

Exploration of Endophytic Actinomycetes from Volcanic Soil Adapted *Pityrogramma calomelanos* as a Novel Source of Antibacterial Agents

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Abstract

The emergence of antibiotic-resistant pathogenic bacteria poses a major threat to public health, emphasizing the importance of discovering new antibiotic compounds. Endophytic microorganisms are known for producing a variety of antibiotics and other bioactive substances. They can generate a broad spectrum of secondary metabolites with potent antibacterial activity. Endophytes are microorganisms that form symbiotic relationships within plant tissues without causing obvious disease to the host plant. Microbes that live in extreme environments develop unique adaptive mechanisms and can produce novel bioactive compounds. Bacteria in such harsh conditions often develop specialized metabolic pathways, resulting in the production of distinctive secondary metabolites. This study aimed to isolate endophytic actinomycetes from the roots of the silver fern (*Pityrogramma calomelanos* (L.) Link), which grows in soil exposed to the eruption of Mount Sinabung in Karo Regency, North Sumatra Province, Indonesia. Antimicrobial screening of these isolates was performed using a dual culture assay to assess their ability to inhibit pathogenic bacteria. The endophytic actinomycete isolate AE9 showed activity against *Staphylococcus aureus*, *Bacillus cereus*, and *Citrobacter freundii*. Morphological, physiological, biochemical, and 16S rRNA gene sequence analyses confirmed that isolate AE9 is related to *Streptomyces* sp. VEL 17. These results suggest that endophytic actinomycetes from silver ferns growing in volcanically affected environments contain antibacterial bioactive compounds.

Keywords: endophytic microbes, actinomycetes, antibacterial, *Pityrogramma calomelanos*

Резюме

Появата на патогенни бактерии, устойчиви на антибиотици, представлява сериозна заплаха за общественото здраве, което подчертава важноста на откриването на нови антибиотични съединения. Ендофитните микроорганизми са известни с производството на разнообразни антибиотици и други биоактивни вещества. Те могат да генерират широк спектър от вторични метаболити с мощна антибактериална активност. Ендофитите са микроорганизми, които образуват симбиотични взаимоотношения в растителните тъкани, без да причиняват видими заболявания на растението гостоприемник. Микробите, които живеят в екстремни среди, развиват уникални адаптивни механизми и могат да произвеждат нови биоактивни съединения. Бактериите в такива сурови условия често развиват специализирани метаболитни пътища, което води до производството на отличителни вторични метаболити. Това проучване имаше за цел да изолира ендофитни актиномицети от корените на сребърната папрат (*Pityrogramma calomelanos* (L.) Link), която расте в почва, изложена на изригването на вулкана Синабунг в Каро Регенци, провинция Северна Суматра, Индонезия. За да се оцени способността на тези изолати да инхибират развитието на патогенните бактерии беше извършен антимикробен скрининг с помощта на двоен културален анализ. Ендофитният актиномицетен изолат AE9 показва активност срещу *Staphylococcus aureus*, *Bacillus cereus* и *Citrobacter freundii*. Морфологични, физиологични, биохимични проучвания, както и 16S rRNA генни секвенционни анализи потвърждават, че изолатът AE9 е свързан със *Streptomyces* sp. VEL 17. Тези резултати показват, че ендофитните актиномицети от сребърни папрати, растящи във вулканично засегнати среди, съдържат антибактериални биоактивни съединения.

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Introduction

Bacterial resistance to antibiotics poses a major threat to public health (Abbas *et al.*, 2024). The paucity in discovery and supply of new novel antimicrobials to treat life-threatening infections by resistant pathogens stands in sharp contrast to demand (Salam *et al.*, 2023). Despite ongoing efforts, the rate at which resistant strains emerge has outpaced the development of new antibiotics, necessitating the exploration of alternative and under-exploited natural sources to uncover novel antimicrobial compounds (Sivalingam *et al.*, 2019).

Natural products continue to represent the primary source for the discovery of novel antibiotics due to their chemical diversity and broad spectrum of biological activity (Bernal *et al.*, 2022). Among these, endophytic actinomycetes are recognized as prolific producers of bioactive metabolites and have been identified as a valuable resource in drug discovery research (Mondal and Thomas, 2022). Endophytic Actinobacteria, particularly from the genus *Streptomyces*, are notable for producing a wide array of bioactive compounds that could serve as alternatives in combating multidrug-resistant pathogens (Anavadiya *et al.*, 2024).

