Biosystems Diversity

ISSN 2519-8513 (Print)
ISSN 2520-2529 (Online)
Biosyst. Divers.

Screening and identification of potential antibiotic-producing Actinobacteria from cemetery soil

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Article info

Received 16.03.2025
Received in revised form 08.04.2025
Accepted 15.05.2025

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Farihati, A. F., Tyastuti, E. M., Agustina, L., Setiyadi, N. A., Sidiq, Y., & Rahayu, T. (2025). Screening and identification of potential antibiotic-producing Actinobacteria from cemetery soil. Biosystems Diversity, 33(2), e2523. doi:10.15421/012523

Actinobacteria which dominated in a cemetery in Surakarta, Central Java, Indonesia has been discovered from a previous study. A total of 29 isolates of Actinobacteria were sucessfully collected. This study is challenging as the information on antibiotics producing Actinobacteria from cemetery soil is restricted. The aim of this research was to identify and characterize Actinobacteria isolated from cemetery soil, also to screen it for its antibiotic producing potential. A total of 29 Actinobacteria isolates collected from cemetery soil were screened for antibiotics using the agar plug diffusion method against the test bacteria *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*. Isolates with strong potential were characterized by colony morphology, spore type, and molecular identification based on the 16S rRNA gene. There are two isolates that can inhibit both *S. aureus* and *B. subtilis*, namely T5 (19.3 and 17.0 mm) and S6 (18.3 and 10.0 mm). Four isolates showed moderate inhibition against *E. coli*, namely T15, T31, T34, and T42. Two selected isolates, T5 and S6, were respectively white and yellow (aerial mycelium), while their vegetative mycelium was yellowish-brown with closed spiral spore type. Based on the analysis of partial sequencing of the 16S rRNA gene isolate, T5 was identified as *Streptomyces* sp. VEL17 (99.9%) and S6 as *Streptomyces* sp. strain ADE 004 (83.5%). Genetic distance of T5 to the 8 nearest strains based on 16S rRNA shows a close relationship range from 0.001 to 0.005, while by contrast genetic distance of S6 to the 8 nearest strains based on 16S rRNA shows a distant relationship range from 1.057 to 1.074. From this research, it can be concluded that the cemetery soil harbors Actinobacteria that show strong antibiotic potential against Gram-positive bacteria and are very close to *Streptomyces* sp. yet less potential against Gram-negative bacteria. Two selected isolates are prospective candidates for the biotechnology of potential antibiotic compounds.

Keywords: Actinobacteria; antibiotic production; cemetery soil; 16S rRNA gene; *Streptomyces*.

Introduction

Actinobacteria are unicellular bacteria, forming mycelium, producing hyphae that support conidia/sporangia, with colony surfaces often powdery, strongly attached to the media, Gram-positive with high G+C content, often producing pigments, heterotrophic or saprophytic, and playing an important role in the decomposition of organic matter (Jose & Jha, 2016; Meenakshi et al., 2024). Their ability as decomposers is very useful in various applications in the field of biotechnology (Meij et al., 2017). This group is well known for the ability to produce some bio-active compounds such as antibiotics, antitumor compounds, anti-inflammatory agents, anticancer agents, biopesticides, biosurfactants, plant growth hormones, and enzymes (Selim et al., 2021; Farda et al., 2022; Meenakshi et al., 2024).

Actinobacteria are known to be aerobic and dominate the soil, representing over 30% of the entire soil microbial population (Silva et al., 2022). Due to their aerobic property, Actinobacteria can be found abundantly in the soil at depths of 10 to 30 cm (Saibi & Tolangara, 2017; Darshit & Pandya, 2018; Arifiyanto et al., 2020). However, Zhao et al. (2021) reported the presence of Actinobacteria at varying soil depths. Some genera are even facultative anaerobes, capable of surviving with or without O_2 (Amin et al., 2020). Meanwhile, metagenomic data analysis actually shows that the Actinobacteria group is commonly found in one of the burial grounds in Surakarta at a depth of more than 50 cm (Rahayu et al., 2024).

