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Antibacterial Actinomycetes from Tropical Ecosystem Karst in South Sulawesi: Isolation, Characterization, and Bioactive Metabolite Profiling

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Abstract

The emergence of antimicrobial-resistant pathogens reinforces the critical need for new antibiotics sourced from unexplored microbial habitats. Actinomycetes remain the most prolific bacterial producers of bioactive secondary metabolites, yet tropical karst ecosystems and plant-associated rhizospheres in Indonesia remain poorly investigated despite their ecological complexity. This study aimed to isolate, screen, and characterize Actinomycetes from the karst and rhizospheric environments of South Sulawesi, Indonesia, and to identify isolates capable of producing antibacterial compounds. A total of 42 isolates were obtained using heat-shock pretreatment and selective Starch Casein Agar. Preliminary antagonistic assays against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 35218 identified four active isolates. Two isolates KMR 1E2 and SDR 2a, exhibited strong inhibition (>30 mm) against *S. aureus*, while SDR 9.9 inhibited *E. coli*. Morphological and phenotypic characterization revealed diverse spore-chain architectures, colony color profiles, carbon and nitrogen utilization patterns, salinity tolerance, melanin production, and variable growth under different pH and temperature conditions. Ethyl acetate extracts of KMR 1E2 and SDR 2a demonstrated potent antibacterial activity, and TLC-bioautography identified three active metabolites (Rf 0.73, 0.48, 0.34), with the Rf 0.34 spot associated with an alkaloid-type compound. The minimum inhibitory concentration (MIC) of KMR 1E2 extract against *S. aureus* was 0.625 mg/mL. This study confirms that tropical karst ecosystems in Indonesia are promising reservoirs of antibiotic-producing Actinomycetes and provides a foundation for future genomic and chemical elucidation studies.

Keywords: Actinomycetes; karst ecosystem; rhizosphere; antibacterial metabolites; TLC-bioautography; MIC.

INTRODUCTION

Antimicrobial resistance (AMR) continues to escalate globally, compromising treatment options for bacterial infections and increasing morbidity, mortality, and healthcare costs. The World Health Organization (WHO) identifies AMR as one of the most urgent global health threats, with resistant pathogens responsible for millions of deaths annually and diminishing the effectiveness of last-resort antibiotics (Ventola, 2020; World Health Organization, 2023). This alarming trend underscores the necessity to discover novel antimicrobial scaffolds, particularly those derived from microorganisms inhabiting unique or poorly characterized environments. Natural products remain a cornerstone in antibiotic discovery, and microbial secondary metabolites continue to provide chemically diverse and structurally complex compounds with therapeutic potential.

Actinomycetes, especially within the genus *Streptomyces*, are recognized as the dominant producers of clinically relevant antibiotics, antifungals, anticancer agents, immunosuppressants, and other specialized metabolites. Their genomes encode extensive biosynthetic gene cluster (BGC) diversity, enabling the synthesis of polyketides, non-ribosomal peptides, terpenoids, alkaloids, and hybrid metabolite classes (Genilloud, 2019; Baltz, 2021). Recent genomic mining has revealed that even well-studied genera harbor numerous cryptic or silent BGCs that can be activated under appropriate environmental stimuli (Federspiel et al., 2022). Consequently, Actinomycetes remain indispensable to modern drug discovery pipelines and continue to attract interest for bioprospecting.

Exploration of new or extreme habitats for bioactive Actinomycetes has become a major focus in natural product research. Microorganisms from caves, deserts, marine sediments, geothermal soils, mangroves, and karst ecosystems frequently display rare phylogenetic lineages and unique biosynthetic repertoires shaped by selective pressures such as nutrient limitation, mineral

stress, darkness, and fluctuating moisture (Wang et al., 2024). Karst regions, in particular, feature limestone formations with high calcium carbonate content, narrow ecological niches, and oligotrophic microhabitats that can induce metabolic diversification and horizontal gene transfer. Culture-dependent and metagenomic studies across Asian karst systems have uncovered high Actinobacterial diversity, including previously undescribed genera and novel BGC architectures (Zhou et al., 2020; Zhang et al., 2021; Li et al., 2022). Despite these promising findings, tropical karst ecosystems especially those in Indonesia remain insufficiently explored, even though Indonesia is a global biodiversity hotspot with high geological and microbial heterogeneity.

Parallel to karst ecosystems, rhizosphere environments represent dynamic microhabitats governed by root exudates, plant microbe interactions, and intense microbial competition. Rhizosphere-associated Actinomycetes often exhibit enhanced antagonistic activity, plant growth promoting traits, and metabolic versatility due to their co-evolution with host plants and exposure to complex chemical signals (Kaur et al., 2020; Huang et al., 2023). Root-derived substrates such as organic acids and phenolics can activate silent BGCs and stimulate secondary metabolite production. Consequently, the rhizosphere serves as an important reservoir for discovering Actinomycetes with robust antimicrobial and biochemical capabilities.