Actinomycetes are classified as Gram-positive bacteria with a high genomic G+C content and are therefore recognized for their ability to produce a wide range of secondary metabolites (Matsumoto and Takahashi, 2017). However, the frequent rediscovery of known compounds has posed a significant challenge, necessitating the search for novel strains from unexplored habitats (Subramani and Sipkema, 2019). Exploration of unique and extreme environments, such as underexplored soils, is critical to discovering new secondary metabolites with potent antibacterial activity (Charousová *et al.*, 2019).

Plants growing in harsh environments, such as under abiotic stress conditions, are promising hosts for novel endophytic actinomycetes (Meklat *et al.*, 2011). Environmental factors such as soil type, climate, and abiotic pressures greatly influence the diversity and biosynthetic potential of microbial endophytes (Przybylska-Balcerek *et al.*, 2020). Particularly, volcanic soils represent a distinctive ecological niche rich in microbial biodiversity and bioactive compound production (Maglangit *et al.*, 2023). Members of the genus *Streptomyces* are well adapted to survive in such environments due to their specialized enzymatic and metabolic adaptations (Lorková *et al.*, 2025).

The silver fern, *Pityrogramma calomelanos* (L.) Link serves as an important host for endophytic

actinomycetes. This pioneer plant species thrives in extreme conditions such as volcanic areas, mining regions, and lead (Pb)-contaminated soils (Anchetta *et al.*, 2020; Yongpisanphop and Babel, 2021). Its remarkable adaptability is attributed to its high flavonoid content, which confers resistance to abiotic stresses like drought and elevated temperatures (Shomali *et al.*, 2022). The study of endophytic actinomycetes from *P. calomelanos* occurring in challenging environments is expected to facilitate the discovery of novel bioactive compounds that could significantly contribute to drug development.

Materials and Methods

Sample collection

Root samples of the silver fern (*P. calomelanos* (L.) Link) were obtained from post-eruption zones of Mount Sinabung, located in Karo District, North Sumatra, Indonesia. Sampling was carried out purposively to target sites most affected by volcanic activity, which are considered extreme habitats with unique ecological pressures. Three representative localities, Gamber Village, Guru Kinayan Village, and Suka Tendel Village, were selected based on their proximity to the eruption sites and their history of ash deposition and soil disturbance. For accurate taxonomic identification and authentication, plant specimens were examined at the Herbarium Bogoriense, Directorate for Scientific Collections Management, National Research and Innovation Agency (BRIN). To safeguard the quality of the samples, freshly collected roots were placed in sterile plastic containers, maintained at low temperatures using ice gel packs, and transported directly to the laboratory for further microbiological examination.

Surface sterilization of plant material

Root samples were cleaned under running tap water to remove adhering soil and debris. Samples were then cut into approximately 2 cm segments and subjected to a surface sterilization protocol: immersion in 1% sodium hypochlorite (NaOCl) solution for 15 seconds, followed by immersion in 70% ethanol for 15 seconds. The samples were then rinsed three times with sterile distilled water to remove residual sterilizing agents. The final rinse water was plated onto sterile media to confirm the efficacy of surface sterilization (Afzal *et al.*, 2019).

Isolation of endophytic actinomycetes

A total of 10 g of surface-sterilized root tissues was aseptically macerated with a sterile mortar and pestle, after which the homogenate was suspended

in 90 mL of sterile distilled water in an Erlenmeyer flask. The suspension was homogenized using a vortex mixer and serially diluted from 10^1 to 10^4 . Aliquots of 0.1 mL from each dilution were plated onto Starch Casein Agar (SCA) supplemented with nystatin (50 µg/mL) to inhibit fungal contamination. The culture plates were incubated at 28°C for a period of up to two weeks. Colonies that developed with morphological characteristics indicative of actinomycetes - such as dry, powdery textures and filamentous structures - were then transferred onto fresh SCA medium to establish pure isolates.

Screening of potential antibacterial activity of endophytic actinomycetes

The antibacterial activity of endophytic actinomycete isolates was evaluated using the dual culture assay method. Three test bacteria were employed: Gram-positive *Staphylococcus aureus* (ATCC 25923), Gram-positive *Bacillus cereus* (ATCC 11778), and Gram-negative *Citrobacter freundii* (ATCC 8090). Tryptic Soy Agar (TSA) medium was used for antibacterial screening. TSA plates were prepared by pouring molten media into sterile Petri dishes and allowing them to solidify. The surface of the solidified TSA was then uniformly swabbed with the test bacterial suspension adjusted to 0.5 McFarland standard turbidity using a sterile cotton swab.

