BIODIVERSITAS
Volume 26, Number 3, March 2025
Pages: 1424-1432

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d260343

Screening of actinobacteria isolated from rice and their potential to inhibit virulence of *Xanthomonas oryzae* pv. *oryzae*

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Manuscript received: 22 December 2024. Revision accepted: 17 March 2025.

Abstract. *Nurwulan UT, Joko T, Arwiyanto T. 2025. Screening of actinobacteria isolated from rice and their potential to inhibit virulence of* Xanthomonas oryzae *pv.* oryzae. *Biodiversitas 26: 1424-1432. Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the pathogen causing bacterial leaf blight, is a major obstacle to improving rice productivity. The virulence factors of *Xoo*, such as motility, biofilm formation, and Extracellular Polysaccharides (EPS), are significant contributors to its pathogenicity. Actinobacteria, known for their beneficial properties, have promising applications in biological control. This study aimed to isolate actinobacteria and evaluate their potential to suppress *Xoo* virulence. Actinobacteria isolates were obtained from paddy fields in Yogyakarta, which have been proven to effectively inhibit the swimming, swarming, and twitching motility of *Xoo*. Isolates KpNg1 and Bs3 showed the most significant suppression, reducing motility to an average diameter of 0.2 cm. All actinobacteria isolates demonstrated the ability to inhibit biofilm formation, as indicated by reduced absorbance values in the crystal violet binding assay, with biofilm inhibitory rates ranging from 16% to 78%. Similarly, the EPS inhibition assay showed that all isolates significantly decreased EPS production, with inhibitory rates ranging from 4% to 49%. The inhibition of *Xoo* virulence factors is associated with quorum quenching mechanisms, particularly in relation to pili and flagella function. Isolates Bs1, KpS, and Bs3 were found to be closely related to *Rhodococcus sovatensis* by phylogenetic analysis based on 16S rRNA gene sequences. At the same time, isolates KpNg1, SGg, and Bs2 were found to be closely related to *Rhodococcus yunnanensis*. These findings underscore the intriguing role of actinobacteria in suppressing Xoo virulence, paving the way for further research and potential applications in biological control.

Keywords: Actinobacteria, bacterial leaf blight, rice, virulence factors, *Xanthomonas oryzae* pv. *oryzae*

INTRODUCTION

Rice is the staple food for the majority of Indonesians despite the availability of alternative carbohydrate sources such as maize and cassava (BPS 2024). However, bacterial leaf blight is caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a major obstacle to improving rice productivity. Yield losses due to *Xoo* infections range from 20-50% and can reach 100% during epidemics. In addition to increasing production costs, disease epidemics threaten global food security (Yang et al. 2020). *Xoo* can infect rice plants at various growth stages, from seedling to reproductive phases. Infections during vegetative phase are considered the most damaging due to their high potential to cause significant plant mortality and yield losses (Chompa et al. 2022).

Xoo shows a high level of genetic diversity, allowing the development of new pathotypes that can infect a wide range of rice varieties, including newly bred resistant cultivars (Irpawa et al. 2024). The virulence factors of *Xoo* are closely related to its ability to infect plants, influencing the severity of the infection and providing protection to the bacterium under unfavorable conditions. Key virulence factors include bacterial motility, biofilm formation, and Extracellular Polysaccharide (EPS) production, which work synergistically to support successful infection (Thepbandit et al. 2021). Biofilm formation provides the pathogen with resistance to extreme environments while EPS production enables it to survive and invade the rice plant. Successful infections often depend on the bacterial ability to attach to and interact with host cells (Büttner and Bonas 2010).

Bacterial motility plays a crucial role in host-pathogen interactions. It directly contributes to virulence by allowing bacteria to move toward nutrient sources, adapt to environmental changes, survive under adverse conditions, and facilitate infection processes (Zegadło et al. 2023). Motility is also essential for biofilm formation, enabling bacteria to locate suitable environments, attach to surfaces, establish colonies, and develop complex biofilm structures (Thepbandit et al. 2021). The primary mechanisms of bacterial motility include swimming, swarming, and twitching. Swimming motility refers to the movement of individual cells powered by the rotation of flagella in liquid environments. Meanwhile, twitching motility occurs on solid surfaces, driven by the extension and retraction of type IV pili, generating thrust that propels the bacteria forward (Colin et al. 2021).

Biofilms are structured bacterial communities that adhere to surfaces and are encapsulated within an extracellular matrix (Lu et al. 2019). Biofilm formation is a natural growth strategy that enables bacteria to compete with other microorganisms and adapt to unfavorable conditions (Azman et al. 2019). Biofilms form through quorum sensing, where bacteria produce autoinducers that accumulate with population growth, regulating gene expression and biofilm development (Urvoy et al. 2021). Bacteria within biofilms exhibit distinct physiological properties compared to their

planktonic counterparts, making them significantly more resistant to antimicrobial treatments (Bjarnsholt et al. 2015; Shafiei et al. 2021).