In Indonesia, several studies have reported the isolation of Actinomycetes with antibacterial, antifungal, antitumor, and enzyme-producing properties from soils, marine sediments, mangrove ecosystems, agricultural rhizospheres, and decaying biomass (Sunaryanto et al., 2010; Ali et al., 2024). However, integrative bioprospecting that connects ecological origin, phenotypic characterization, antimicrobial profiling, and preliminary chemical elucidation is still limited. Notably, little is known about Actinomycetes inhabiting tropical karst landscapes combined with rhizosphere zones two ecologically contrasting yet complementary habitats that may harbor distinct microbial communities and untapped BGC diversity.

The present study aims to fill this knowledge gap by exploring Actinomycetes from tropical Indonesian karst and rhizosphere ecosystems. Through isolation, taxonomic screening, bioactivity assays, and partial chemical characterization, this work provides new ecological and biochemical evidence that these environments constitute a promising reservoir of novel antibiotic-producing Actinomycetes, contributing to the global search for new antimicrobial leads in the post-antibiotic era.

MATERIALS AND METHODS

Sampling Sites and Sample Collection

Actinomycetes were collected from two locations: (1) karst ecosystems in Pangkep and Maros, South Sulawesi, and (2) the rhizosphere of cassava (*Manihot utilissima*). Karst plant roots, stems, and surrounding soils were sampled aseptically. Samples were transported in sterile containers at ambient temperature and processed within 24 hours (Zhang et al., 2021). Isolation procedures of strains used two methods. The methods were chosen based on whether the samples were endophytic and rhizospheric microbes.

Isolation from Rhizosphere Soil

Five grams of soil were suspended in 45 mL of sterile distilled water, homogenized, and subjected to heat-shock at 70°C for 1 hour to suppress non-sporulating bacteria while enriching Actinomycetes (Genilloud, 2019). Serial dilutions were spread onto Starch Casein Agar (SCA) supplemented with nystatin (25 μg/mL) to inhibit fungal contamination.

Isolation from Endophytic Plant Tissues

In the endophytic procedures, the plant tissue were immersed in 70% ethanol for 15 minutes and followed in NaOCl 5% for 8 seconds for surface sterilization. Finally, samples were rinsed in sterile water and dried under a laminar flow hood. The plant tissue was crushed with NaCl physiological solution aseptically in mortar and pestle. The crushed tissue were spread (0.1 mL) onto the SNA media plate, and then incubated at 37^{o}C for 2 weeks. The actinomycetes colony was characterized by the morphological features and color was recultured on new media to obtain a pure colony. The pure colony was inoculated in SNA test tube slant media maintained as a spore suspension in 20% glycerol at - 20°C for further investigation.

Screening of Antibacterial Activity of Strains

Strains were screened for antibacterial activity by using agar block assay against *Staphylococcus aureus* ATCC 25923 (Gram positive), and *Escherichia coli* ATCC 35218 (Gram negative). Each of the strains was inoculated in SNA media plate, incubated at 37^{o}C for 7 days. A colony with a diameter of 5 mm was punctured in the media plate by using sterile cork borer, then transferred on the top of NA media plate containing test bacteria. The plates were incubated at 37^{o}C for 24 h, and the antibacterial activity was recorded. The clear zone in surrounding the strain colony was considered as endophytic actinomycetes of antibacterial activity, measured in terms of the diameter of inhibition zone with triplicate (mm). The agar block was inoculated with the strain and placed in the centre of the NA media plate, which was used as control.

Selection of Potential Strain

All strains that inhibited test bacterial growth in primary screening were considered potential strains. The active metabolites and strain characteristics were further analyzed. Strain selection was based on the ability of metabolite to inhibit test bacteria using the agar diffusion method. Each strain was grown in 150 mL of SN broth medium (pH 7.0±0.5, temperature 33-34ºC) in a 250 mL Erlenmeyer flask under agitated conditions. Fermentation was carried out for 9 days, after that the biomass was separated from the fermentation liquid using a thin cloth. The supernatant liquid was extracted with ethylacetate in a ratio of 1: 1.5. Furthermore, the extract obtained was tested for antibacterial activity. The strain was declared potential if it showed the diameter of the inhibition to the growth of the test bacteria more than 20 mm inhibition zone diameter.