Endophytic actinomycete isolates, previously cultivated on Starch Casein Agar (SCA) for 7 days at 28°C (Alhialy and Thanoon, 2022), were prepared for the assay. Agar plugs (6 mm diameter) containing actively growing actinomycete colonies were aseptically cut using a sterile cork borer (Xu *et al.*, 2024). These plugs were carefully placed onto the TSA plates that had been pre-inoculated with the test bacteria.

The plates were incubated at 28°C for 24 hours. The antibacterial potential of each isolate was indicated by the formation of a clear inhibition zone surrounding the agar plug. The diameter of the inhibition zones was measured in millimeters with a digital caliper (Santos-Beneit *et al.*, 2022). A control plate containing only the test bacteria without actinomycete inoculation was included to verify baseline growth conditions.

Morphological and cultural characterization

Macroscopic characteristics of endophytic actinomycetes, including colony shape, surface texture, margin, pigmentation, and diffusible pigment production, were observed after 7 days of incubation on (SCA) at 28°C (Holt *et al.*, 2000).

Microscopic features were examined using Gram staining (Cappuccino and Sherman, 2014) and the slide culture technique to assess hyphal structure and spore chain morphology (Ali *et al.*, 2022).

Genetic characterization

Temperature tolerance was evaluated by growing isolates at 28°C, 37°C, and 40°C on SCA for 7 days (Mondal and Thomas, 2022). pH tolerance was assessed on SCA adjusted to pH 5, 7, 9, and 11, while salinity tolerance was determined on SCA supplemented with 0%, 4%, 10%, and 13% NaCl concentrations (Siddharth *et al.*, 2020).

Biochemical tests included Triple Sugar Iron Agar (TSIA) fermentation, citrate utilization, catalase production, urease activity, and motility in SIM medium, all performed according to standard protocols (Cappuccino and Sherman, 2014). Positive reactions were indicated by color changes or diffusion patterns characteristic of each test.

Genomic characterization

Genomic DNA was extracted from bacterial isolates using the Quick-DNA Magbead Plus Kit (Zymo Research, D4081) according to the manufacturer's instructions (PT. Genetika Science Indonesia). The extraction process involved cell lysis, DNA binding to magnetic beads, washing, and elution to obtain high-purity genomic DNA suitable for PCR amplification. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal primers: 27F: 5'-AGAGT-TTGATCMTGGCTCAG-3', 1492R: 5'-TACG-GYTACCTTGTTACGACTT-3' (Tedsree *et al.*, 2022). PCR amplification was carried out in a 25 µL reaction mixture containing a PCR master mix, 10 pmol of each primer, DNA template, and nuclease-free water. The thermocycling conditions were as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 30 min, annealing at 55°C for 30 min, and extension at 72°C for 90 min; and a final extension at 72°C for 7 min.

PCR products were checked by electrophoresis on a 1% agarose gel. PCR products were purified using a standard PCR purification kit according to the manufacturer's protocol. The purified products were sequenced bidirectionally using an Applied Biosystems 3730XL DNA Analyzer. The nearly full-length 16S rRNA gene sequences obtained were analyzed using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) website (<https://www.ncbi.nlm.nih.gov>) to determine sequence similarity against the GenBank database. Phylogenetic trees

were reconstructed using the Neighbour-Joining (NJ) method (Saitou and Nei, n.d.) in MEGA version 11.0 software (Tamura *et al.*, 2021). The stability of the tree topology was evaluated by bootstrap analysis on 1000 replications. Sequence similarity percentages between strains were calculated based on pairwise alignments using the EzTaxon-e server (Kim *et al.*, 2012).

Results

A total of nine strains of endophytic actinomycetes were successfully isolated from the roots of *P. calomelanos* collected from post-eruption volcanic soils of Mount Sinabung. The exploration of microbial diversity in extreme environments, such as volcanic ecosystems, is crucial for uncovering novel bioactive compounds (Huang *et al.*, 2024). All isolated strains exhibited antibacterial activity against at least one of the tested pathogenic bacteria. Specifically, all nine strains inhibited *B. cereus* ATCC 11778, seven strains inhibited *C. freundii* ATCC 8090, and seven strains inhibited *S. aureus* ATCC 25923. Notably, six strains demonstrated broad-spectrum antibacterial activity by inhibiting all three pathogenic bacteria simultaneously. Among the isolates, strain AE9 exhibited the most prominent antibacterial activity compared to other isolates and was thus selected for further study (Fig. 1).

