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Antimicrobial Activity of *Actinomyctes* from the Rhizosphere of Pandanus Plants (*Pandanus* spp.)

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ABSTRACT. This quantitative descriptive research aimed to describe the antimicrobial activity and phylogenetic relationships of Actinomycetes from the rhizosphere of pandan (Pandanus spp.). Soil samples were taken from three different villages in Gorontalo Regency, including Bontula Village, Asparaga Sub-district, Lombongo Village, Suwawa Tengah Sub-district, and Moutong Village, Tilongkabila Sub-district, Bone Bolango Regency. Isolation was carried out using SCA media, resulting in six Actinomycetes isolates with different colony morphologies. Antimicrobial activity tests were carried out using the cross-streak method against Escherichia coli, Staphylococcus aureus, Candida albicans, Fusarium oxysporum, and Neocosmospora solani. The results showed that only one isolate, IRzP-at.k, showed significant antimicrobial activity, against Escherichia coli and Staphylococcus aureus with inhibition zones of 13,31 mm and 13,67 mm, respectively. The IRzP-at.k isolate also showed inhibition against Candida albicans and Fusarium oxysporum with inhibition zones of 34,12 mm and 11,25 mm, but did not show inhibition against Neocosmospora solani. Phylogenetic analysis based on 16S rRNA gene sequences identified the isolate as closely related to Streptomyces vinaceusdrappus (97.42% similarity). This research indicated the potential of the IRzP-at.k isolate as a source of new antimicrobial compounds and strengthens the role of the pandan rhizosphere as a habitat rich in Actinomycetes.

Keywords : *Actinomycetes, Antimicroba, Identification, Pandanus spp., Rhizosphere*

INTRODUCTION

Antimicrobials play an important role in human and veterinary medicine, livestock health management, and crop protection (Mejias *et al.,* 2023). However, the effectiveness of these agents continues to decline due to misuse and inappropriate use, leading to an increase in antimicrobial resistance (AMR) cases globally (Serwecinska, 2020; Qiu *et al.,* 2022; Xiao & Li, 2016). This resistance poses serious challenges in the treatment of infectious diseases, causing therapy failure, and increasing morbidity and mortality (Breijyeh *et al.,* 2020).

The World Health Organization (WHO) in 2017, has identified a number of priority pathogens that are resistant to various types of antibiotics and require serious attention in the development of new therapies. Several pathogens included in the tested group, including *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter species*, have shown multiple resistance and are a serious threat in the treatment of infections (Nova *et al.,* 2024). *Staphylococcus aureus* and *Escherichia coli* are classified as disease-causing bacteria (pathogens) that often cause various types of diseases. Both bacteria are known to have shown resistance to various types of antibiotics. For example, *Staphylococcus aureus* has been reported to be resistant to several antibiotics such as penicillin, erythromycin, and cefoxitin (Schulte & Munson, 2019). Meanwhile, *Escherichia coli* also shows resistance to antibiotics such as Penicillin G, Cefotaxime, and Tetracycline (Agustin & Ningtyas, 2022). On the other hand, *Candida albicans* is a type of pathogenic fungus commonly found in

humans and can cause a variety of infections, ranging from superficial mucosal infections to potentially fatal systemic infections (Pfraller & Diekema, 2007). According to the World Health Organization (2022), *Candida albicans* is not only a common fungal pathogen, but is included in the category of the deadliest fungi, which has caused the deaths of more than 1,6 million people. The problem of resistance to antifungal drugs not only occurs in fungi in the critical category, but also in groups of fungi with high and medium risk. It is estimated that antimicrobial resistance caused around 1,27 million direct deaths and contributed to around 5 million deaths in 2019 globally (Murray *et al.*, 2022).

In an effort to face the challenge of AMR, exploration of new sources of antimicrobial compounds is very important. One potential source is the Actinomycetes group of bacteria, which are known to produce bioactive secondary metabolite compounds such as antibiotics, antivirals, antifungals, anticancer, and immunosuppressant agents (Salam *et al.*, 2023). Around 80% of antibiotics currently available come from Actinomycetes, especially from the genera *Streptomyces* and *Micromonospora* (Mohamedsalih & Sabir, 2020). Lihaawa, *et al.*, (2024), reported that *Streptomyces aegyptia* strain KSLI and *Streptomyces* sp. strain KSIc from plant rhizospheres in karst ecosystems showed anticandidal activity with an inhibition zone diameter of 17,5 mm.