Primary Screening of Antibacterial Activity

Antibacterial screening employed the agar block diffusion assay using *Staphylococcus aureus* ATCC 25923 (Gram-positive) and *Escherichia coli* ATCC 35218 (Gram-negative). Agar plugs (5 mm) from 7-day cultures were placed onto nutrient agar seeded with test bacteria and incubated at 37°C for 24 h. Clear zones surrounding the plug were measured in millimeters (Heng et al., 2021). Isolates showing inhibition ≥15 mm were cultured in 150 mL Starch Nitrate Broth (SNB) in 250 mL Erlenmeyer flasks. Fermentation proceeded for 8-9 days at 28°C with shaking at 150 rpm, following established procedures for Actinomycete metabolite production (Baltz, 2021; Santos et al., 2021). Fermentation broth was filtered to separate biomass and culture supernatant.

Antibacterial Bioassay of Metabolite

Supernatants were extracted with ethyl acetate (1:1.5 v/v), while biomass was extracted with methanol. Organic fractions were concentrated using rotary evaporation at 40°C (Hu et al., 2020). Dried extracts were stored at 4°C. Extract activity was evaluated via disc diffusion assay using 20 µL extract per 6 mm disc. After diffusion at 4°C (2 h), plates were incubated at 37°C for 48 h. Inhibition zones were measured as described previously (Chen et al., 2021).

Morphological, Biochemical, and Physiological Characterization

Morphology was examined using slide culture and observation of spore-chain structures at 400× magnification. Colony characteristics on ISP media (ISP1-ISP7), SCA, SNA, and PDA were recorded following updated taxonomic guidelines (Komaki et al., 2020). Physiological assays followed methods standardized for Actinomycetes (Li et al., 2024; (Cruz-Morales et al., 2019).

Spore chains were examined using slide culture methods. Growth on ISP media (ISP1-ISP7), SNA, SCA, PDA, NA, TSA, and Czapek-Dox agar determined colony color, soluble pigment, and aerial/substrate mycelia. Biochemical characterization included carbon and nitrogen utilization, hydrolysis of starch, casein, gelatin, tween 80, melanin production, tolerance to phenol, NaCl, crystal violet, and pH/salinity/temperature tolerance.

Chromatographic Profiling and TLC-Bioautography

Ethyl acetate extracts were resolved on silica gel TLC plates using ethyl acetate:methanol mobile phases (10:0; 9:1; 8:2). Active fractions were further separated using silica column chromatography with gradient elution (Awad et al., 2020). For TLC-bioautography, developed plates were overlaid onto agar seeded with *S. aureus*, and zones of growth inhibition were visualized under UV 254/366 nm using established protocols (Wang et al., 2023).

Minimum Inhibitory Concentration (MIC)

MIC values were determined using two-fold serial dilution (0.3125-40 mg/mL) applied onto discs followed by diffusion assays. MIC was defined as the lowest concentration producing measurable inhibition (WHO, 2023; Hu et al., 2020).

RESULTS AND DISCUSSION

Isolation and Primary Antibacterial Screening of Actinomycetes

A total of 42 Actinomycetes isolates were successfully obtained from different sampling sites in South Sulawesi, comprising 17 isolates from cassava rhizosphere soils and 25 isolates from karst-associated environments, of which 11 originated from karst rhizospheres and 14 from symbiotic tissues of karst plants (Figure 1).

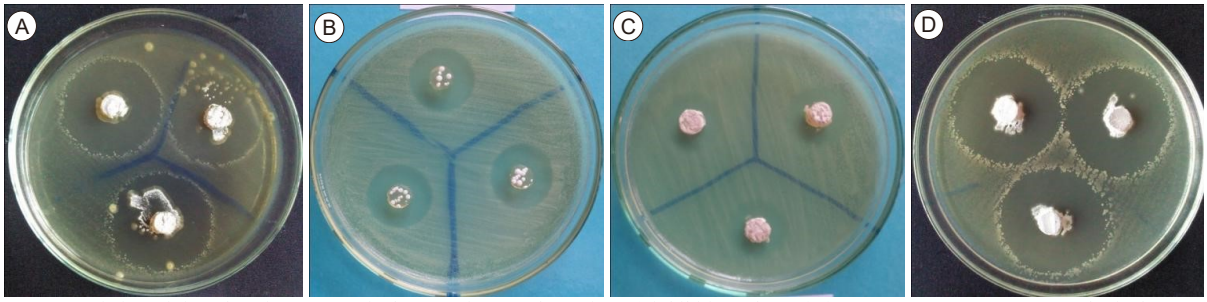

Figure 1. Antagonistic activity of Actinomycetes isolates against test bacteria: (A) inhibition of *Staphylococcus aureus* ATCC 25923 by isolate KMR 1E2; (B) inhibition of *S. aureus* ATCC 25923 by isolate SMT 1; (C) inhibition of *Escherichia coli* ATCC 35218 by isolate SDR 9.9; and (D) inhibition of *S. aureus* ATCC 25923 by isolate SDR 2a.