Strain AE9 exhibited strong antimicrobial activities against *B. cereus* ATCC 11778 (38.6 mm), *S. aureus* ATCC 25923 (32.8 mm), and *C. freundii* ATCC 8090 (26.9 mm). These results indicate

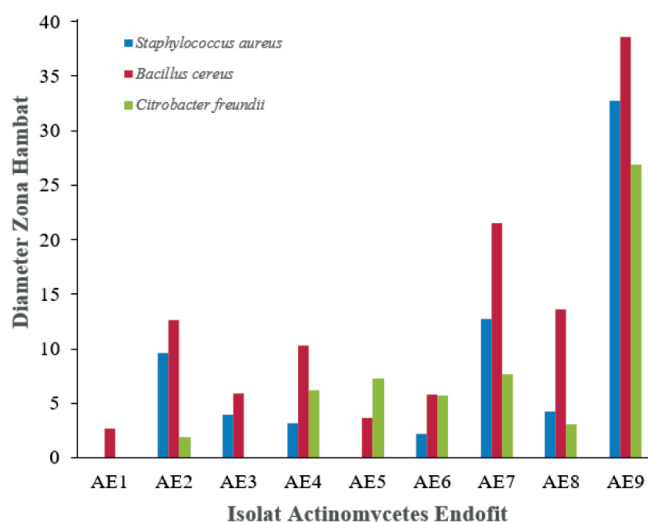


Fig. 1. Inhibition zone shown by actinomycete isolates from volcanic soil after the eruption of Mount Sinabung on pathogenic test bacteria.

that AE9 possesses strong and broad-spectrum antibacterial properties (Fig. 2). These findings are consistent with previous studies demonstrating that endophytic actinomycetes, particularly those from unexplored or extreme habitats, are prolific sources of antimicrobial agents (Hui *et al.*, 2021).

Identification of endophytic actinomycete strain AE9

Figure 2 illustrates the morphological characteristics of the endophytic actinomycete strain AE9. After 2 weeks of incubation at 28 °C on tryptic soy agar (TSA), the colonies exhibited a red pigmentation with a round, convex, and smooth surface. Early stage filamentous structures observed under light microscopy, indicating hyphal-like growth.

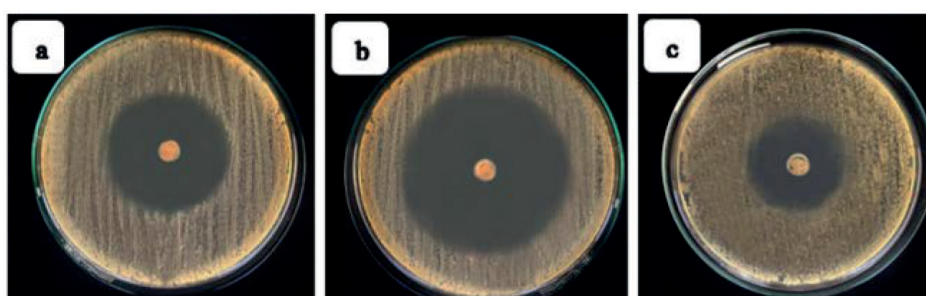


Fig. 2. Clear zone formation by AE9 against (a) *S. aureus* ATCC 25923, (b) *B. cereus* ATCC 11778, and (c) *C. freundii* ATCC 8090