Xoo is a bacterium capable of producing EPS. EPS is critical in forming xanthan gum or xanthomonadin pigment, which appears as a yellow, mucus-like substance on *Xoo* bacterial colonies. EPS secreted from xanthomonadin is vital for bacterial growth, biofilm formation, and survival under desiccation, and it acts as a key virulence factor (Li et al. 2015). The EPS matrix provides a moist environment to sustain biofilm integrity, and it can also obstruct toxic compounds, such as antibiotics, from penetrating the biofilm cells (Thepbandit et al. 2021).

Actinobacteria are potential biological agents for controlling bacterial leaf blight in rice, offering a safe and sustainable alternative to harmful agents (Ham and Kim 2018). They can inhibit *Xoo* both in vitro by suppressing bacterial growth and in vivo by reducing disease symptom development (Ashwini et al. 2023). Actinobacteria are Gram-positive, unicellular bacteria with peptidoglycan cell walls and DNA rich in guanine and cytosine (Barka et al. 2016). Most species within this phylum are non-motile and lack flagella (Zhu et al. 2023). Certain groups of actinobacteria have been reported to suppress various pathogens and produce intriguing beneficial secondary metabolites (Azman et al. 2019; Ebrahimi-Zarandi et al. 2022).

This study aimed to isolate actinobacteria from rice rhizosphere, which have the potential to control *Xoo*, particularly by targeting its virulence factors. The actinobacteria isolates obtained were further analyzed to evaluate their potential to control *Xoo* infections, offering a promising solution to this agricultural challenge.

MATERIALS AND METHODS

Isolation of Actinobacteria

Actinobacteria were isolated from three regions in Yogyakarta, Indonesia, namely Sleman, Kulon Progo, and Bantul. Sampling parameters focused on the soil surrounding the roots of rice plants exhibiting bacterial leaf blight symptoms. The soil samples were air-dried to suppress the growth of unwanted bacteria. A total of 10 g of soil was placed in an Erlenmeyer flask, and sterile distilled water was added to make up 100 mL suspension. The suspension was then heated at 50°C for 60 minutes (El Karkouri et al. 2019). A 100 µL aliquot of the suspension was spread onto a Water Yeast Extract (WYE) medium supplemented with 100 ppm Benlate and 50 ppm kanamycin. The plates were then incubated at room temperature for 3-7 days. The resulting isolates were subsequently sub-cultured on PSA medium (Priyadarshini et al. 2016).

Hypersensitivity and pathogenicity test

Hypersensitivity tests on tobacco leaves and pathogenicity tests on rice were conducted to determine whether the isolated actinobacteria were plant pathogens. A suspension of actinobacteria was prepared by transferring a single colony from a 3-5-days-old culture into 1 mL of sterile water in an Eppendorf tube, followed by homogenization. As a control, a 24-hour-old suspension of *Xoo* BaK_2 was used. For the hypersensitivity test, 0.5 mL of the suspension was infiltrated into tobacco leaves using a needleless syringe. Two-week-old rice plants were inoculated for the pathogenicity test by clipping the leaves with scissors dipped in the actinobacteria suspension. Positive controls consisted of leaves inoculated with *Xoo* BaK_2. Necrosis on tobacco leaves and rice leaves, which spread after 3-7 days of treatment, indicated that the isolate was pathogenic to the host plant (Wahyudi et al. 2019).

Antagonism test

The actinobacterial isolates were tested for inhibitory potential using the Xoo BaK_2 isolate, which was collected from the previous study (Irpawa et al. 2024). The isolates were cultured on Potato Sucrose Agar (PSA), comprising 200 g of potato extract (per 1 L), 2 g $Na_2HPO_4 \cdot 12H_2O$, 0.5 g $Ca(NO_3)_2 \cdot 4H_2O$, 15 g sucrose, 5 g peptone, and 18 g agar (Adachi et al. 2012). The actinobacteria were spot-inoculated symmetrically and incubated at room temperature for 24 hours. Subsequently, 1 mL of chloroform was added to the Petri dish lid, and the Petri dish was incubated in an inverted position for 1 hour. PSA medium containing 1% agar and a suspension of *Xoo* were poured over the plates, forming two layers. After another 24-hour incubation at room temperature, the inhibition zones were identified as clear zones around the actinobacterial colonies.

Suppression of virulence factors

Preparation of Actinobacterial culture filtrate

Actinobacterial isolates were cultured in liquid PSA and shaken at 150 rpm for seven days. Pellets and supernatant were separated by centrifugation at 3,250 rpm for 15 minutes at 4°C. The supernatant was filtered through a 0.22 µm sterile filter and stored at -20°C for further tests (Kaari et al. 2022).

Motility test

Swimming, swarming, and twitching motility tests were performed using Wakimoto medium (ferrous sulphate 0.05 g, calcium nitrate 0.5 g, sodium phosphate 0.82 g, peptone 5 g, sucrose 20 g, and distilled water 1 L) containing 0.25%, 0.5%, and 1.5% agar (EPPO 2007). A 100 µL aliquot of actinobacterial filtrate was added to 9.9 mL of medium supplemented with 10 µL TZC (10,000 ppm). A 2 µL suspension of *Xoo* (10^8 CFU/mL) was inoculated onto the medium. *Xoo* colonies were pricked vertically into the agar using an inoculation needle for the twitching motility test. Each treatment was made into three replications, and bacterial motility diameters were measured daily for three days. The control consisted of *Xoo* without actinobacteria treatment (Yu et al. 2017; Thepbandit et al. 2021).