Actinobacteria have been proven to be the largest producers of antibiotics (two-thirds) of all antibiotics, especially the genus *Streptomyces* (Selim et al., 2021; Ambarwati et al., 2023; Meenakshi et al., 2024). There are even claims that the genus produces about 80% of the total antibiotic products (Alam et al., 2022). Antibiotics are some of the secondary metabolites produced by microorganisms that function to combat other microorganisms (Khadse & Titirmare, 2020). Some antibiotics derived from Actinobacteria include ampicillin, penicillin, meropenem, gentamicin, tetracycline, and streptomycin (Shrestha et al., 2021). According to the WHO, around 700,000 people die each year due to antibiotic resistance (De Simeis & Serra, 2021). Inappropriate use of antibiotics, including administration at high doses or without a prescription, causes bacteria to undergo spontaneous mutations due to selective pressure, so that antibiotic-susceptible bacteria will be killed while resistant bacteria will survive and proliferate (Endale et al., 2023). In the study of Budhathoki & Shrestha (2020), it was reported that at least some Actinobacteria isolates with antibiotic producing potential exhibited inhibitory effect against one or all of the tested pathogenic bacteria.

The high incidence of antibiotic resistance is causing the potential of antibiotics to save human lives from pathogens to decline. Therefore, we must immediately begin the development of new antibiotics. We must identify the Actinobacteria group, a major producer of antibiotics, from unexplored locations to uncover new sources of antibiotic secondary metabolites. Based on the literature review, there have been many studies researching the exploration of the antibiotic producing potentialof Actinobacteria from various soil samples, but information regarding the antibiotic producing potential of Actinobacteria from cemetery soil is still very limited. We collected a total of 22 isolates from a depth of 30 cm and 7 isolates from a depth of 70 cm From these isolates, we expected that they would belong to rare Actinobacteria, which are groups of Actinobacteria that are rarely isolated as the genus *Streptomyces* (Parra et al., 2023), such as *Micromonospora* and *Nocardia* (Sapkota et al., 2020). Without relying on the genus *Streptomycetes*, rare antibacterial agents can be beneficial as new antibiotic producers. Thus, this research aims to identify and characterize Actinobacteria isolated from burial ground soil and also screen for their antibiotic potential.

Materials and methods

Preparation of test bacteria. The test strains for determination of antibacterial activity were *Escherichia coli*, *Staphylococcus aureus*,

and *Bacillus subtilis*. The cultures of the three test bacteria strains were tested for their resistance to 5 antibiotics: ampicillin, cefepime, meropenem, oxacillin, and vancomycin. The determination of antibiotic resistance was assessed based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (Aditi et al., 2017; Naldi & Hanum, 2021; Al-Busaidi et al., 2024).

Rejuvenation of Actinobacteria isolates. A total of 29 Actinobacteria isolate collections were subcultured on actinomycetes isolation agar (AIA) media with the addition of 25 mg/L cycloheximide. The preparation of the AIA medium was carried out by dissolving 21.7 g of the medium in 1 liter of aquades and adding cycloheximide at 25 mg/L. After incubation for about a week, its antibiotic producing potentialwas tested (Millang, 2020).

Antagonistic activity test. The antagonistic activity test was conducted using the agar plug diffusion method (Balouiri et al., 2016), which began with the growth of the three test bacteria (*Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*) in NB media with overnight shaking (Rosmania & Yanti, 2020). The test bacteria were evenly inoculated onto the NA medium using a sterile cotton swab. Next, the Actinobacteria isolate was plugged using a sterile cork punch with a diameter of 0.5 mm and placed on the NA medium that had already been inoculated with the test bacteria. The test petri dishes were incubated at 37 ˚C for 24 hours. The clear zone indicating inhibition against the test bacteria (Arie et al., 2020) was measured using a ruler in millimeters (mm). The wider the clear zone formed, the stronger and greater the potential of the bio-active compounds in the Actinobacteria isolate (Seko et al., 2021). The antibiotic producing potential is categorized as "very strong" (> 20 mm), "strong" (10–20 mm), "moderate" (5–10 mm), "no response" (< 5 mm) (Ouchari et al., 2019). Isolates with "strong" and "very strong" potential were selected for molecular identification based on the 16S rRNA gene (Messaoudi et al., 2020).

Morphological and microscopic characterization of selected isolates. The morphological characterization of selected Actinobacteria was performed macroscopically, including the color of aerial and vegetative mycelium, colony shape, colony elevation, colony margin, diffuse pigments, and colony texture. Observation of spore type was conducted under a binocular microscope and Scanning Electron Microscope (SEM) (Kamarudheen et al., 2019).