Morphological Characterization of Antibacterial-Producing Isolates

Four isolates that displayed consistent antibacterial activities were subjected to detailed morphological characterization. Microscopic examination (400×) revealed distinct spore-chain morphologies, including spiral, straight, and reticulate patterns, typical of diverse Actinomycetes groups. These morphological traits are presented in Figure 2, highlighting structural differences among isolates KMR 1E2, SDR 2a, SDR 9.9, and SMT 1.

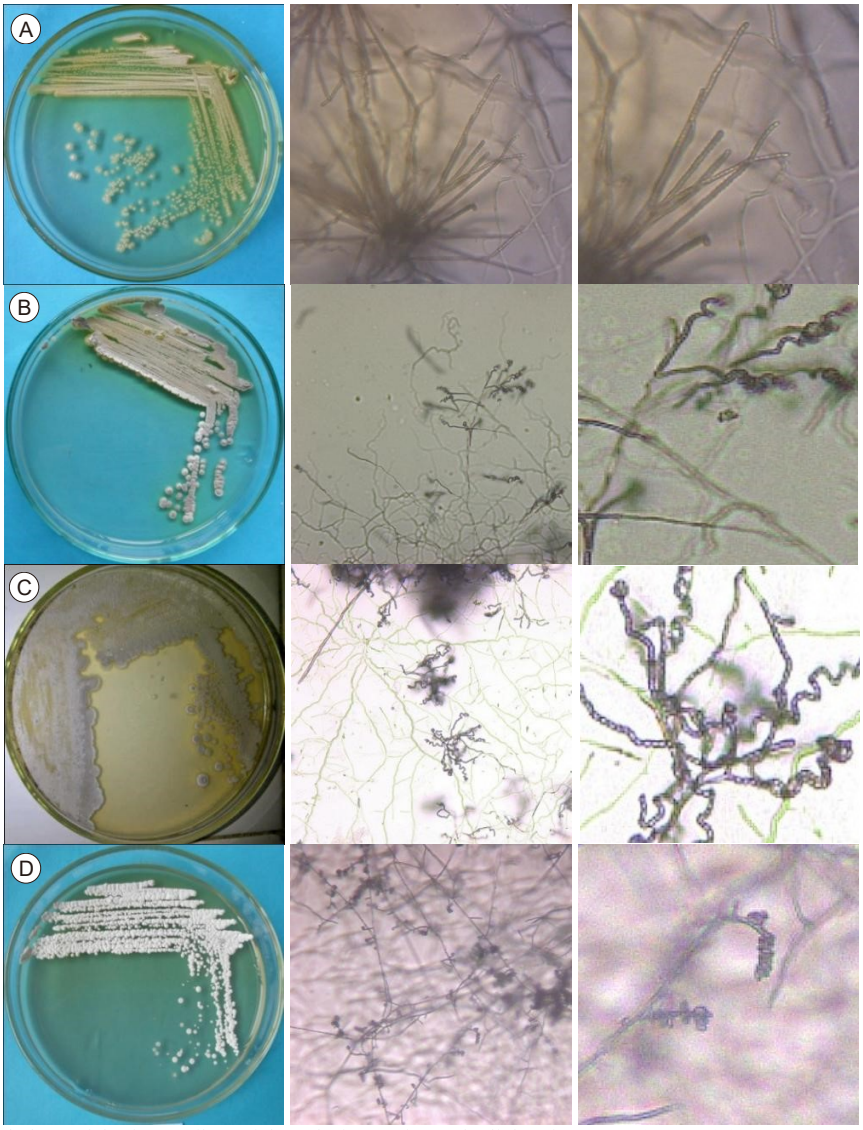

Figure 2. Spore-chain morphology of the four antibacterial-producing strains under light microscopy (400×). (A) KMR 1E$_2$, (B) SDR 2a, (C) SDR 9.9 and (D) SMT 1.

Colony Pigmentation and Aerial/Substrate Mycelium Profiles on Diverse Media

To further discriminate the active isolates, each was grown on a panel of 12 media, including ISP 1-5, SCA, SNA, PDA, NA, TSA, Czapek-Dox, and Bennett's agar. Colony features such as aerial mycelium color, substrate mycelium color, and soluble pigment production showed broad variability across media and isolates.

Distinct pigmentation patterns were observed, reflecting medium composition and intrinsic metabolic differences. These characteristics are summarized in Table 1, which documents the RAL color code (Harmony Color), pigment solubility, and growth intensity for each isolate on all tested media.

Table 1. Pigmentation and growth characteristics of four Actinomycetes isolates across different culture media.