Actinomycetes are native soil bacteria that are widely found associated with plants in the rhizosphere. The rhizosphere is a zone around plant roots with root exudate content that provides essential nutrients for microorganisms, thus encouraging complex microbial diversity and interactions (Jog *et al.*, 2014; Fauziah & Djide, 2022). Microbes in the rhizosphere play an important role in decomposition, nutrient cycling, and plant protection against pathogens (Braga *et al.*, 2016). Therefore, the rhizosphere is a strategic location for isolating microbes that produce bioactive compounds.

Pandanus (*Pandanus spp.*) is native to Indonesia and is widely distributed from the coast to the mountains. Pandan roots, which have been underutilized and often discarded as waste, contain various bioactive compounds, including alkaloids, saponins, flavonoids, tannins, and polyphenols that have the potential as antimicrobial, antioxidant, anticancer, and immunostimulant agents (Sukandar *et al.*, 2009; Sumastuti *et al.*, 2002; Tan *et al.*, 2010; Bhuyan & Sonowal, 2021). However, the potential of the pandan root rhizosphere has not been widely studied, especially regarding its antimicrobial activity. Studies on antimicrobial activity in Actinomycetes from the rhizosphere of pandan (*Pandanus spp*.) are still very limited, even though this area has a significant level of biodiversity. Thus, this research aims to describe the antimicrobial activity and phylogenetic relationships of Actinomycetes from the rhizosphere of pandan (*Pandanus spp.*).

MATERIALS AND METHODS

Research site

The location of Actinomycetes sampling came from the rhizosphere of pandan found in three geographically different locations, including Bontula Village, Asparaga Sub-district, Lombongo Village, Suwawa Tengah Sub-district, and Moutong Village, Tilongkabila Sub-district, Bone Bolango Regency. The location coordinates and environmental conditions are listed in Table 1.

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Figure 1. Actinomycetes sampling locations in the rhizosphere of Pandanus plants (*Pandanus spp.*) ket: (a) Bontula Village, Asparaga Sub-district, Gorontalo Regency, (b) Lombongo Village, Suwawa Tengah Sub-district, Gorontalo Regency, and (c) Moutong Village, Tilongkabila Sub-district, Bone Bolango Regency

Table 1. Environmental parameters of Pandanus plants *(Pandanus* spp.*)*

Location	**Coordinate Point**	**Host Plant**	**Pandanus *(Pandanus* spp.*)* Environmental Matters**	
			Avarege pH	**Average Humidity**
Bontula Village, Asparaga Sub-district, Gorontalo Regency	(0°80'74.77''N; 122°43'68.45''E)	Pandan wangi *(Pandanus ammaryllifolius)*	4,4	5,7%
Lombongo Village, Suwawa Tengah Sub-district, Gorontalo Regency	(0°54'81.38''N; 123°18'06.98''E)	Pandan duri *(Pandanus tectorius)*	3,4	6,1%
Moutong Village, Tilongkabila Sub-district, Bone Bolango Regency	(0°54'91.84''N; 123° 13'04.72''E)	Pandan wangi *(Pandanus ammaryllifolius)*	7.9	6,8%

Tools and Materials

This research employed various tools and materials, including soil tester, small shovel, sample plastic, labels, books, pencils, cellphone camera, laminar air flow, incubator, oven, autoclave, incubator shaker, analytical balance, water bath, hot plate, Bunsen burner, petri dish, Erlenmeyer, ose needle, beaker glass, stirring rod, test tube, test tube rack, micropipette, culture bottle, centrifuge, spectrophotometer, cuvette, mortar pestle, measuring cup, vortex, distilled water, alcohol, SCA (Starch Casein Agar), Agar Powder, Nystatin/Cycloheximide, ringer, NA (Nutrient Agar), NB (Nutrient Broth), and test pathogens: *Escherichia coli, Staphylococcus aureus, Candida albicans, Fusarium oxysporum* and *Neocosmospora solani*.