Identification of selected Actinobacteria isolates based on the 16S rRNA gene. This stage begins with DNA extraction. DNA extraction was performed using the spin column method based on the protocol from the Presto™ Mini gDNA Bacteria Kit (Geneaid) with the addition of glass beads (Maillet et al., 2021).

The next step is the amplification of the 16S rRNA gene using two universal primers, namely 27F (5'-AGA GTT TGA TCM TGG CTG AG-3') and 1492R (5'-CGG TTA CCT TGT TAC GAC TT-3') (Palkova et al., 2021) and a few modifications to the program (Ambarwati et al., 2023). A total of 25 µL of PowerPol 2x PCR mix (ABclonal), 22 µL of ddH_2O, and 1 µL of each primer were mixed and added to 1 µL of DNA template. Amplification was performed using a miniPCR® thermal cycler from miniPCR bio™ (Ampylus, MA, USA) with the following programs: initial denaturation at 96 ˚C (180s), denaturation at 95 ˚C (30 s), annealing at 55 ˚C (30 s), elongation at 72 ˚C (60 s), and final extension at 72 ˚C (300 s), and with initial denaturation at 96 ˚C (120 s), denaturation at 96 ˚C (30 s), annealing at 50 ˚C (40 s), elongation at 72 ˚C (60 s), and final extension at 72 ˚C (240 s). Each program was set for 30 cycles. The amplification results were then subjected to electrophoresis (Advance Mupid-EXu) using 0.8% agarose, 100 volts for 30 minutes, along with a 1 kb ladder DNA marker. The appropriate PCR product, which is approximately 1500 bp in size, was sequenced at 1st Base Singapore. The sequencing results were analyzed using the database available at www.ncbi.nlm.nih.gov using BLAST. This aims to make the nucleotide sequences visible so that similarities between DNA sequences can be obtained (Sjafaraenan et al., 2018). The phylogenetic relationship of the selected isolate with other strains that have been deposited in GenBank was constructed using MEGA 11 (Tamura et al., 2021).

Data analysis. Antibiotic producing potential data were obtained from the average of two replications and was expressed as mean (x) ± standard deviation (SD). The obtained data were analyzed using excel then were prepared in a table. The most two potential isolates were marked with asterik (*).

Results

Antibiotic test. The results of the antibiotic screening of Actinobacteria isolates showed their potential with a "strong" category, namely T1, T5, T9, T10, T22, T25, T34, T43, S6, S22, S23 against *S. aureus* and isolates T5 and S6 against *B. subtilis*. The range of the inhibition zone diameters formed is 10.5 ± 0.5 to 19.3 ± 0.8 mm. There are two isolates that can inhibit both *S. aureus* and *B. subtilis*, namely T5 and S6, and 4 isolates that show moderate inhibition against *E. coli*, namely T15, T31, T34, T42 (Table 1).

Table 1
Screening of antibiotic producing potential from Actinobacteria collection

Isolate code	Diameter of inhibition zone (mm)		
	Escherichia coli	*Staphylococcus aureus*	*Bacillus subtilis*
T1	0	14.0 ± 3.0	0
T3	0	0	0
T5*	0	19.3 ± 0.8	17.0 ± 0.0
T8	0	0	0
T9	0	13.3 ± 1.3	0
T10	0	15.5 ± 7.8	0
T15	5.5 ± 1.0	0	0
T22	0	10.5 ± 0.5	9.3 ± 0.8
T23	0	0 ± 0	0
T25	0	11.0 ± 0.0	8.0 ± 0.0
T27	0	0	0
T28	0	0	0
T31	9.5 ± 4.8	0	0
T32	0	7.0 ± 1.0	0
T33	0	0	0
T34	6.5 ± 3.3	14.0 ± 1.5	7.0 ± 0.0
T35	0	0	0
T36	0	0	0
T41	0	0	0
T42	6.8 ± 0.3	6.0 ± 0.0	9.0 ± 2.0
T43	0	15.3 ± 0.3	5.5 ± 0.5
T46	0	6.0 ± 0.0	5.5 ± 0.5
S1	0	0	0
S6*	0	18.3 ± 1.3	10.0 ± 0.0
S17	0	0	0
S19	0	0	0
S20	0	0	0
S22	0	14.5 ± 7.3	0
S23	0	17.5 ± 7.5	0

Note: * – isolates with strong potential to inhibit *S. aureus* and *B. subtilis*.