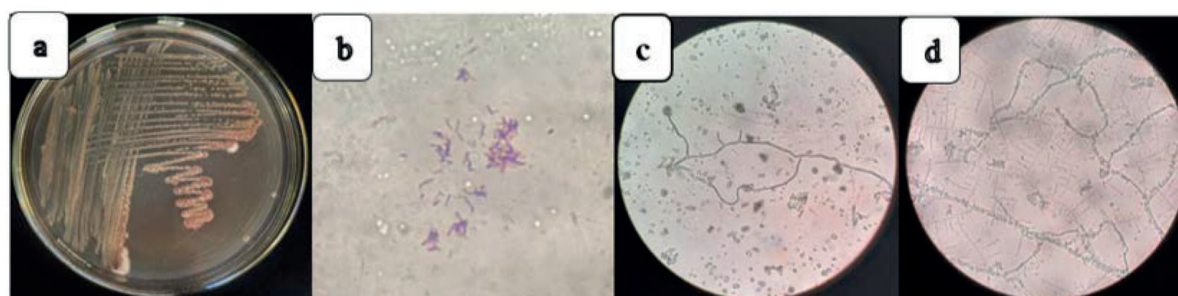


Fig. 3. Morphology and micrograph of the bacterial isolate; (a) Morphology of the isolate; (b) Gram staining; (c) Hyphal structure; (d) Spore chains at 100× magnification.

Extensive branching filamentous network resembling actinomycete morphology, suggesting affiliation with filamentous bacteria such as *Streptomyces* spp. (Fig. 3).

Isolate AE9 was characterized taxonomically based on morphological and physiological characters (Table 1). Microscopic observation of Gram staining and spore chain morphology of strain AE9 revealed that the cells are Gram-positive and filamentous, with spore chains forming an open spiral structure (Fig. 3c and d).

Morphological characterization on various ISP (International Streptomyces Project) media showed significant variations in growth and pigment production. Isolate AE9 showed good growth on most media. The color variations of the air and substrate mycelium are typical, such as whitish gray in ISP2 and dark gray in ISP3. Diffuse pigment production was only observed in ISP3 media, while melanin formation occurred in ISP6 and ISP7. These variations reflect complex and specific

metabolic capabilities to different nutritional conditions. The absence of melanin production on most media, except on certain media, is also an important feature in the differentiation of *Streptomyces* species (Table 2).

The amplification product of the 16S rRNA gene from strain AE9 was visualized using 1% agarose gel electrophoresis (Fig. 4). A single distinct DNA band was observed at approximately 1427 base pairs (bp), corresponding to the expected size of the partial 16S rRNA gene commonly used in bacterial taxonomic and phylogenetic analyses (Trujillo and Dedysh, 2022).

The phylogenetic tree indicates that the bacterial isolate AE9 is most closely related to *Streptomyces* spp., as evidenced by its clustering within the same clade as *Streptomyces* sp. VEL17 from the GenBank database. This close relationship supports the hypothesis that the isolate belongs to the genus *Streptomyces*, a group of Gram-positive bacteria widely known for their ability to produce

Table 1. The phenotypic characterization of isolate AE9 revealed several macroscopic, microscopic, and physiological traits consistent with members of the genus *Streptomyces*

Characterization	Isolate AE9
Microscopic Features	
Cell morphology	rod-shaped
Gram's	positive (+)
Hyphal structure	flexible
Pigments in hyphae	blue
Spore chain	open spiral
Aerial mycelium	white aerial mycelium
Growth Conditions	
Temperature tolerance	
Excellent growth at 28°C	++
Moderate growth at 37°C and 40°C	+
pH range	
Optimal growth at pH 7.0	++
Tolerant at pH 5.0, 9.0, and 11.0	+
Salt tolerance (NaCl concentration)	
Optimal growth in 0% NaCl	++
Tolerant in 4%, 10%, and 13% NaCl	indicating halotolerance, (all+)
Biochemical Tests	
Triple sugar iron agar (TSIA) test:	
Glucose fermentation	positive (+)
Lactose and sucrose fermentation	negative (-)
Gas and H ₂ S production	negative (-)
Citrate utilization	negative (-)
Catalase activity	positive (+)
Urease activity	negative (-)
Motility	negative (-)

Table 2. Morphological characteristics of AE9 strains on ISP media; positive (+) growth, negative (-) growth, moderate (+) growth

Media ISP (International Streptomyces Project)	Growth	Aerial mycelium	Substrate mycelium	Reverse color	Diffusible pigment	Melanin formation
ISP2 (Yeast–malt agar)	(+)	whitish-gray	cream	yellowish	no	no
ISP3 (Oatmeal agar)	(+)	dark gray	light brown	brown	yes	no
ISP4 (Inorganic salts–starch agar)	((+))	white	greenish cream	yellowish-green	no	no
ISP5 (Glycerol–asparagine agar)	(+)	light gray	yellowish-brown	brownish-yellow	no	no
ISP6 (Peptone–yeast extract iron agar)	(+)	pale gray	cream	gray	no	yes
ISP7 (Tyrosine agar)	((+))	white	brown	dark brown	no	yes