Biofilm formation test

Xoo was cultured in liquid Wakimoto at 150 rpm for two days to reach a density of 10^8 CFU/mL. In a 96-well microplate, 100 µL of liquid Wakimoto, 50 µL of *Xoo* culture, and 50 µL of actinobacterial filtrate were added. For the control, 150 µL of liquid Wakimoto and 50 µL of *Xoo* culture were used. Plates were incubated for 72 hours.

After incubation, wells were washed and air-dried. Crystal violet 1% (250 µL) was added to each well and incubated for 15 minutes. Excess dye was washed off, and wells were treated with 250 µL of 96% ethanol. A purple ring in the wells indicated biofilm formation (Joko et al. 2024), which was then quantified using a microplate reader at 530 nm (Kaari et al. 2022).

EPS production test

Xoo was cultured in liquid Wakimoto at 150 rpm for 48 hours. A suspension of *Xoo* (10^8 CFU/mL) was prepared, and 100 µL of *Xoo* culture, 100 µL of actinobacteria filtrate, and 9.8 mL of Wakimoto medium were combined. The suspension was incubated at 150 rpm and 28°C for 72 hours. After centrifugation at 12,000 × g for 15 minutes at 4°C, the supernatant was mixed with three volumes of 95% ethanol to precipitate EPS. The pellet was dried and quantified using phenol-sulfuric acid, with absorbance measured at 490 nm (Dubois et al. 1956; Thepbandit et al. 2021).

Data analysis

The suppression of virulence factors was evaluated using a Completely Randomized Design (CRD) with three replicates for each treatment. Data analysis was performed using RStudio software and subjected to Analysis of Variance (ANOVA). Treatment significance was determined based on the F-value at a significance level of $p < 0.05$. Mean differences were further analyzed using Tukey's Honestly Significant Difference (HSD) test to identify significant variations between treatments.

Molecular identification of Actinobacteria

DNA was extracted using the Geneaid DNA Kit. PCR was performed with primers S-C-Act-235-a-S-20 (CGCGGCCTATCAGCTTGTTG) and S-C-Act-878-a-A-19 (CCGTACTCCCCAGGCGGGG), targeting a 640-660 bp product (Stach et al. 2003). The PCR reaction mixture consists of 25 µL Bioline My Taq HS Red Mix, 9 µL nuclease-free water, 4 µL forward primer, 4 µL reverse primer, and 8 µL DNA template. PCR conditions consisted of an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 1 minute, 65.5°C for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 5 minutes. Visualized DNA using 1% agarose gel electrophoresis stained with ethidium bromide. Sequencing was performed by PT. Genetika Science Indonesia. The nucleotide sequences were analyzed using BLAST and compared with GenBank references. Phylogenetic trees were constructed in MEGA 11 using the maximum-likelihood algorithm and bootstrap analysis with 1,000 replicates (Trianom et al. 2019).

RESULTS AND DISCUSSION

Isolation of Actinobacteria

Six isolates were obtained from three different sampling locations, representing the collection sites, and their morphological characteristics suggested that they belong to the class Actinobacteria (Table 1; Figure 1). The colony morphology was circular, with a grayish-green color, white edges, and an uneven colony surface. The isolates formed spore masses on the colony surface resembling a powdery texture, except for isolate Bs1, which exhibited a smooth colony surface that adhered to the growth medium. Additionally, the colonies emitted a distinctive earthy odor associated with volatile compounds such as geosmin, a secondary metabolite produced by certain actinomycetes (Araujo-Melo et al. 2019). Yellow pigmentation of the PSA medium was observed for isolates KpNg1, Bs2, and Bs3. This pigmentation change is attributed to the production of pigments by aerial hyphae and spores, which dissolve and diffuse into the medium, altering its color (Minas et al. 2000). Pigment production is influenced by several factors, including pH and the carbon and nitrogen sources present in the culture medium (Abraham and Chauhan 2018).

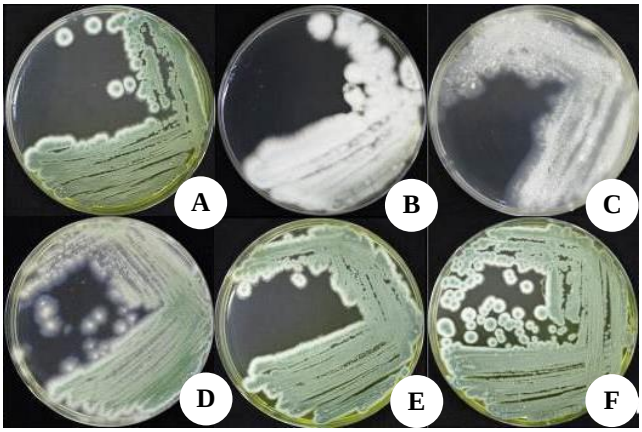

Figure 1. Actinobacterial colonies isolated from the rhizosphere of rice plants on PSA medium after 5 days of incubation: A. isolate KpNg1; B. Bs1; C. SGg; D. KpS; E. Bs2; and F. Bs3. The colonies exhibited white-grayish-green pigmentation and some isolates induced color changes in the growth medium