Media	Strain codes	Aerial mycelium		Substrate mycelium		Growth	Soluble Pigment (SP)
		Color Harmonical codes	Color of observation	Color Harmonical codes	Color of observation		
ISP1	KMR 1E$_2$	RAL 1013	Oyster White	RAL 8000	Green Brown	+	-
	SDR 2a	RAL 9002	Grey White	RAL 2000	Yellow Orange	+	-
	SDR 9.9	RAL 1015	Light Ivory	RAL 1002	Sand Yellow	+	-
	SMT 1	RAL 9010	Pure White	RAL 1023	Traffic Yellow	++	-
ISP2	KMR 1E$_2$	RAL 9001	Cream	RAL 3005	Wine Red	++	+
	SDR 2a	RAL 9001	Cream	RAL 1003	Signal Yellow	++	+
	SDR 9.9	RAL 1012	Lemon Yellow	RAL 1012	Lemon Yellow	+	-
	SMT 1	RAL 9010	Pure White	RAL 1018	Zink Yellow	+	-
ISP4	KMR 1E$_2$	RAL 9003	Signal White	RAL 1032	Broom Yellow	++	-
	SDR 2a	RAL 9003	Signal White	RAL 2004	Pure Orange	++	-
	SDR 9.9	RAL 1013	Oyster White	RAL 1011	Brown Beige	+	-
	SMT 1	RAL 9000	Cream	RAL 1000	Green Beige	+	-
ISP5	KMR 1E$_2$	RAL 1023	Traffic Yellow	RAL 1002	Sand Yellow	+	-
	SDR 2a	RAL 7030	Stone Grey	RAL 7044	Silk Gray	+	-
	SDR 9.9	RAL 1013	Oyster White	RAL 1024	Ochre Yellow	+	-
	SMT 1	RAL 9010	Pure White	RAL 1006	Maize Yellow	+	-
SCA	KMR 1E	RAL 1001	Beige	RAL 2008	Bright Red Orange	++	-
	SDR 2a	RAL 9003	Signal White	RAL 1032	Broom Yellow	++	-
	SDR 9.9	RAL 9002	Grey White	RAL 1023	Traffic Yellow	++	-
	SMT 1	RAL 9010	Pure White	RAL 1007	Chrome Yellow	++	-
PDA	KMR 1E$_2$	RAL 9001	Cream	RAL 1012	Lemon Yellow	+	-
	SDR 2a	RAL 1002	Sand Yellow	RAL 1006	Maize Yellow	++	-
	SDR 9.9	RAL 9001	Cream	RAL 1005	Honey Yellow	+	-
	SMT 1	RAL 9010	Pure White	RAL 1014	Ivory	+	-
NA	KMR 1E$_2$	RAL 1013	Oyster White	RAL 3020	Traffic Red	++	-
	SDR 2a	RAL 9010	Pure White	RAL 1007	Chrome Yellow	++	-
	SDR 9.9	RAL 9001	Cream	RAL 1016	Traffic White	+	-
	SMT 1	RAL 9000	Cream	RAL 1018	Zinc Yellow	+	-
Oatmeal	KMR 1E$_2$	RAL 1013	Oyster White	RAL 1006	Maize Yellow	+	+
	SDR 2a	RAL 1013	Oyster White	RAL 1007	Chrome Yellow	+	+
	SDR 9.9	RAL 1024	Ochre Yellow	RAL 1027	Curry	+	-
	SMT 1	RAL 9003	Signal White	RAL 7039	Quartz Grey	+	-
TSA	KMR 1E$_2$	RAL 9016	Traffic White	RAL 1006	Maize Yellow	+	+
	SDR 2a	RAL 9003	Signal White	RAL 8024	Beige Brown	+	-
	SDR 9.9	RAL 1016	Sulfer Yellow	RAL 1016	Sulfur Yellow	+	-
	SMT 1	RAL 9010	Pure White	RAL 1023	Traffic Yellow	+	-
SNA	KMR 1E$_2$	RAL 9000	Cream	RAL 1003	Signal Yellow	++	+
	SDR 2a	RAL 7044	Silk Grey	RAL 2004	Pure Orange	++	+
	SDR 9.9	RAL 9002	Grey White	RAL 1018	Zinc Yellow	++	-
	SMT 1	RAl 9010	Pure White	RAL 8007	Fawn Brown	+	-
Czapek'	KMR 1E$_2$	RAL 9016	Sulfur Yellow	RAL 7036	Platinum Grey	+	-
s-Dox	SDR 2a	RAL 7032	Pebble Grey	RAL 1005	Honey Yellow	+	-
Agar	SDR 9.9	RAL 7035	Light Grey	RAL 7044	Silk Grey	+	-
	SMT 1	RAL 9010	Pure White	RAL1018	Zinc Yellow	+	-
Benetts	KMR 1E$_2$	RAL 1005	Honey Yellow	RAL 1027	Curry	+	-
agar	SDR 2a	RAL 1005	Honey Yellow	RAL 1004	Golden yellow	+	-
	SDR 9.9	RAL 1016	Sulfur Yellow	RAL 1016	Sulfur Yellow	+	-
	SMT 1	RAL 9010	Pure White	RAL 1017	Saffron Yellow	+	-

Note: ++: Indicates very strong isolate growth. +: Indicates good isolate growth and/or positive production of soluble pigments. – : Indicates absence of growth and/or no soluble pigment production.