Soil sampling

Soil rhizosphere sampling was carried out using the exploration method. Rhizosphere soil samples were taken with a hand scoop to a depth of 20-30 cm on each plant found. Then, the sample was taken and placed in a plastic clip coded with a paper label and stored in a coolbox. After that, the coordinates of the sampling location were taken using a GPS Map Camera. When taking soil samples, measurements of the physicochemical characteristics of the soil were carried out, including soil pH and soil moisture.

Isolation and Purification of Actinomycetes

The soil suspension was carried out in a series of dilutions of 1 ml of suspension and added to a test tube containing 9 ml of ringer's solution or a 10^{-1} tube then vortexed and 1 ml of solution from the first tube was taken and transferred gradually to a test tube with a dilution level of 10^{-2} to a test tube 10^{-5}. A total of 200 µl of soil suspension from each dilution level of 10^{-4} to 10^{-5} was inoculated onto the surface of the SCA medium using the surface/spread plate technique with the Duplo method. Furthermore, the incubation process took place at a temperature of 37°C for 7-14 days. SCA was added with clomid (50 µl/ml) or nystatin (50 µl/ml) to prevent the growth of contaminant colony fungi during incubation (Baskaran *et al.*, 2011). Actinomycetes colonies that grow on SCA with different morphologies were inoculated into new plates containing SCA to be purified based on the single-cell colony method using a streak plate and incubated for 1 week at a temperature of 37°C.

Screening of Isolates that Show Antimicrobial Activity

All selected Actinomycetes were tested for their ability to inhibit the growth of microorganisms grown on Natrium Agar (NA) and Potato Dextrose Agar (PDA) for seven days using the cross-streak method to streak the Actinomycetes isolate vertically on half of the petri dish (vertical) (Kurniawati *et al.*, 2015). The test pathogenic bacteria used were first cultured purely, the pathogenic strains used as test organisms, including *Escherichia coli*, *Staphylococcus aureus* were cultured on Nutrient Broth (NB) while *Candida albicans*, *Fusarium oxysporum* and *Neocosmopora solani* were cultured on Potato Dextrose Broth (PDB) and incubated in a shaker incubator at 37ºC with 120 rpm for 24 hours to obtain a bacterial culture, after which the OD 1,5 density was measured using a spectrophotometer. Then, the test bacteria were inoculated on the side of the plate that did not contain the isolate, with a horizontal scratch direction perpendicular to the Actinomycetes scratch. The incubation process was then carried out at a temperature of 37ºC for 2x24 hours. Antimicrobial activity was determined based on the size of the inhibition zone formed between the growth of the Actinomycetes isolate and the test bacteria.

Identification of phylogenetic relationships among Actinomycetes with potential antimicrobial properties

Actinomycetes isolates that show antimicrobial activity were then subjected to molecular characterization of Actinomycetes isolates through several stages. First, genomic DNA extraction began with spore cultivation in ISP2 broth media, then cell pellets were processed using lysis solution, proteinase K and lysozyme enzymes, and organic solvents such as phenol and chloroform to separate DNA. DNA was then precipitated using ethanol and purified. After that, genomic DNA was examined through 2% agarose gel electrophoresis to ensure its quality. The next stage included 16S rRNA gene amplification through PCR techniques using universal primers 27F and 1492R, with confirmation of amplification results carried out through electrophoresis. Then, the next stage included 16S rRNA gene sequencing using the ABI PRISM 3730xl automatic sequencer, followed by bioinformatics analysis. The

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sequence results were analysed with BioEdit and matched with the GenBank database via BLAST to determine phylogenetic closeness. Sequence alignment was performed using ClustalW and phylogenetic tree reconstruction was performed using the Neighbor Joining method using 1000x bootstrap through MEGA 11 (Retnowati *et al.*, 2017).

