). The highest antibacterial activity showed by RTB 1 isolate (31.6 mm against *S. aureus*, 23 mm against *Bacillus subtilis*) and RTB 26 isolate has a broad spectrum (34.6 mm against *S. aureus*, 14.6 mm against *E. coli*) (Table 2).

The antibacterial tests showed that *S. aureus* and *B. subtilis* were sensitive to actinobacteria. Both of these bacteria are Gram-positive. On the other hand, Gram-negative bacteria, i.e., *E. coli* and *S. typhimurium*, have smaller inhibition zone. *Salmonella typhimurium* was not inhibited by actinobacteria. The results suggest that actinobacteria isolates are more sensitive to Gram-positive bacteria. The inhibitory mechanism of actinobacteria is probably by disrupting bacterial cell walls. The cell wall of Gram-negative bacteria contained more lipopolysaccharides. Therefore, it is more difficult for antibacterial agents to penetrate. According to Raningsih et al. (2015), the antimicrobial mechanism of actinobacteria affects plasma membranes and their integrity. Increased permeability and leakage of cells due to the damage of the cell membrane causes the release of intracellular material.

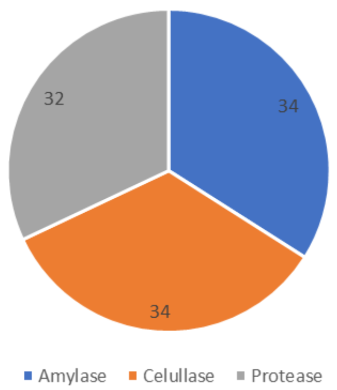

Figure 1. Enzyme production of 34 actinobacteria isolates from *Ficus deltoidea* rhizosphere. Amylase produced by 34 isolates, Cellulase produced by 34 isolates, and Protease produced by 32 isolates

Table 1. Actinobacteria isolates with high producing enzymes

Isolate code	Diameter (mm)		
	Amylase	Cellulase	Protease
RTB 1	13	22.5	12.5
RTB 2	12.5	14.5	17.8
RTB 3	12	13	24.6
RTB 6	18	27.5	26.5
RTB 8	20	27.5	18.5
RTB 12	19	25	25.6
RTB 16	24	27	16
RTB 17	21.5	21	21.5
RTB 20	28.5	28	24.8
RTB 33	16	30	28
RTB 34	11	21.5	16.8

Table 2. The actinobacteria isolates that are active for antibacterial activity

Isolate code	Zone inhibition (mm)			
	S. aureus	*S.typhimurium*	*B. cereus*	*E. coli*
RTB 1	31.6	-	23	-
RTB 2	-	-	16	-
RTB 3	-	-	-	-
RTB 4	11.5	-	28.5	-
RTB 15	15.5	-	-	11.5
RTB 19	32	-	-	12.6
RTB 26	34.6	-	-	14.6
RTB 27	25	-	34	-
RTB 31	-	-	29	-
RTB 32	28.4	-	28	-
RTB 33	29	-	35	-
RTB 34	21.5	-	27	-

Note: Not active (-).

As reported in the previous studies showed that actinobacteria from the rhizosphere have antimicrobial activity. Sulistyanto and Trimulyono (2019) reported that actinomycetes from the rhizosphere of sugarcane (*Saccharum officinarum*) could inhibit the growth of *E. coli and S. aureus.* A study by Retnowati et al. (2019) showed that actinobacteria from the paddy rhizosphere have strong antibacterial activity against *S. aureus* (14.7 mm) and *E. coli* (13.1 mm) (Retnowati et al. 2019). This study showed that actinobacteria from *F. deltoidea* rhizosphere had higher inhibition against *S. aureus* (34.6 mm) and *E. coli* (14.6 mm) than other rhizospheres. It may be due to the root exudate released by *F. deltoidea* affecting secondary microbial metabolites in the rhizosphere. Therefore, it can be concluded that the actinobacteria from *F. deltoidea* rhizosphere are a rich source of broad-spectrum antimicrobial agents. Further investigations are needed to determine the secondary metabolites of these isolates.

Actinobacteria are the primary producer of pharmaceuticals and agricultural pesticides due to their ability to produce antibiotics. Actinobacteria contributes 80% of antibiotics available in the market (Bhakyashree and Kannabiran 2020). All isolates from *F. deltoidea* were screened for their ability to produce antifungal compounds. The antifungal activity of endophytic actinobacteria isolates is shown in Figure 4 and Table 3. Most isolates produce secondary metabolites that displayed antifungal activity against the four fungal tested. There were 22 isolates (64.7%) which have antifungal properties, 12 isolates (35.3%) had no inhibitory activity.