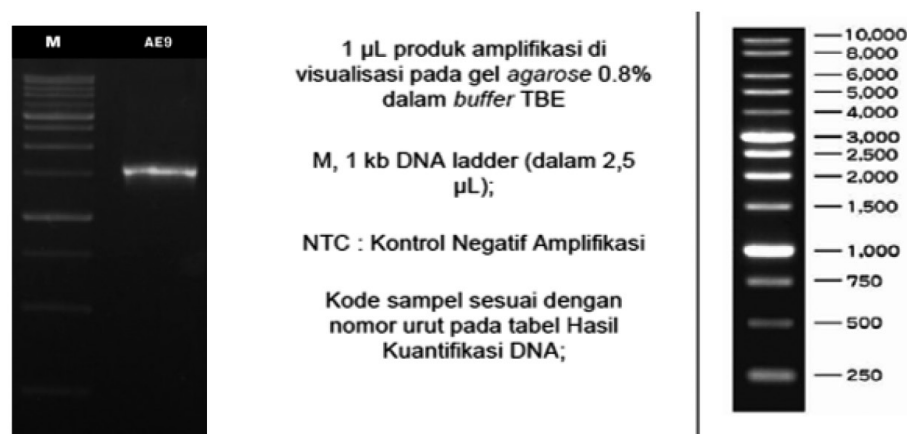


Fig. 4. Gel electrophoresis image of the PCR product of the 16S rRNA gene from isolate AE9. A single DNA band appeared at approximately 1427 bp, indicating specific amplification of the target gene.

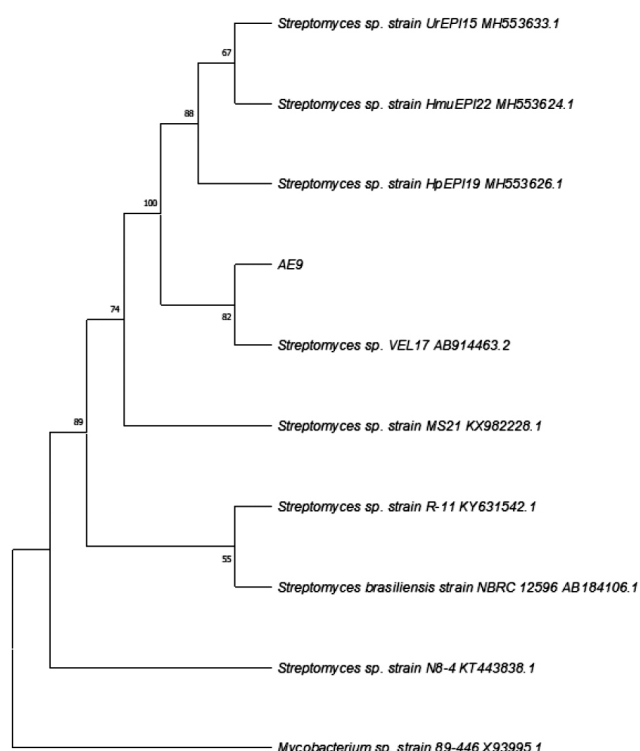


Fig. 5. Phylogenetic tree based on 16S rRNA gene sequences showing the evolutionary relationship between the bacterial isolate AE9 and reference strains retrieved from GenBank. The tree was constructed using the Neighbor-Joining method with 1000 bootstrap replications

various secondary metabolites, including antibiotics, enzymes, and anticancer compounds (Nguyen *et al.*, 2020). The isolate exhibited a more distant relationship with bacterial groups such as *Microbacterium*, which appeared as an outgroup with lower bootstrap values and a distinct branching pattern, indicating a significant phylogenetic distance. Therefore, the isolate can be confidently classified within the phylum Actinobacteria, specifically in the genus *Streptomyces* (Fig. 5).

Discussion

The isolation of endophytic actinomycetes from plant roots represents a promising avenue in the discovery of novel antibacterial agents. Previous studies have demonstrated that endophytic actinomycetes are abundant in root tissues, likely due to the intimate interaction between roots and rhizospheric microbial communities (Delbari *et al.*, 2023). The root environment, rich in nutrients and water, serves as an ideal gateway for microbial colonization. Several reports confirm that root tissues harbor higher endophytic actinomycetes populations compared to stems, leaves, or petioles (Alzahrani *et al.*, 2022). Entry of these microbes into

plant tissues is facilitated through natural openings such as root hairs, lateral root emergence sites, and wounds (Bennett *et al.*, 2018).