Table 1. A list of actinobacterial isolates collected

Code	Isolate code	Sample location	Coordinates
A	KpNg1	Jatisarono, Nanggulan, Kabupaten Kulon Progo, DIY	-7.751659, 110.201359
B	Bs1	Argorejo, Sedayu, Kabupaten Bantul, DIY	-7.824938, 110.254712
C	SGg	Ambarketawang, Gamping, Kabupaten Sleman, DIY	-7.804605, 110.319434
D	KpS	Banguncipto, Sentolo, Kabupaten Kulon Progo, DIY	-7.818112, 110.225819
E	Bs2	Argorejo, Sedayu, Kabupaten Bantul, DIY	-7.824938, 110.254712
F	Bs3	Argorejo, Sedayu, Kabupaten Bantul, DIY	-7.824938, 110.254712

Hypersensitivity and pathogenicity test

None of the six actinobacterial isolates exhibited a hypersensitive reaction in tobacco plants at 3 days post-inoculation (Figure 2.A), indicating that the isolates are not plant pathogens. The pathogenicity test on rice plants showed yellowing at the wound site; however, this symptom did not progress within 10 days post-inoculation (Figure 2.B). This suggests that the actinobacterial isolates are not capable of infecting rice plants. The positive control with *Xoo* treatment exhibited necrosis in tobacco leaves and elongated lesions on rice leaves. The symptoms observed on the rice leaves were consistent with bacterial leaf blight disease (Chompa et al. 2022). The occurrence of necrosis is a plant response aimed at protecting the tissue from further infection. The death of plant tissue around the injection site is considered a hypersensitive reaction, which can also be associated with plant resistance responses.

Antagonism test

All actinobacterial isolates exhibited growth inhibition of *Xoo*, with varying sizes of clear zones (Figure 3). The KpS isolates displayed the largest clear zones. The clear zone represents the area of bacterial inhibition, indicating the presence of antibiosis or growth inhibition mechanisms by the antagonistic bacterial isolates against the pathogenic bacteria, preventing their growth around the antagonistic bacteria. The observed inhibition of *Xoo* growth suggests an antibiosis mechanism employed by the actinobacterial isolates. Differences in the inhibition mechanisms of the bacteria can influence bacterial growth, leading to variations in the diameter of the inhibition zone (Nellawati et al. 2016).

Bacterial virulence suppression test

Motility test

Over three days, observation of three bacterial motility mechanisms (swimming, swarming, and twitching motility) showed that the actinobacterial isolates could suppress bacterial motility with varying degrees of suppression (Table 2; Figure 4). The isolates KpNg1 and Bs3 consistently showed the best suppression, indicated by minor motility diameter in swimming, swarming, and twitching. Meanwhile, other actinobacterial isolates showed smaller motility zones compared to the control. According to Tukey's HSD test, all isolates showed a significant difference from the control in swimming motility. However, Bs1 did not significantly differ from the control in swarming motility, while in twitching motility, KpNg1, Bs2, and Bs3 exhibited significant differences from the control. The suppression of motility can be interpreted as a reduction in the pathogen's ability to spread and cause disease. The inhibition of swimming, swarming, and twitching motility by actinobacteria indicates its potential as a quorum quenching agent. Quorum quenching does not kill pathogens but suppresses or inhibits pathogenic trait expression (Kapadia et al. 2022).

Biofilm formation test

Six actinobacterial isolates inhibited *Xoo* biofilm formation, indicated by a decrease in crystal violet binding and a lower average absorbance value compared to the control (Figure 5; Table 3). Isolate Bs2 showed the best inhibitory effect, with a 78.43% reduction in biofilm formation and showed a significant difference from the control based on Tukey's HSD test. Each treatment showed different levels of crystal violet binding. The amount of crystal violet bound is directly proportional to biofilm formation by the bacteria. In this study, biofilm formation was inhibited by adding actinobacteria to *Xoo* suspensions to prevent bacterial adhesion to surfaces. The metabolites from actinobacteria were expected to inhibit the biofilm formation. Biofilm formation can be inhibited through several mechanisms, including preventing bacterial colonization by inhibiting growth, preventing bacterial attachment to surfaces, disrupting biofilm formation gene expression, and degrading the EPS matrix (Roy et al. 2018; Azman et al. 2019). The ability of actinobacteria to suppress *Xoo* biofilm formation affects the reduction of its virulence, and it can enhance the effectiveness of control measures.