RESULTS AND DISCUSSION

Results

In the three locations, different types of pandan were found, including *Pandanus ammaryllifolius* and *Pandanus tectorius*. Actinomycetes from the rhizosphere of pandan were obtained through isolation and purification, resulting in 6 isolates. The results of the Actinomycetes isolates were coded IRzP-pk.k, IRzP-at.k, IRzP-okh.p, IRzP-p.p, IRzP-ag.kh, IRzP-ka.pa.

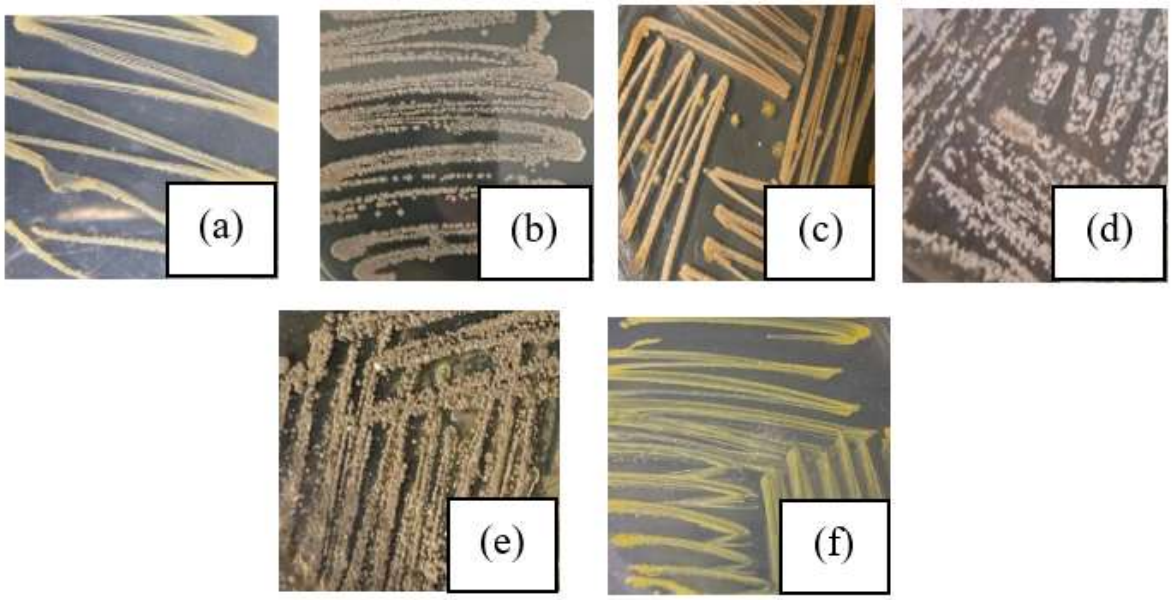

Figure 2. Purification *Actinomycetes* (a) IRzP-pk.k, (b) IRzP-at.k, (c) IRzP-okh.p, (d) IRzP-p.p, (e) IRzP-ag.kh, (f) IRzP-ka.pa

These isolates were then tested for antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, *Fusarium oxysporum* and *Neocosmopora solani.* The ability of antimicrobial activity is indicated by the formation of an inhibition zone in the area around the Actinomycetes colony.