The antibiotic producing potential screening from the 29 Actinobacteria isolates showed more effective inhibition against Gram-positive bacteria (*S. aureus* and *B. subtilis*) compared to Gram-negative bacteria (*E. coli*, Fig. 1). The results of the resistance test of the test bacteria against 5 antibiotics showed that *E. coli* and *S. aureus* were resistant to three types of antibiotics, namely meropenem, oxacillin, and vancomycin, while *B. subtilis* was only resistant to one type of antibiotic, namely oxacillin.

Morphological and microscopic characterization of selected isolates. Two selected isolates, T5 and S6, were respectively white and yellow (aerial mycelium), while the vegetative mycelium of both was yellowish-brown. The colony shape of the T5 isolate was punctiform, with a convex elevation, undulate margin, and a powdery texture with a white surface pigment, unlike S6, which had an irregular shape, umbonate elevation, undulate margin, and wrinkled texture. In addition, both also produced diffuse pigments with a closed spiral spore type (Fig. 2 and 3).

Identification based on the 16S rRNA gene. Two potential isolates, T5 and S6, were identified molecularly based on the 16S rRNA gene, starting with DNA genome extraction followed by amplification of the 16S rRNA gene, which is 1500 bp in size (Fig. 4). T5 has the closest match with the genus *Streptomyces* with an identity percentage of 99.86% as *Streptomyces* sp. VEL17, while S6 is 83.50% as *Streptomyces* sp. strain ADE 004 (Table 2).

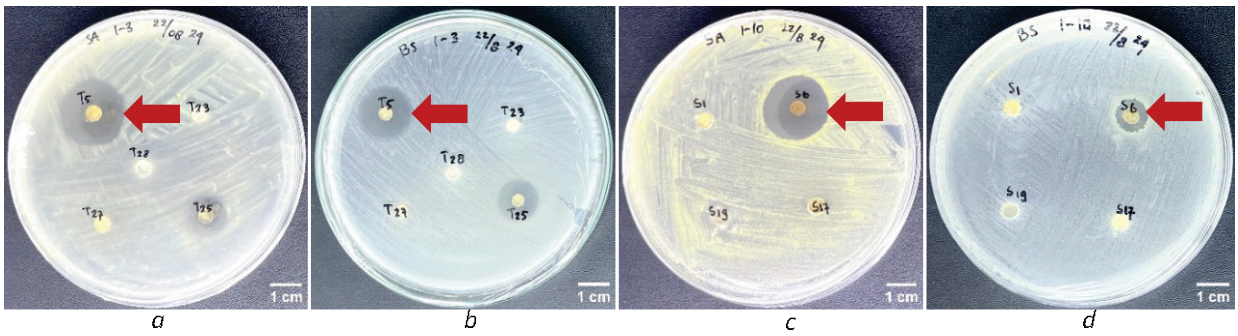

Fig. 1. Results of screening potential antibiotic isolates incubated for 24 hours:
a – T5 against *S. aureus*; *b* – T5 against *B. subtilis*; *c* – S6 against *S. aureus*; *d* – S6 against *B. subtilis*