Biochemical and Physiological Characterization

To evaluate metabolic versatility, the four isolates were tested for their ability to utilize different carbon and nitrogen sources and to hydrolyze various biomolecules. Most isolates exhibited strong utilization of glucose, lactose, galactose, mannitol, and several amino acids. Hydrolysis of casein, starch, and Tween was positive in all isolates, while cellulose utilization was consistently negative. The biochemical profiles are summarized in Table 2.

Table 2. Biochemical characteristics of the four selected antibacterial-producing Actinomycetes isolates.

Biochemical properties	Strain codes			
	KMR 1E$_2$	SDR 2a	SDR 9.9	SMT 1
Carbon sources (C)				
D- glucose (+ Control)	+	++	++	++
D- lactose	+	+	+	+/-
Sucrose	+	+	+	+
D- galactose	++	+	++	++
Fructose	+	+	+	+
L (+) arabinose	++	++	++	+
D- mannitol	++	++	++	++
Trehalose	++	++	+	+/-
Cellobiose	+	+	+	-
Maltose	+	+	+	+/-
Dextrose	++	++	+	+
Myo-inositol	+	+	+/-	+/-
Dulcitol	+	+	+	+/-
L (+) sorbitol	+	+	+	-
Meso-erythritol	++	+	+	+/-
Cellulose	-	-	-	-
Nitrogen (N) sources				
ISP 2	++	++	++	++
L-valine	+	++	++	+
L-asparagine	++	++	++	++
L-tyrosine	+	+	+	+
L-glutamate	++	++	+	-
L-cysteine	+	+	++	+
$(NH_4)_2SO_4$	++	++	++	++
$NaNO_3$	++	++	++	++
Urea	++	++	++	++
Isoleusine	++	++	++	+
KNO_3	++	++	++	+
Glycine	++	++	++	+
DL-amino-n-butyric acid	++	+	+	+
Hidrolysis				
Casein	+	+	+	+
Gelatin	-	-	-	+
Starch	+	+	+	+
Tween	+	+	+	+
Melanin production	+	+	-	-

Note:
++ : Isolate growth on the carbon/nitrogen source is greater than that of the positive control.
+ : Isolate growth on the carbon/nitrogen source is comparable to the positive control.
+/– : Isolate growth on the carbon/nitrogen source is ambiguous or uncertain.
– : No isolate growth observed (negative).

Physiological tolerance was also assessed, including salt tolerance, growth at different pH values, and temperature optima. All isolates tolerated NaCl up to 5%, with SDR 9.9 displaying higher tolerance up to 8%. Growth was optimal at pH 7-8 and temperatures between 25-35 °C. Detailed data are presented in Table 3.

Table 3. Physiological growth responses of the four isolates under different environmental conditions.

Test	Strain codes			
	KMR 1E$_2$	**SDR 2a**	**SDR 9.9**	**SMT 1**
Crystal violet concentration (%)				
Control	+	+	+	+
0.02	-	-	-	-
0.04	-	-	-	-
0.06	-	-	-	-
0.08	-	-	-	-
0.1	-	-	-	-
Phenol concentration (%)				
Control	++	+	++	+
0.2	++	+	+	+
0.4	++	+	+	+
0.6	++	+	+	+
0.8	++	+	+	+
1	++	+	-	+
NaCl concentration (%)				
4	+	+	+	+
5	+	+	+	-
6	-	-	+	-
7	-	-	+	-
8	-	-	++	-
Growth pH				
3	-	-	-	-
4	-	-	-	-
5	-	-	+	+
6	+	-	+	+
7	+	+	++	++
8	+	+	++	++
9	-	+	++	+
10	-	+	-	-
Growth temperature (º C)				
4	-	-	-	-
25	+	++	++	++
30	++	++	++	+
35	++	+	++	++
37	+	+	+	++
45	-	-	-	-/+

Note:
++ : Isolate growth on the carbon/nitrogen source is equivalent to the positive control.
+ : Isolate growth on the carbon/nitrogen source is good and comparable to the positive control.
+/– : Isolate growth on the carbon/nitrogen source is uncertain or weak but still detectable.
– : No isolate growth observed (negative).