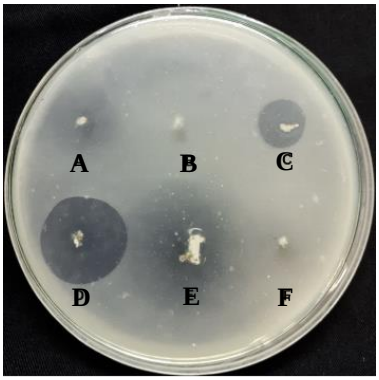

Figure 3. The clear zones representing the inhibition of *Xoo* growth by actinobacterial isolates: A: KpNg1, B: Bs1, C: SGg, D: KpS, E: Bs2, F: Bs3 after 24 hours of incubation

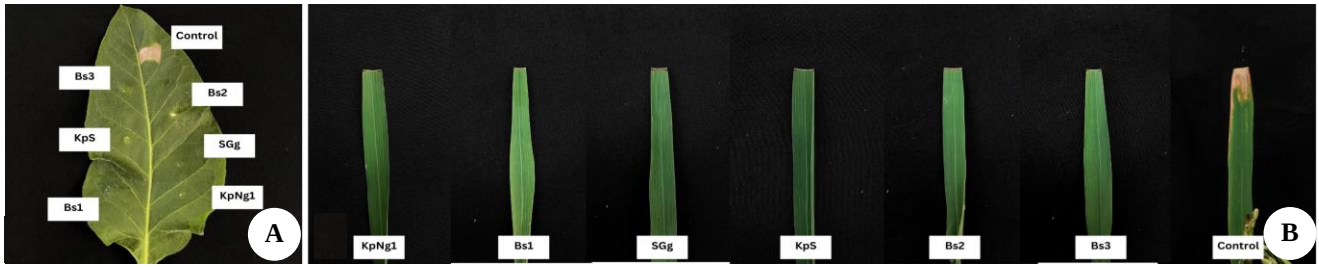

Figure 2. A. Hypersensitivity test on tobacco plants; B. Pathogenicity test of actinobacterial isolates on rice plants; *Xoo* BaK_2 was used as a control, showing a hypersensitive reaction on tobacco plants and bacterial leaf blight symptoms on rice

Extracellular Polysaccharides (EPS) production test

Six actinobacterial isolates inhibited EPS formation based on the phenol-sulfuric acid method (Figure 6). The reaction between the sugars in the exopolysaccharide and phenol under strongly acidic conditions resulted in a yellow-to-brown color. The intensity and color formed can be a visual indicator to determine the amount of sugar produced. A more intense or brown color indicates a higher concentration of EPS produced by the bacteria. The best suppression of EPS production was demonstrated by isolate Bs3, with a 49.83% reduction in EPS production and showed a significant difference from the control based on Tukey's HSD test. The suppression of EPS formation is one way to reduce the virulence of *Xoo*. Inhibiting the formation of the EPS matrix can block biofilm formation and spread, as well as optimize control since planktonic bacteria can be exposed to antibacterial agents or antibiotics (Roy et al. 2018).

Molecular identification

Based on the PCR amplification results, the actinobacteria isolates showed a DNA band of 643 bp in size (Figure 7). This amplification result indicates that all isolates belong to the actinobacteria group. Based on DNA sequencing results, the isolates showed a close genetic relationship with *Rhodococcus sovatensis* and *Rhodococcus yunnanensis*. According to Táncsics et al. (2017), *R. sovatensis* sp. nov. is non-motile, and its colonies produce orange pigments on TSA media. Meanwhile, *R. yunnanensis* sp. nov. is non-motile, and it forms hyphal structures that fragment into short segments and have colony diameters ranging from 0.5-1 mm after 3 days of incubation (Zhang et al. 2005). These characteristics are similar to those observed in the isolates in this study.

Discussion

The class Actinobacteria is significant in the phylum Actinobacteria and is essential in various ecosystems. Most actinobacteria form filamentous structures similar to fungi and are considered transitional groups between fungi and bacteria. Actinobacteria have diverse habitats, including soil, rhizosphere, and marine or aquatic environments (Miao and Davies 2010). In this study, six isolates with morphologies characteristic of the actinobacteria group were collected from the rhizosphere of rice plants (Figure 1). The isolates on PSA media exhibited distinctive features, including the formation of spore masses on the colony surface. The spore mass formed is a collection of spores at the hyphal tips, accumulating like flour on the colony's surface. According to Kawuri (2016), this flour-like structure represents aerial spores produced by mycelium when the colony matures. Molecular identification confirmed that all six isolates belong to actinobacteria, specifically *R. sovatensis* and *R. yunnanensis*.

The six actinobacterial isolates did not exhibit pathogenicity in tobacco and rice plants (Figure 2.B). This indicates that these isolates are not pathogenic to the test plants and can be used as biocontrol agents. In laboratory tests, all six actinobacterial isolates inhibited the growth of *Xoo*, demonstrating their potential in controlling pathogens (Figure 3). According to Serdani et al. (2018), some antagonistic microbes inhibit the growth of pathogens by producing compounds with antibiotic properties or growth inhibitors, such as antibiotics, siderophores, hydrolytic enzymes that can lyse pathogen cells, and volatile compounds that are toxic to plants. Actinobacteria capable of inhibiting the growth of *Xoo* were expected to reduce the virulence of *Xoo*. Virulence refers to the ability of pathogens to cause disease. Reducing *Xoo* virulence may mitigate disease severity and optimize control efforts.