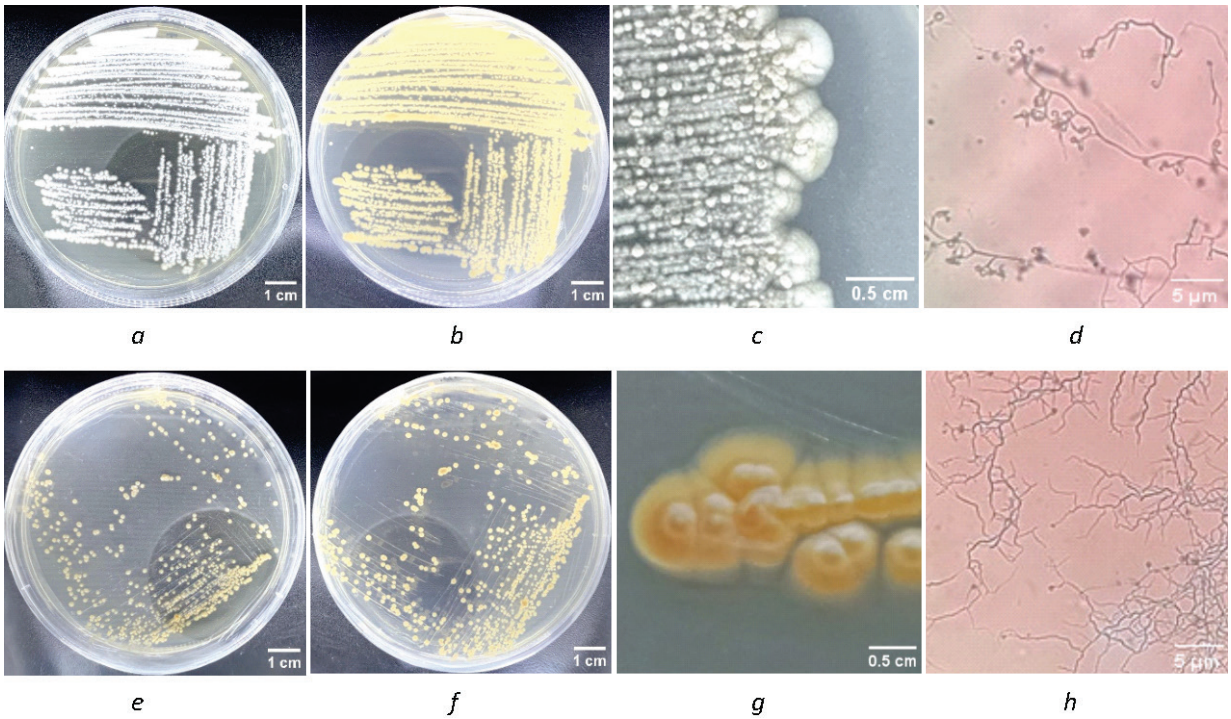

Fig. 2. Morphology and spores type of Actinobacteria isolates (T5 and S6): *a* – aerial mycelium of T5; *b* – vegetative mycelium of T5; *c* – colony of T5; *d* – spore type of T5; *e* – aerial mycelium of S6; *f* – vegetative mycelium of S6; *g* – colony of S6; *h* – spore type of S6

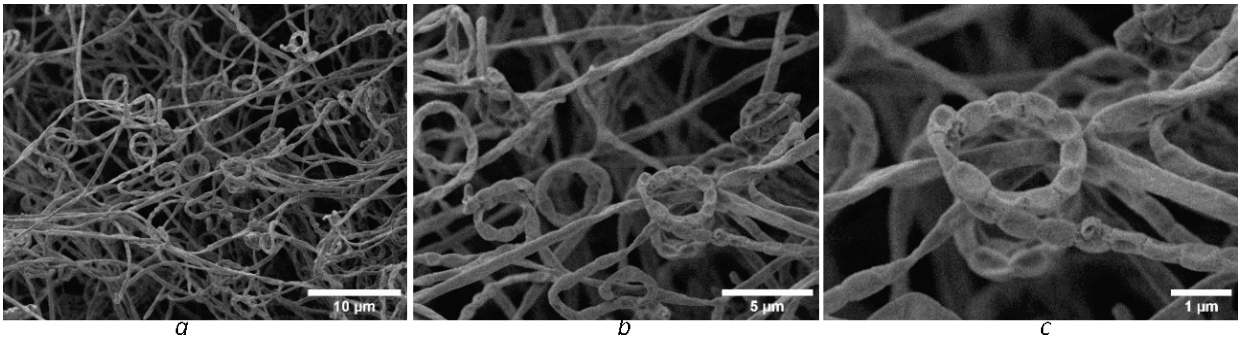

Fig. 3. Scanning electron microscope (SEM) of spore and mycelium structure of isolate T5

The phylogenetic tree is constructed as a form of examination of the kinship relationships between species. The construction of the phylogenetic tree was based on the Neighbor-Joining algorithm using 1000 bootstrap consensus repetitions. The alignment of the isolate sequences with several related sequences taken from Gene Bank data was performed using the CrustalW technique to indicate the distance between their relatives by constructing a phylogenetic tree based on the MEGA 11 software program. Isolates T5 and S6 are in the same clade, closely related to *Streptomyces* sp. VEL17, *Streptomyces indiaensis* strain IF5, and *Streptomyces* sp. MAH25 (Fig.5). The genetic distance of T5 is closest to *Streptomyces* sp. Isolate T5 is similar to *Streptomyces* sp., where the genetic distance displayed towards the other 8 strains shows a range of 0.001 to 0.005, which means it is indeed related to those species (Table 3). For isolate S6, the genetic distance from the other 8 *Streptomyces* sp. strains is very great, ranging from 1.057 to 1.074, which means S6 may indeed not be related to those species (Table 4).