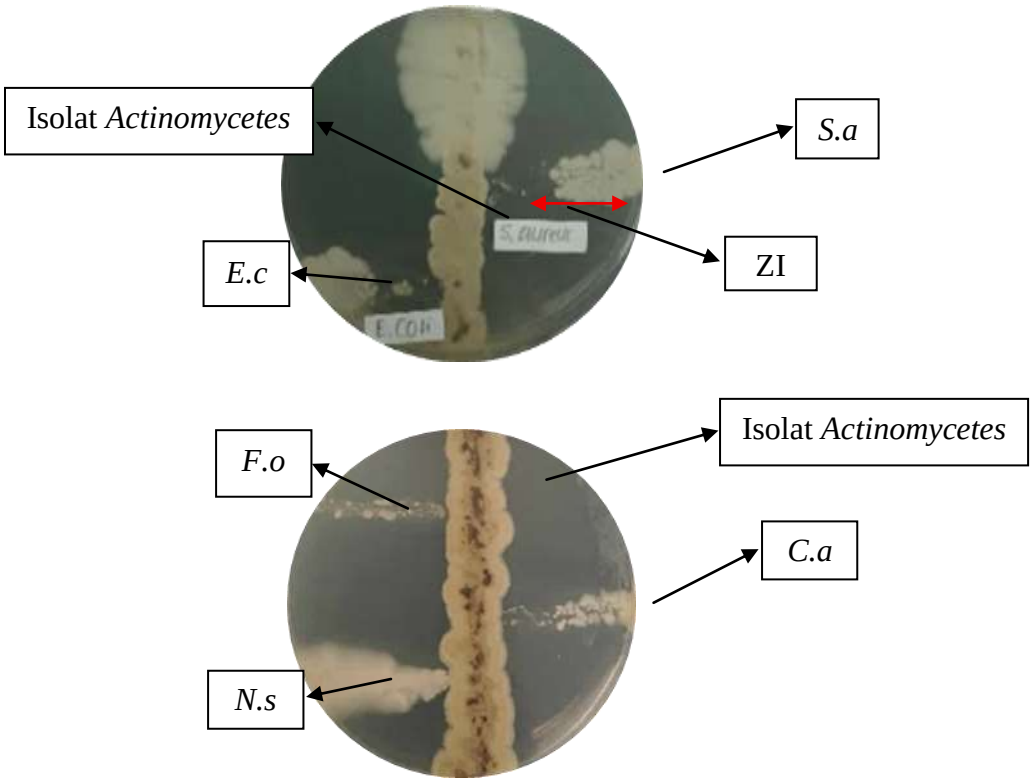

Figure 3: Zone of inhibition formed around the *Actinomycetes* isolate
(E. c: *Escherichia* coli, S.a: *Staphylococcus aureus*, C.a: *Candida albicans*, F.o: *Fusarium oxysporum*, N. s: *Neocosmospora solani*, ZI: Zona Inhibition)

The results indicated that not all Actinomycetes isolates exhibited antimicrobial potential. Out of the six isolates obtained, only one coded IRzP-at.k demonstrated such potential and was associated with the rhizosphere of pandan (*Pandanus spp.*).

Table 2. Antimicrobial activity testresult of *Actinomycetes* isolates

Isolat	**Zona hambat (mm)**				
	Escherichia coli	*Staphylococcus aureus*	*Candida albicans*	*Fusarium oxysporum*	*Neocosmopora solani*
IRzP-pk.k	-	-	-	-	-
IRzP-at.k	13,31	13,67	34,12	11,25	-
IRzP-okh.p	-	-	-	-	-
IRzP-p.p	-	-	-	-	-
IRzP-ag.kh	-	-	-	-	-
IRzP-ka.pa	-	-	-	-	-

The Actinomycetes isolate with antimicrobial potential, coded IRzP-at.k, was further analyzed to identify its phylogenetic relationship. Sequence data analysis revealed that IRzP-at.k is related to the genus Streptomyces.

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Table 3. BLAST results of 16S rRNA sequence of *Actinomycetes* isolate IRzP-at.k

Nearest phylogenetic neighbor	**Percent identity (%)**	**E-value**	**Genus**
Streptomyces enissocaesilis strain NRRL B-16365 (NR_115668)	99.78%	0.0	*Streptomyces*
Streptomyces geysiriensis strain NRRL B-12102 (NR_043818)	99.71%	0.0	*Streptomyces*
Streptomyces mutabilis strain NBRC 12800 (NR_112281)	99.50%	0.0	*Streptomyces*
Streptomyces vinaceusdrappus strain NRRL 2363 (NR_041091)	99.42%	0.0	*Streptomyces*
Streptomyces tuirus strain NBRC 15617 (NR_041190)	99.28%	0.0	*Streptomyces*