Environmental conditions surrounding the host plant significantly influence the diversity and composition of endophytic actinomycetes (Xu *et al.*, 2024). Factors such as plant age, soil type, seasonal variation, geographical location, and environmental stressors have all been shown to affect endophytic communities (Thenappan *et al.*, 2024). In this study, *Streptomyces* sp. AE9 was isolated from the roots of *P. calomelanos* growing in a volcanic eruption-affected area, an extreme environment known to harbor highly diverse and unique microbial populations (Djuidje *et al.*, 2022).

The endophytic actinomycete isolate AE9 demonstrated notable antibacterial activity against three pathogenic bacteria: *S. aureus*, *B. cereus*, and *C. freundii*. Variations in inhibition zones among these pathogens suggest differential production or diffusion of secondary metabolites by AE9 (Najm *et al.*, 2023). The observed antibacterial potency is consistent with earlier findings, highlighting that actinomycetes isolated from extreme or stress-prone environments tend to produce potent and structurally novel bioactive metabolites (Quinn and Dyson, 2024). The selection of promising actinomycete isolates for further metabolite extraction is often guided by the diameter of their inhibition zones (Abdelaziz *et al.*, 2023). Consistently, AE9's strong antibacterial activity positions it as a candidate for further chemical characterization and metabolite profiling.

Molecular identification using 16S rRNA gene sequencing confirmed that isolate AE9 is closely related to *Streptomyces* sp. VEL 17. Morphologically, AE9 exhibited a distinctive brown pigment diffused into the culture medium, a common characteristic among *Streptomyces* strains producing secondary metabolites (Rammali *et al.*, 2024). Pigment production in actinomycetes is often associated with colony maturation or specific nutritional or stress conditions (Bennett *et al.*, 2018).

Interestingly, microscopic examination revealed that AE9 produces a blue pigment in its vegetative hyphae, indicative of actinorhodin production, a well-known secondary metabolite with wide-ranging pharmaceutical applications (Hoskisson *et al.*, 2024). The absence of aerial mycelia in AE9, a rare trait in *Streptomyces* spp., aligns with previous reports where mutations in regulatory genes such as *bldA* inhibit aerial hyphae formation (Stone *et al.*, 2025).

Previous studies have extensively documented the antibacterial potential of *Streptomyces* species. Approximately 80% of known actinomycete-derived natural products originate from *Streptomyces* (Alam *et al.*, 2022). Recent analyses also reveal that from 2015 to 2022, a substantial number of newly discovered secondary metabolites were attributed to *Streptomyces* strains isolated from diverse environments (Donald *et al.*, 2022).

Streptomyces are widely found in terrestrial and marine environments, with the greatest diversity seen in soil habitats (Donald *et al.*, 2022). However, the microbial diversity in volcanic environments remains largely unexplored. Recent studies show that extremophilic actinomycetes from marine, desert, volcanic, and mangrove ecosystems are promising new sources of antimicrobial agents (Subramani and Sipkema, 2019). Importantly, *Streptomyces* strains from these unique niches often contain novel biosynthetic gene clusters, highlighting their biotechnological potential (Sivakala *et al.*, 2021).

Overall, the findings of this study add to the growing evidence that endophytic *Streptomyces* isolated from extreme or underexplored environments are valuable sources of new antibacterial compounds. Future research should focus on purifying and structurally identifying the active metabolites produced by AE9, as well as investigating its biosynthetic pathways.

Conclusion

The present study showed that endophytic actinomycetes isolated from the roots of the silver fern (*P. calomelanos* (L.) Link), which grows in post-eruption soils of Mount Sinabung, have significant antibacterial activity against *S. aureus* (ATCC 25923), *B. cereus* (ATCC 11778), and *C. freundii* (ATCC 8090). Among the isolates, *Streptomyces* sp. AE9 displayed the strongest inhibitory effect, with inhibition zone diameters categorized as highly active. Phenetic and phylogenetic analyses confirmed that isolate AE9 shares a 99.30% sequence similarity with *Streptomyces* sp. VEL 17 based on the 16S rRNA gene sequence. These findings indicate that endophytic *Streptomyces* from ferns living in extreme environments are promising candidates for discovering new antibacterial compounds.

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