Discussion

The responses of Gram-negative and Gram-positive bacteria showed differences in antibiotic screening. The Gram-negative bacteria group has three layers of cell wall structure: the outer membrane, peptidoglycan, and the inner membrane (Tavares et al., 2020; Salam

et al., 2023) the Gram-positive bacteria group, on the other hand, only has two layers and no outer membrane. The three-layer structure becomes a strong permeability barrier that prevents antibacterial activity from getting through to the Gram-negative bacterial membrane (Lehman & Grabowicz, 2019). Nevertheless, the antibiotic screening results showed that the Actinobacteria isolate was most potent against *S. aureus*, followed by *B. subtilis* and *E. coli*. This is possible because the type of antibiotic produced by the Actinobacteria isolate differs from the antibiotic used for the resistance test. Each type of antibiotic has an inhibition spectrum against certain types of bacteria. For example, streptomycin, gentamicin, ciprofloxacin, and levofloxacin are effective in inhibiting the growth of *E. coli*. Then amoxicillin, which is effective in inhibiting the growth of *E. coli*, *S. aureus*, and *B. subtilis* bacteria. Additionally, streptocycline, validamycin, and chloramphenicol are effective in inhibiting *B. subtilis*. Amphomycin, aspartochins, and erythromycin are effective in inhibiting *S. aureus* (Kendre et al., 2021; Siddiq & Azizah, 2022; Meenakshi et al., 2024).

The discovery of new antibiotics must continue to address resistance that impacts human health. The cases of resistance in the world today have become an urgent problem due to the prevalence of antibiotics that are no longer effective in killing bacteria. Resistance testing (Salam et al., 2023) is needed to find superbug-resistant strains so that better antibiotics can be made. Reports related to antibiotic resistance have estimated that by 2050, the global death toll will reach 8.22 million people (Naghavi et al., 2024).

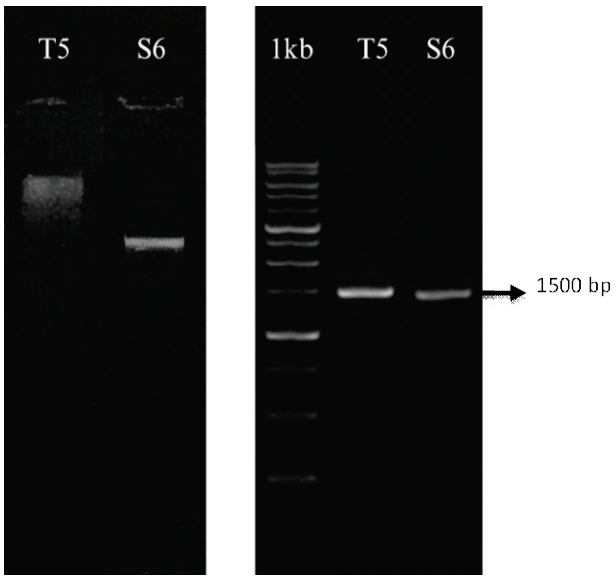

Fig. 4. Electropherogram of DNA genome extraction results (*a*) and PCR product of the 16S rRNA gene from Actinobacteria isolates (T5 and S6) (*b*)

Table 2
Molecular identification of potential antibiotic isolates based on the 16S rRNA gene

Isolates	16S rRNA identification	Query cover, %	Percent identity, %
T5	*Streptomyces* sp. VEL17	99	99.86
S6	*Streptomyces* sp. strain ADE 004	99	83.50

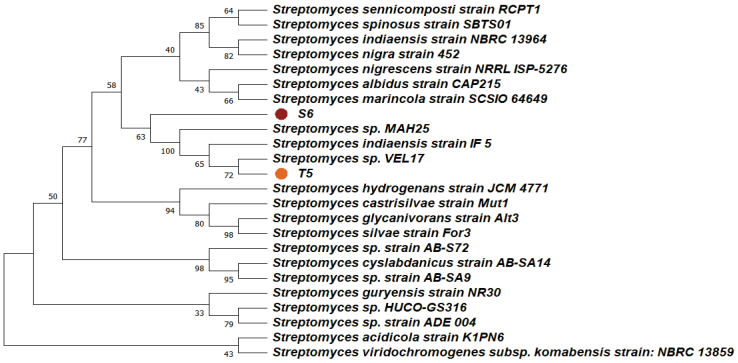

Fig. 5. Place of studied strains of Actinobacteria – potential producers on phylogenetic tree of *Streptomyces* genus