Table 2. The average motility diameter of *Xoo* treated with actinobacterial isolates on the third day after incubation. Three replications of the experiment were made, and the results ($p<0.05$) showed significant differences compared to the control, as evaluated using Tukey's HSD test

Treatment	Diameter (cm)		
	Swimming motility	Swarming motility	Twitching motility
KpNg1	0.20 e	0.20 c	0.20 b
Bs1	0.97 b	0.87 ab	0.70 a
SGg	0.33 de	0.83 b	0.70 a
KpS	0.70 c	0.77 b	0.63 a
Bs2	0.43 d	0.30 c	0.20 b
Bs3	0.20 e	0.20 c	0.20 b
Control	1.23 a	0.97 a	0.77 a

Note: Values followed by the same letters within the same column are not significantly different based on Tukey's HSD test at α = 5%

Table 3. Effects of actinobacteria on biofilm formation and Extracellular Polysaccharide (EPS) production by *Xoo* measured by absorbance values; the inhibitory rate of each treatment was compared to that of the control. Three replications of the experiment were made, and the results ($p<0.05$) showed significant differences compared to the control, as evaluated using Tukey's HSD test

Treatment	Average absorbance value at 530 nm	Biofilm inhibition rate (%)	Average absorbance value at 490 nm	EPS inhibition rate (%)
KpNg1	0.43 ab	62.96	3.72 a	4.75
Bs1	0.76 ab	34.62	2.86 ab	26.81
SGg	0.97 ab	16.82	2.28 b	41.50
KpS	0.36 ab	68.76	2.29 b	41.36
Bs2	0.25 b	78.43	2.90 ab	25.70
Bs3	0.46 ab	58.25	1.96 b	49.83
Control	1.16 a	0	3.91 a	0

Note: Values followed by the same letters within the same column are not significantly different based on Tukey's HSD test at α = 5%

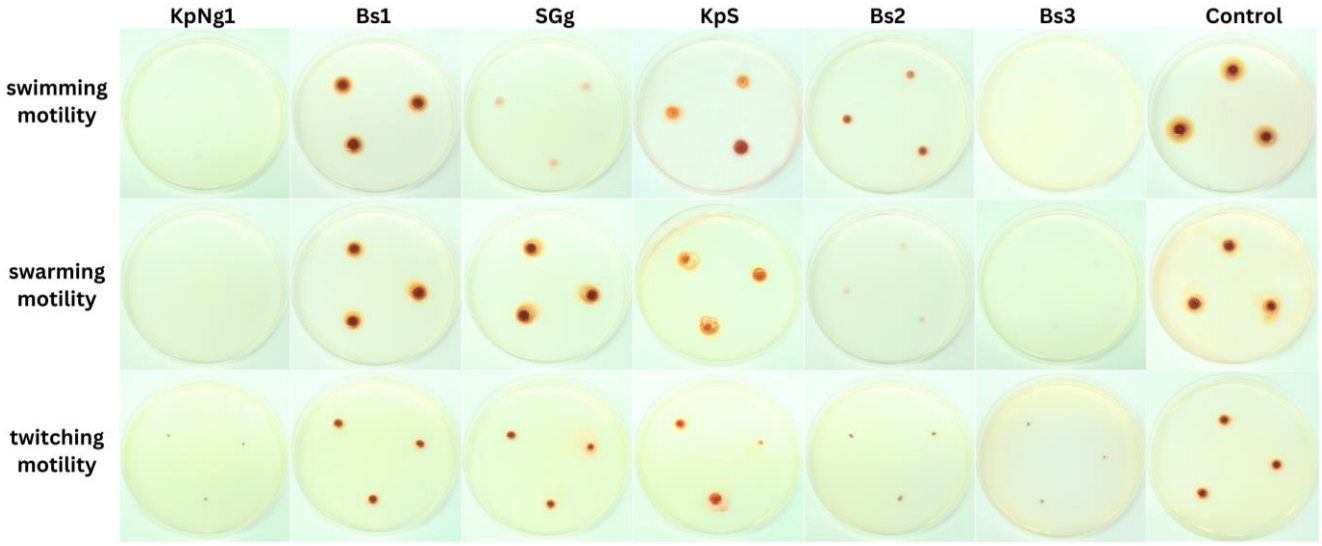

Figure 4. Swimming, swarming, and twitching motility of *Xoo* treated with actinobacteria on Wakimoto medium after 3 days of incubation; motility was measured by recording the diameter of bacterial movement from the center of inoculation. Larger motility diameters indicate higher bacterial motility capabilities, while smaller diameters suggest inhibition of movement due to actinobacterial treatment

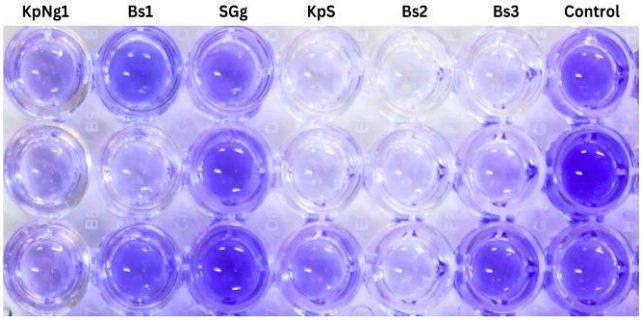

Figure 5. Biofilm formation by *Xoo* treated with actinobacteria was assessed using crystal violet staining after 3 days of incubation. More intense crystal violet binding on the microplate indicated enhanced biofilm formation