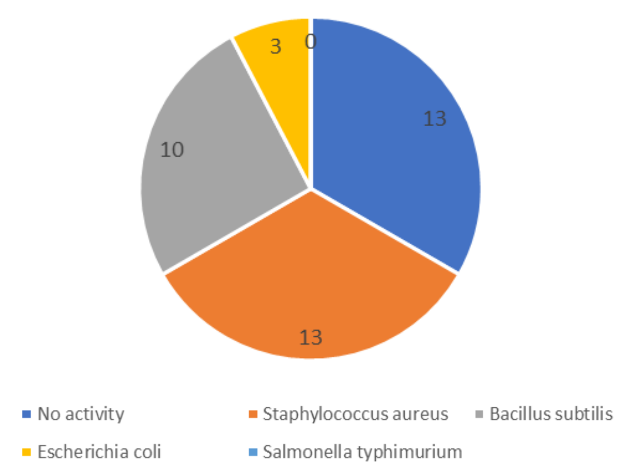

Figure 2. Antimicrobial activity of actinobacteria from *Ficus deltoidea* rhizosphere

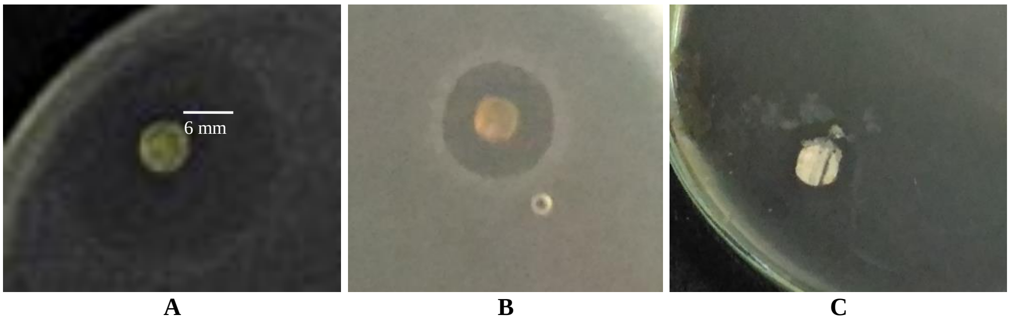

Figure 3. The inhibitory zone of actinobacteria from *Ficus deltoidea* rhizosphere against *Staphylococcus aureus* (RTB 27) (A), *B. cereus* (RTB 2) (B), and *Escherichia coli* (RTB 15) (C)

Table 3. The active actinobacteria isolates for antifungal activity

Isolate code	% inhibition			Diameter (mm)
	Fusarium oxysporum	***Sclerotium roflsii***	***Colletotrichum capsici***	***Candida albicans***
RTB 1	55.0	-	75.0	22
RTB 2	50.0	-	62.0	25
RTB 3	42.5	-		13
RTB 6	13.3	-	27.5	14
RTB 8	44.4	-	50.0	-
RTB 19	67.5	-	37.6	13.4
RTB 20	-	-	62,5	12.5
RTB 21	12.5	-	50.0	-
RTB 23	-	-	55.0	15
RTB 30	52.5	-	-	-
RTB 34	75.0	-	75.0	15.5

Note: No activity (-).

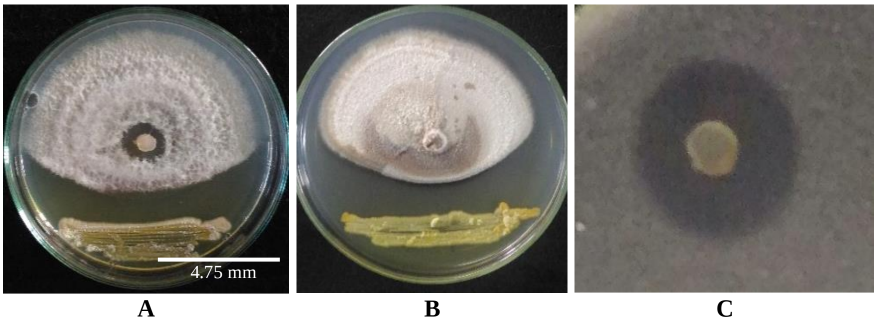

Figure 4. Antifungal activity of actinobacteria against *Fusarium oxysporum* (RTB 1) (A), *Colletotrichum capsici* (RTB 34) (B), and *Candida albicans* (RTB 1) (C)