Table 3
Genetic distance of T5 to the 8 nearest strains based on 16S rRNA

No	Species	1	2	3	4	5	6	7	8	9
1	T5	–	–	–	–	–	–	–	–	–
2	*Streptomyces* sp. VEL17	0.001	–	–	–	–	–	–	–	–
3	*Streptomyces indiaensis* strain IF 5	0.004	0.003	–	–	–	–	–	–	–
4	*Streptomyces* sp. MAH25	0.003	0.002	0.005	–	–	–	–	–	–
5	*Streptomyces* sp. VITTKGB	0.005	0.005	0.007	0.006	–	–	–	–	–
6	*Streptomyces* sp. VEL27	0.003	0.002	0.005	0.000	0.006	–	–	–	–
7	*Streptomyces* sp. KOD10	0.001	0.000	0.003	0.002	0.005	0.002	–	–	–
8	*Streptomyces exfoliatus* strain F4	0.005	0.005	0.007	0.005	0.002	0.005	0.005	–	–
9	*Streptomyces* sp. strain A-JW-02	0.001	0.000	0.003	0.002	0.005	0.002	0.000	0.005	–

Table 4
Genetic distance of S6 to the 8 nearest strains based on 16S rRNA

No	Species	1	2	3	4	5	6	7	8	9
1	S6	–	–	–	–	–	–	–	–	–
2	*Streptomyces* sp. strain ADE 004	1.057	–	–	–	–	–	–	–	–
3	*Streptomyces* sp. strain AB-SA9	1.062	0.014	–	–	–	–	–	–	–
4	*Streptomyces cyslabdanicus* strain AB-SA14	1.062	0.014	0.000	–	–	–	–	–	–
5	*Streptomyces kagawaensis*	1.066	0.013	0.011	0.011	–	–	–	–	–
6	*Streptomyces guryensis* strain NR30	1.068	0.016	0.019	0.019	0.018	–	–	–	–
7	*Streptomyces viridochromogenes subsp. komabensis* strain: NBRC 13859	1.074	0.015	0.019	0.019	0.019	0.012	–	–	–
8	*Streptomyces* sp. HUCO-GS316	1.071	0.013	0.018	0.018	0.013	0.015	0.018	–	–
9	*Streptomyces* sp. strain AB-S72	1.066	0.015	0.001	0.001	0.012	0.020	0.019	0.019	–

Table 5
Nucleotide composition of the 16S rRNA gene

Species	Nucleotides composition, %						Total
	T(U)	C	A	G	G+C	A+T	
T5	19.87	24.17	24.98	30.98	55.15	44.85	1233
Streptomyces sp. VEL17	19.81	24.11	25.00	31.09	55.19	44.81	1232
Streptomyces indiaensis strain IF 5	19.87	22.99	25.29	31.86	54.84	45.16	1218
S6	22.38	30.38	20.81	26.53	56.81	43.19	1278
Streptomyces sp. strain ADE 004	18.38	25.47	22.51	33.64	59.11	40.89	1213
Streptomyces sp. strain AB-SA9	18.63	25.31	22.59	33.47	58.78	41.22	1213

Analysis of the nucleotide composition of 16S rRNA isolates T5 and S6 with their closest relatives taken from GenBank data shows that these isolates have a GC composition of more than 55% (Table 5).