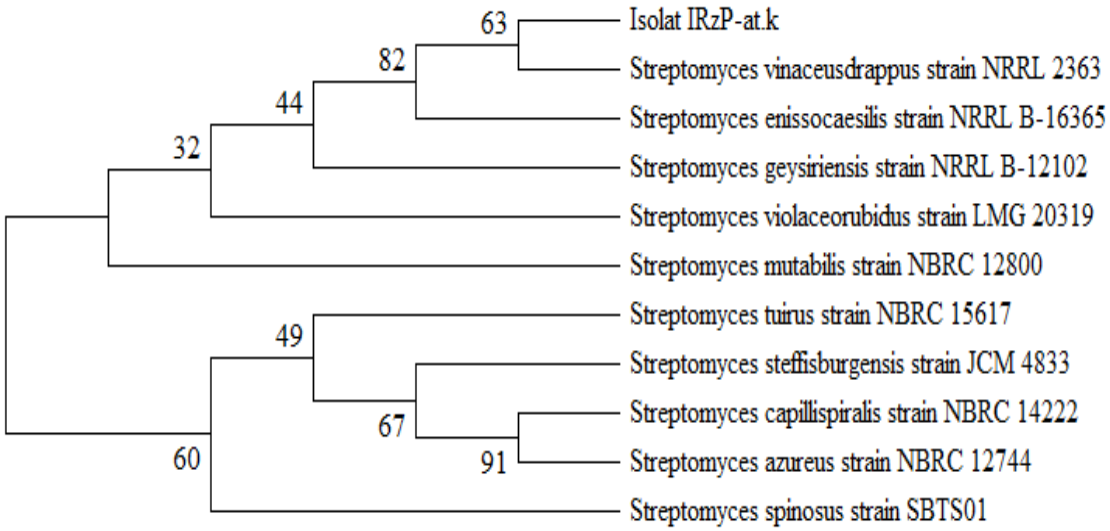

Figure 4. Phylogenetic tree recontruction of isolate IRzP-at.k

Based on the phylogenetic test results displayed on the phylogenetic tree, the isolate was successfully identified through 16S rRNA gene sequence similarity analysis. The analysis showed that IRzP-at.k has the highest phylogenetic closeness to *Streptomyces vinaceusdrappus* strain NBRC 13099, with a 100% similarity level, supported by its adjacent position on the tree and a high bootstrap value. This strong phylogenetic relationship confirms that IRzP-at.k belongs to the genus Streptomyces, which is widely known for producing bioactive compounds such as antibiotics and antifungal agents.

Discussion

Actinomycetes are gram-positive bacteria that have morphological characteristics resembling fungi and are widely known as the main producers of natural antimicrobials (Sapkota *et al.*, 2020). Actinomycetes can form spores in response to nutrient deficiencies, allowing them to survive until the environment again supports growth (Mitra *et al.*, 2022). The diversity of Actinomycetes is greatly influenced by environmental factors such as chemical, biological, and physical factors, including soil pH, humidity, temperature, and soil chemical compounds (George et al, 2012).

The isolate with the code IRzP-at.k was obtained at the location of Lombongo Village, Suwawa Tengah Sub-district, Gorontalo Regency (0°54'81.38''N; 123° 18'06.98''E) with a soil pH of 3,4 and soil moisture of 6,1%. The acidic soil conditions (pH 3.4) at the isolation site are considered one of the factors that trigger the production of antimicrobial compounds by the IRzP-at.k isolate. According to Penggele (2021), the existence of the diversity of Actinomycetes species is greatly influenced by soil type, soil physical properties, organic matter content, and the acidity level (pH) of the environment. Although Actinomycetes generally show optimal growth in the neutral and alkaline pH range, the presence of an environment with an acidic pH does not completely inhibit their growth, although the growth rate tends to decrease under these conditions.

A study by Golinska & Hanna (2011), suggested that Actinomycetes can still be successfully isolated from the forest rhizosphere with high acidity levels, pH range 4.0-4.3. The abundance of Actinomycetes in forest ecosystems is related to the characteristics of forest soil because forests are able to regulate groundwater, which has an effect on nutrient circulation and biological activity. Cornell & Joseph (1981), obtained 126 Actinomycetes isolates from the rhizosphere of the Cumberland National Forest (New South Wales) from 3 locations with acidity levels (pH) (4,91; 4,83; 4,60) and all of them belonged to the Streptomyces group. This activity indicates that the acidic pH conditions of the IRzP-at.k isolate can play a role in inducing the biosynthesis of antimicrobial compounds.