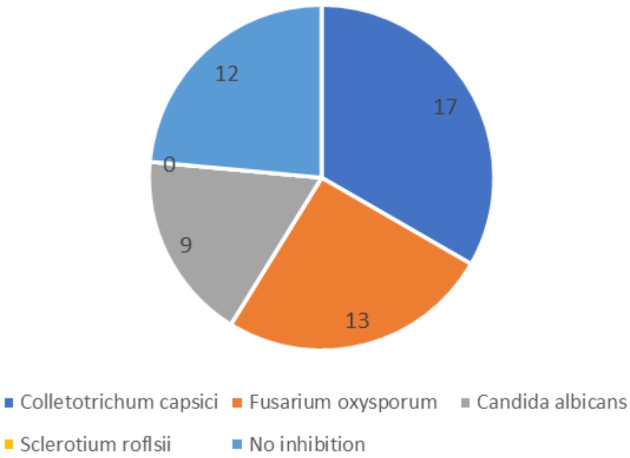

Figure 5. Antifungal activity of 34 actinobacteria isolates from *Ficus deltoidea* rhizosphere

The antifungal activity was performed against three multicellular fungi, i.e., *F. oxysporum, S. roflsii, C. capsici*, and one unicellular fungus that is *C. albicans*. Thirteen actinobacteria isolates have antifungal activity against *F. oxysporum.* RTB 34 has the highest activity in inhibiting *F. oxysporum*, equal to 75% growth inhibition of the fungi (Table 3). This fungal species can be found among the communities of soilborne fungi in every type of soil (Hermann and Lecomte 2019). *Fusarium oxysporum* is saprophytic, which colonizes plant roots. It survives on organic matter in soil or rhizosphere plants for extended periods (Lamo and Takken 2020). Half of the total isolates can inhibit *C. capsici*. RTB 1 and RTB 34 have the highest inhibition against *C.capsici* up to 75%. *Colletotrichum* is one of the most widespread and important genera of plant pathogenic fungi worldwide. Anthracnose caused by *C. capsici* is the most destructive disease of pepper and chili (Gowtham et al. 2018). *Candida albicans* is a normal human fungus, but it can be a systemic pathogen. Pathogenic *C. albicans* is found predominantly as pseudo-hyphae in the digestive tract, mouth, vagina, and rectum (Gow et al. 2012). From 34 actinobacteria isolates, nine isolates have activity against *C. albicans*. RTB 1 and RTB 2 had the highest inhibitory zone for *C. albicans* up to 22 and 25 mm (Figure 5).

Due to their highest antibacterial and antifungal activities and enzyme production, RTB 1 and RTB 2 were selected for morphological characterization and molecular identification. Morphological examination showed that both isolates belong to the *Streptomyces* genera, i.e., based on spore chain with coiling and branching. Both isolates were cultivated in 3 different agar mediums: ISP 2, ISP 4, and YSA (Table 4). It showed that RTB 1 has a white-grey aerial hyphae color with yellowish substrate hyphae in all media. RTB 34 has gray aerial hyphae with ivory as the substrate color (Figure 6).

The molecular identification of 16S rRNA

Based on the 16S rRNA, RTB 1 has a similarity > 99% with *Streptomyces sasae*, and RTB 34 has a homology of 98.51% with *Streptomyces shenzhenensis*. *Streptomyces sasae* has been isolated from the rhizosphere soil of bamboo (*Sasa borealis*) in Damyang, Korea. *Streptomyces sasae* has morphological characters as aerobic actinobacteria, Gram-positive, non-motile. It extensively branched substrate mycelium, and aerial hyphae differentiate into flexuous (Rectiflexibiles) spiral spore chains, smooth-surfaced spores, and yellow-pigmented (Lee and Whang 2015). These characteristics are similar to the macroscopic and microscopic characteristics features of RTB 1. In addition, previous research found that *S. sasae* has antifungal activity against *F. oxysporum* and antimicrobial ability against some positive and negative pathogenic bacteria (Sudiana et al. 2020; Srivastava et al. 2021).

Table 4. Morphological characteristics of selected actinobacteria isolates

Medium	Characteristic	Code of isolates	
		RTB 1	RTB 2
ISP 2	Aerial	Traffic white	Yellow grey
	Substrate	Sun yellow	Ivory
ISP 4	Aerial	Traffic white	Yellow grey
	Substrate	Daffodil yellow	Ivory
YSA	Aerial	Traffic white	Yellow grey
	Substrate	Daffodil white	Ochre yellow
	Pigmentation	Traffic yellow	Green brown
	Spore chain	Spiral	Spiral