Despite extensive research on antibiotic-producing Actinobacteria, no publications have yet identified potential antibiotic-producing Actinobacteria from cemetery soil. Cemetery soil harbors abundant microorganisms, especially decomposer bacteria, due to the active decomposition of buried bodies (Majgier et al., 2014). The bacterial population count in the Surakarta, Indonesia, cemetery soil shows that there are between 10^6 to 10^7 CFU/g of soil living there (Adityaradja et al., 2023; Rahmawati et al., 2023). Additionally, the results of the metagenomic analysis of cemetery soil samples in Surakarta showed the abundance of Actinobacteria at depths > 50 cm (Rahayu et al., 2024). Interestingly, this group of bacteria is known to produce several bio-active compounds, including antibiotics. Some of the antibiotics that *Streptomyces* sp. produces are tetracycline, anthracyclines, erythromycin, penicillin, streptomycin, carbapenem, rifamycin, kanamycin, actinomycins, amphomycin, aspartocins, and chloramphenicol. Then there is *Micromonospora* sp., which produces antibiotics, such as anthracycline, gentamicin, sagamicin, rosamicin, verdamicin, sisomicin, everninomicin, and netamicin. As for *Nocardia* sp., it produces the antibiotics transvalencin, neocitramycin, nocardicin, nocathiacin, myomicin, and nocardicyclin. *Actinoplanes* sp. is responsible for producing the antibiotics purpuromycin and teicoplanin. *Streptomyces* has become a genus frequently explored due to its effective potential for producing antibiotics. Two-thirds of the antibiotics known today are derived from *Streptomyces* (Fu et al., 2020; Mebrat, 2024; Meenakshi et al., 2024).

Actinobacteria are a dominant bacterial phylum in soils, including cemetery soils, playing crucial roles in decomposition and soil fertility (Schrempf, 2013; Grigoryan & Bataeva, 2023). In cemetery soils of Surakarta, Indonesia, Actinobacteria were found to be the second most abundant phylum at 20 cm depth (21.6%) and the most abundant at 140 cm depth (34.2%) (Rahayu et al., 2024). Similarly, Actinobacteria were identified as a major phylum in Revolutionary War, Civil War, and modern graveyards on Long Island, NY (Caputo et al., 2019). These bacteria produce various secondary metabolites with antibiotic, antimicrobial, and other beneficial properties, making them valuable for biotechnological applications (Grigoryan & Bataeva, 2023). Their presence in cemetery soils is significant due to their potential interactions with decomposing remains and embalming fluids, which can impact soil and groundwater composition (Caputo et al., 2019). Understanding the ecological features of Actinobacteria in various soil types, including polluted soils, is crucial for developing biological products for agroecosystems (Grigoryan & Bataeva, 2023).

The phylogenetic analysis evaluated by bootstrap based on 1000 replications actually shows the close relationship between S6 and T5 along with their relatives (especially *Streptomyces* sp. MAH25) and not with the S6 relative strain itself, which is *Streptomyces* sp. strain ADE 004. Meanwhile, T5 was proven to be in the same clade as *Streptomyces* sp. VEL17, supported by a 72% bootstrap value. In another group, strains were also used as a comparison to determine how closely related the strain is to the isolated isolate. The revealed phylogenetic distance indicates the number of nucleotide base changes in the DNA or the genetic distance between related species (Huber et al., 2022). Genetic variation causes genetic distance in the target taxa where similarity strains occur because the nucleotide sequences come from the same species or fewer mutations have occurred in T5. In this case, mutations have occurred extensively in S6 where its genetic distance exceeds 1.057 from *Streptomyces* sp., which was identified as the closest strain in the BLAST search. However, Actinobacteria have more than 55% G+C content (Amin et al., 2020; Meenakshi et al., 2024). The examination of the nucleotide composition in T5 and S6 showed a high content of guanine and cytosine (>55%), thus meeting the criteria indicating that both belong to the Actinobacteria group. Low similarity and high genetic distance of S6 to *Streptomyces* sp. allow it to be reclassified into a new species. Further research is needed as a form of confirmation or refutation of the argument regarding the classification of S6, which is suspected to be a new species, for example, through long sequencing.

Conclusion

Actinobacteria successfully isolated from the Bonoloyo cemetery soil show strong antibiotic producing potential against Gram-positive bacteria, especially *Staphylococcus aureus* and *Bacillus subtilis*, namely T5 and S6, which are closely related to *Streptomyces* sp. The antibacterial activity of both candidates is promising for further research in the development of potential antibiotic agents. Likewise, the follow-up regarding the discovery of a new species that may be related to the potential antibiotics. The research results can provide an additional assumption that the soil in the cemetery contains a type of Actinobacteria that has antibiotic compounds.

The study was supported by Fundamental Regular Research of Direktorat Jenderal Pendidikan Tinggi, Riset dan Teknologi Kementerian Pendidikan, Kebudayaan, Riset dan Teknologi with contract number : 007/LL6/PB/AL.04/2024, 196.63/A.3-III/LRI/2024.

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