Fermentation, Extraction, and Antibacterial Activity of Crude Extracts

Based on broad-spectrum activity, two isolates KMR 1E2 and SDR 2a were selected for metabolite extraction. Fermentation broths were extracted using ethyl acetate and methanol. Both extracts displayed antibacterial activity against *S. aureus* ATCC 25923, with inhibition zones visibly larger than the solvent control. These results are shown in Figure 3.

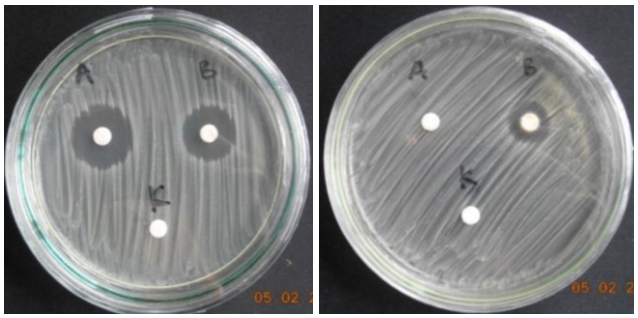

Figure 3. Antibacterial activity of ethyl acetate and methanol extracts of isolates KMR 1E2 and SDR 2a against S. aureus. **A)** Ethyl acetate extracts: inhibition of *Staphylococcus aureus* ATCC 25923 by isolates KMR 1E2 (A) and SDR 2a (B), with solvent control (K). **(B)** Methanol extracts: inhibition of *S. aureus* ATCC 25923 by isolates KMR 1E2 (A) and SDR 2a (B), with solvent control (K).

Bioautography and Determination of Active Rf

Bioautographic TLC revealed three principal antibacterial spots with Rf values of 0.73, 0.48, and 0.34, confirming that multiple metabolites contribute to the antibacterial activity observed. These results are shown in Figure 4.

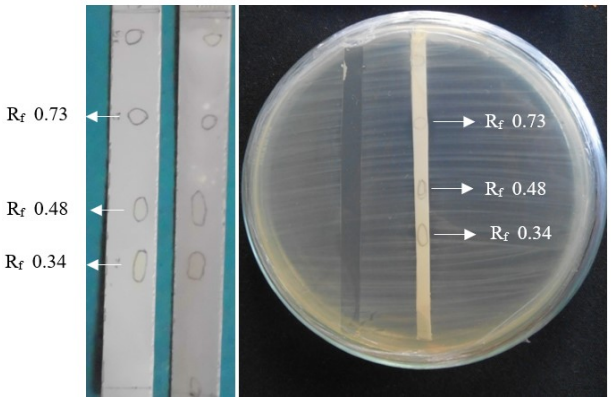

Figure 4. Bioautography profile showing active antibacterial spots and their corresponding Rf values.

Minimum Inhibitory Concentration (MIC) Assay

Ethyl acetate extracts from isolate KMR 1E2 were further evaluated for MIC against *S. aureus*. Inhibition was observed at concentrations as low as 0.625 mg/mL, although no inhibition occurred at 0.3125 mg/mL and below. The complete MIC data are presented in Table 4.

Table 4. Minimum inhibitory concentrations (MIC) of ethyl acetate extract from isolate KMR 1E2 against S. aureus ATCC 25923.

Concentration (mg/mL)	Concentration (mg/paperdisc)	Diameter of Inhibitory Zone (mm) (*Staphylococcus aureus*)
40	1.6	15
20	0.8	16
10	0.4	15
5	0.2	12
2.5	0.1	11.5
1.25	0.05	10.3
0.625	0.025	8
0.3125	0.0125	-

Discussion

The present study highlights the considerable antibacterial potential of Actinomycetes isolated from tropical karst and rhizospheric environments in South Sulawesi, Indonesia. The successful recovery of 42 isolates, of which four displayed measurable antibacterial activity, underscores the ecological richness and biochemical diversity of these habitats. Recent studies have emphasized that microbial communities in extreme or mineral-rich environments often harbor previously unrecognized Actinomycete taxa capable of producing unique bioactive metabolites (Genilloud, 2019; Zhang et al., 2023). The karst system examined in this study appears to support this observation, particularly considering the strong inhibition exhibited by isolates KMR 1E2 and SDR 2a.