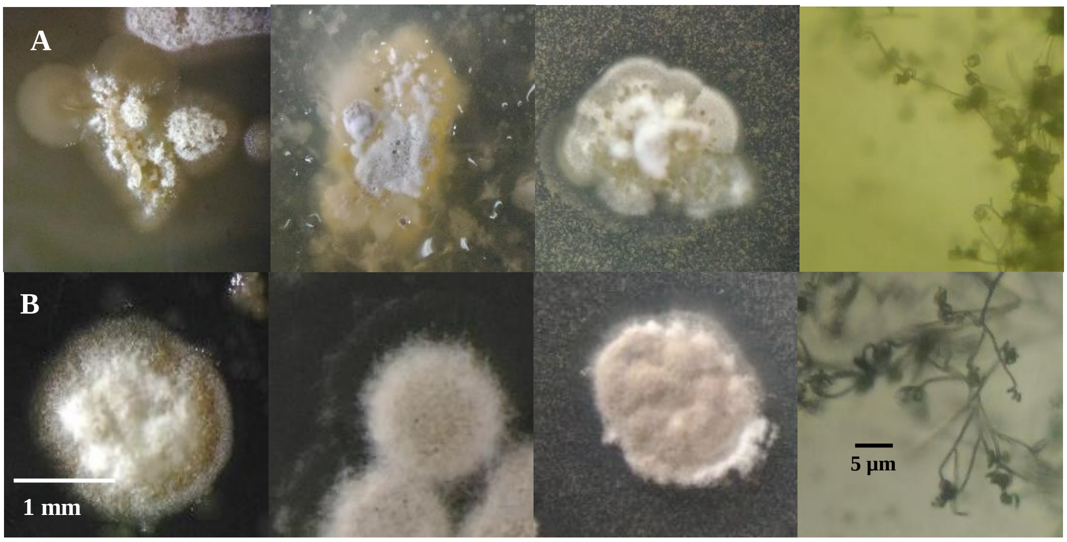

Figure 6. Macroscopic morphology of RTB 1 (A) and RTB 34 (B) colony at three different media: ISP 2, YSA, ISP 4, and microscopic observations of actinobacteria (right). Isolates observed after 7 days of incubation at room temperature (Olympus Optilab, 400x magnification)

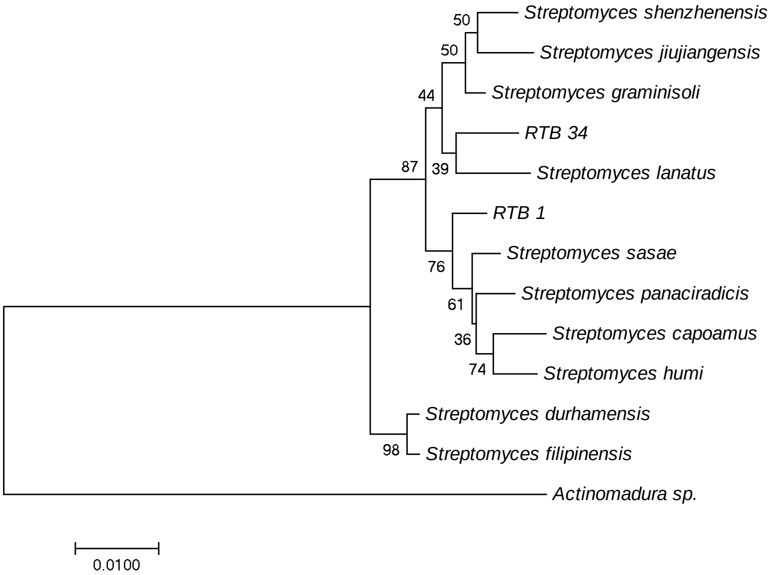

Figure 7. Phylogenetic tree of 16S rRNA gene of actinobacteria constructed using MEGA 7 software (Boostrap 1000 replicate)

Streptomyces shenzhenensis was isolated from different rice plant components and field locations in Malaysia (Hata et al. 2015). The report showed that *S. shenzhenensis* treatments successfully suppressed *Xanthomonas oryzae* pv. oryzicola infection. Morphological observation of the 15-day old culture grown on ISP 3 agar revealed that it produces extensively branched substrate and aerial hyphae that carried spiral or looped spore chains. The spore surfaces are smooth, and spores are elliptical or shaped like short rods. It has grayish-white aerial hyphae, white subtree hyphae, and no pigmentation. This species has the capability against pathogenic bacteria and produces secondary metabolites for plant growth promotion hormone (PGPR) (Hata et al. 2021). These characters are different from the result of the morphological characterization of RTB 34. It is due to the similarity of RTB 34 with *S. shenzhenensis* <99%. Therefore, it suggests RTB 34 might be a new species.

In conclusion, a total of 34 actinobacteria from the rhizosphere of *F. deltoidea* were isolated. Almost all isolates can produce protease amylase and cellulase enzyme. 61,76% of actinobacteria isolates have antibacterial activity against 4 pathogenic bacteria tested. An antifungal test resulted in 64.7% of actinobacteria isolates inhibiting pathogenic fungi such as *C. albicans*, *S. rolfsii*, *F. oxysporum*, and *C. capsici.* RTB 1 and RTB 34 have high antibacterial and antifungal activity. Based on the morphological character and identification of 16S rRNA, RTB 1 has high similarity with *S. sasae* and RTB 34 is similar with *S. shenzhenensis* <99%. This indicates that there is a possibility that RTB 34 and *S. shenzhenensis* is a different species.

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