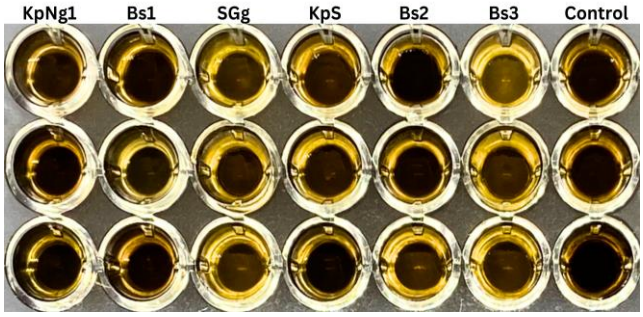

Figure 6. EPS production by *Xoo* treated with actinobacteria analyzed using the phenol-sulfuric acid method; A more intense brown coloration indicates higher EPS production

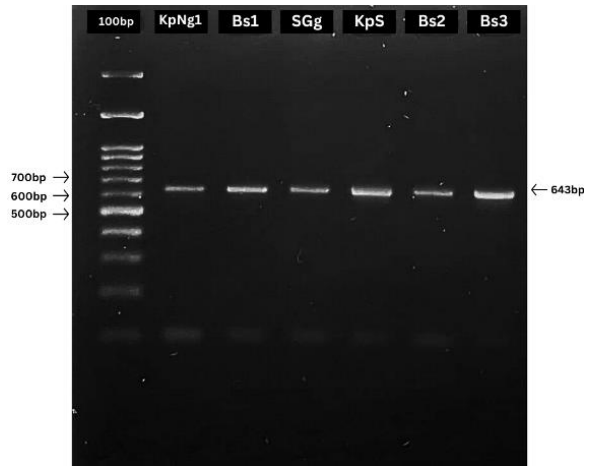

Figure 7. PCR amplification results of actinobacterial isolates with 16s rRNA primers on a 1% agarose gel, with a target size of approximately 643 bp

The six actinobacteria tested in this study reduced *Xoo* virulence by inhibiting swimming, swarming, and twitching motility (Figure 4), biofilm formation (Figure 5), and EPS production (Figure 6). Each isolate exhibited varying inhibitory rates. The suppression of swimming, swarming, and twitching motility by actinobacteria suggests their potential quorum-quenching activity. The quorum-quenching mechanism can attenuate or inhibit the expression of pathogenic traits (Kapadia et al. 2022). According to Thepbandit et al. (2021), the inhibition of *Xoo* swimming motility may result from treatments affecting the expression of genes required for flagellar assembly or function, thereby impairing flagella-driven motility. Actinobacterial isolates KpNg1, Bs2, and Bs3 consistently inhibited all three motility mechanisms. In contrast, isolates Bs1, SGg, and KpS failed to inhibit twitching motility, which is likely due to insufficient production of specific antimicrobial compounds capable of suppressing type IV pili function. The inhibition of *Xoo* twitching motility by actinobacteria

may occur through the disruption of bacterial adhesion mediated by type IV pili, which also affects microcolony formation (Thepbandit et al. 2021).

The inhibition of biofilm formation may occur because actinobacteria introduced to the *Xoo* suspension can suppress the growth and development of surface-attached bacteria, thereby preventing bacterial colonization (Azman et al. 2019). Additionally, actinobacteria can interfere with signaling molecules that regulate biofilm formation by degrading the EPS matrix (Roy et al. 2018). The phenol-sulfuric acid method was used to assess EPS production by measuring the polysaccharide content, a class of complex carbohydrates, in *Xoo* aliquots treated with actinobacteria. Concentrated sulfuric acid hydrolyzes polysaccharides into simple monosaccharides, which subsequently react with phenol to produce a yellowish-brown color complex (Saad et al. 2023). EPS production in bacteria is regulated by 12 key genes, *gumB* to *gumM*, which are involved in the synthesis and secretion of pentasaccharide polymers. Additionally, the *xanA* and *xanB* genes are responsible for providing nucleotide sugar precursors required for EPS biosynthesis (Thepbandit et al. 2021). Pathogenic bacteria utilize cell-to-cell communication to regulate the expression of factors contributing to various biological functions, including pathogen virulence (Urvoy et al. 2021). Quorum sensing enhances *Xoo* virulence by regulating motility, biofilm expansion, and Extracellular Polysaccharide (EPS) production (Liang et al. 2018).