In addition to environmental factors, differences in the spectrum of antimicrobial activity in each isolate are also thought to be related to the diversity of enzymes and secondary metabolite compounds produced by each isolate. This is in accordance with Lestari (2019), suggesting that differences in the working spectrum of Streptomyces isolates are caused by variations in secreted secondary metabolites. In this research, the IRzP-at.k isolate showed antimicrobial potential against *Escherichia coli* and *Staphylococcus aureus* as indicated by inhibition zones of 13,31 mm and 13,67 mm, respectively. This isolate also inhibited *Candida albicans* and *Fusarium oxysporum* with inhibition zones of 34,12 mm and 11,25 mm, but did not show inhibition against *Neocosmospora solani.* The presence of inhibition zones around the colonies indicates that the metabolites produced by the IRzP-at.k isolate are able to effectively suppress the growth of target microbes, possibly through mechanisms such as cell membrane damage, protein synthesis disorders, or inhibition of vital enzymes of microorganisms.

The IRzP-at.k isolate was marked with a black circle in the phylogenetic analysis and grouped together with *S. vinaceusdrappus*, indicating evolutionary closeness between them. This indicates that the IRzP-at.k isolate may be a new species that has a close relationship with the group based on genetic similarities. Analysis of the 16S rRNA gene is a very effective method for taxonomic classification and determining the evolutionary relationships of microorganisms (Chun *et al.,* 2018). This diversity is reflected in the number of long-branched branches and bootstrap values that indicate evolutionary variation. Streptomyces is known to have a large and flexible genome, which allows adaptation to various environments, and several species of the genus Streptomyces produce antibiotic compounds that are effective against plant pathogens, such as *S. griseoviridis* and *S. lydicus* (Purnamasari, 2024). The IRzP-at.k isolate, which is close to species such as *S. vinaceusdrappus*, potentially has a similar biosynthetic pathway, opening up opportunities for exploring new metabolites. This is very important in the development of new antibiotics to overcome antimicrobial resistance.

The rhizosphere is a complex environment and strongly supports the formation of diverse microbial communities (Amin *et al.,* 2018). The composition of microorganisms in the rhizosphere microbiome is influenced by various biotic and abiotic factors, including environmental parameters, soil physicochemicals, plant biological activity, and chemical

signals produced by plants and microorganisms associated with the root system. Actinomycetes have secondary metabolites that are widely used for the production of antibiotics, enzymes, antitumor agents, enzyme inhibitors, biosurfactants and so on (Selim *et al.*, 2021). Actinomycetes play an important role for a plant because they are able to produce antibiotics in vitro that inhibit the growth of pathogenic fungi around the root system (Torres *et al.*, 2022).

This research succeeded in isolating six Actinomycetes isolates from the rhizosphere of pandan plants (*Pandanus* spp.) and only one isolate, IRzP-at.k, showed significant antimicrobial activity against several test pathogens. This shows that isolates from environments with extreme pH conditions have high potential as a source of new antimicrobial compounds.

CONCLUSION

This research concluded that the Actinomycetes isolate coded IRzP-at.k, obtained from the rhizosphere of pandan in Lombongo Village, Suwawa Tengah Sub-district, Gorontalo Regency, exhibited strong antimicrobial potential against several test microorganisms, including *Escherichia coli, Staphylococcus aureus, Candida albicans,* and *Fusarium oxysporum*. The acidic soil conditions at the isolation site (pH 3,4) were thought to play a significant role in stimulating the production of antimicrobial secondary metabolites by the isolate. Phylogenetic analysis based on the 16S rRNA gene sequence revealed that IRzP-at.k shares a high degree of similarity with *Streptomyces vinaceusdrappus*, indicating its potential as a source of novel metabolites for antibiotic development. Variations in antimicrobial activity among the isolates are likely influenced by differences in the enzymes and secondary metabolites they produce.

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