Research on *R. sovatensis* and *R. yunnanensis* in biological control remains limited. However, several *Rhodococcus* species have been extensively studied for their biocontrol potential. Based on the study by Hu et al. (2024), *Rhodococcus* possesses broad biosynthetic capabilities and produces various secondary metabolites with strong antibacterial activity. *Rhodococcus erythropolis* has been shown to inhibit biofilm formation and development through quorum-quenching mechanisms, which involve disrupting microbial communication (Bourigault et al. 2021). *Rhodococcus pyridinivorans* exhibit acylase enzyme activity, capable of degrading N-Acyl Homoserine Lactone (AHL) into fatty acids and homoserine lactone (Zhou et al. 2022). AHL molecules serve as signaling compounds used by bacteria for communication through a mechanism known as quorum sensing. When bacterial populations reach a quorum, AHL molecules trigger the activation of specific genes, including those involved in motility and biofilm formation. Enzymatic reactions mediated by lactonase and acylase enzymes can disrupt interbacterial communication by degrading AHL molecules, thereby weakening bacterial infections and suppressing pathogenicity without directly killing the bacteria (Bourigault et al. 2021; Zhou et al. 2022). These findings highlight the potential of *Rhodococcus* as a promising source of novel antibacterial compounds, particularly those associated with metabolites that can be developed for disease control.

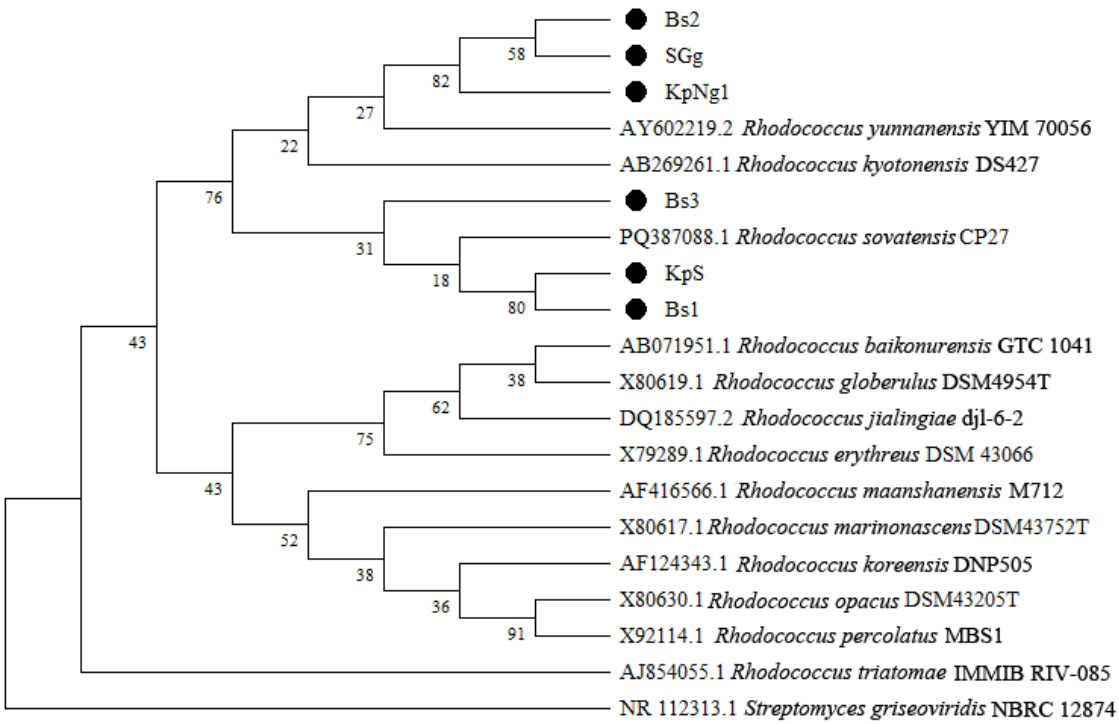

Figure 8. The phylogenetic tree of actinobacterial isolates KpNg1, Bs1, SGg, KpS, Bs2, and Bs3 based on 16S rRNA primers. The tree was constructed using the maximum likelihood method with the Tamura model and tested with 1,000 bootstrap replicates

In conclusion, this study demonstrates that the six actinobacterial isolates effectively reduce the virulence of *Xoo*, as evidenced by the inhibition of motility, biofilm formation, and Extracellular Polysaccharide (EPS) production following actinobacterial treatment. The most significant inhibitory effect was observed in isolates Bs3 and Bs2, both of which consistently reduced bacterial motility, biofilm formation, and EPS production. The reduction in *Xoo* virulence may be attributed to actinobacterial culture filtrates, which contain various bioactive metabolites capable of attenuating *Xoo* pathogenicity. Further research is needed to identify the specific secondary metabolites produced by these actinobacteria, as this is crucial for understanding the precise mechanisms involved in *Xoo* inhibition.

ACKNOWLEDGEMENTS

This research was funded by The Directorate of Higher Education, Research, and Technology through the National Competitive Research Scheme for Master's Thesis Research based on Decree No. 0459/E5/PG.02.00/2024 and Agreement/Contract No. 048/E5/PG.02.00.PL/2024; 2877/ UN1/DITLIT/PT.01.03/2024. The authors would like to express gratitude to all parties who have contributed regarding guidance, facilities, and technical support throughout this research. Their support is invaluable in completing this study.

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