Karst ecosystems are characterized by oligotrophic conditions, fluctuating humidity, and high mineral content, all of which create selective pressures that shape microbial evolution and secondary metabolite production. Recent metagenomic surveys have demonstrated that karst environments contain specialized microbial consortia with high biosynthetic gene cluster (BGC) diversity, especially among Actinobacteria (Zhou et al., 2020; Li et al., 2022). The findings here confirm that tropical karst ecosystems remain underexplored microbial reservoirs, as indicated by the presence of multiple isolates displaying distinct spore-chain morphologies and pigmentation patterns. The recovery of isolates with unique physiological profiles including tolerance to elevated salinity and phenolic compounds suggests that these microorganisms have adapted to the physicochemical stresses typical of karst substrates, a feature also widely reported in recent studies from Asian karst zones (Zhang et al., 2021; Wang et al., 2024).

The antibacterial activity exhibited by isolates KMR 1E2 and SDR 2a (>20 mm inhibition zones against *Staphylococcus aureus*) demonstrates their strong bioactivity relative to Actinomycetes isolated from conventional soils. Comparative analyses reveal that Actinomycetes isolated from non-extreme terrestrial environments typically produce inhibition zones ranging from 8-18 mm when tested against *S. aureus* (Sharma et al., 2020; Heng et al., 2021). Thus, the broader inhibition observed in this study suggests that the ecological constraints of karst habitats may stimulate increased production of defensive or competitive metabolites.

The ability of SDR 9.9 to inhibit *Escherichia coli* further supports the presence of metabolites with broad-spectrum activity. While Gram-negative bacteria are often more resistant due to their outer membrane, several recent studies highlight that karst-derived Actinomycetes can synthesize metabolites active against both Gram-positive and Gram-negative pathogens (Huang et al., 2023; Ma et al., 2022). This aligns with the competitive dynamics of nutrient-poor ecosystems, where microbial species evolve potent antagonistic traits to acquire resources.

The morphological characteristics, particularly spore-chain architecture, and pigment production provide valuable taxonomic insights and correlate with biosynthetic capacity. Spore-chain variability observed in this study, including rectus (straight), spiral, and retinaculiaperti forms, is consistent with recent taxonomic revisions suggesting that morphological diversity may reflect distinct evolutionary trajectories among Streptomyces-like Actinomycetes (Komaki et al., 2020; Li et al., 2024). Melanin production in isolates KMR 1E2 and SDR 2a is particularly noteworthy, as melanogenic Actinomycetes are often associated with

increased stress tolerance and are known to allocate more metabolic resources toward secondary metabolite biosynthesis (Santos et al., 2021; Tao et al., 2023).

Physiological assays revealed that isolates could utilize a wide range of carbon and nitrogen sources, tolerate moderate salinity, and grow across a broad pH spectrum. These findings are congruent with recent investigations demonstrating that metabolically flexible Actinomycetes often possess enriched biosynthetic gene clusters encoding non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS), which are primarily responsible for the synthesis of antibacterial metabolite (Cruz-Morales et al., 2019; Baltz, 2021). The ability of SDR 9.9 to grow in up to 8% NaCl highlights its halotolerance, a trait frequently linked with the production of osmotically regulated antibiotics such as ectoine derivatives and modified polyketides (Kalam et al., 2020).

One of the most notable findings of this study is the detection of an alkaloid-associated antibacterial compound at Rf 0.34 using TLC-bioautography. Alkaloids are less commonly reported from Actinomycetes than polyketides, macrolides, or peptide-based antibiotics; however, recent discoveries indicate that Actinomycetes from specialized habitats including caves, deserts, and thermal springs can produce previously uncharacterized alkaloid frameworks (Awad et al., 2020; Al-Dhabi et al., 2022). The alkaloid signature detected in KMR 1E2 reinforces the high novelty of this isolate and warrants further genomic and structural elucidation using LC-MS/MS and NMR.

The presence of multiple active bands (Rf 0.73, 0.48, and 0.34) suggests that the isolates synthesize a complex mixture of antibacterial agents. This indicates the production of multiple, rather than single, active compounds. Such multi-metabolite profiles are widely reported among Actinomycetes with high BGC diversity, particularly those sourced from geologically unique environments (Wang et al., 2023; Kyeremeh et al., 2021).

The MIC value of 0.625 mg/mL obtained for KMR 1E2 against *S. aureus* compares favorably with early-stage antibiotic discovery benchmarks. Recent natural product screens from environmental Actinomycetes report MIC values typically ranging between 0.5 and 4 mg/mL for crude extracts, while purified compounds may achieve 0.01-0.1 mg/mL (Hu et al., 2020; Chen et al., 2021). The low MIC reported here suggests that KMR 1E2 produces metabolites of considerable potency, aligning with karst-derived Actinomycetes known to generate strong antimicrobial scaffolds.

Given the increasing prevalence of methicillin-resistant *S. aureus* (MRSA), the discovery of metabolite-rich isolates from understudied regions such as South Sulawesi is highly valuable. Recent WHO reports emphasize the need for new antibiotics targeting resistant Gram-positive pathogens (World Health Organization, 2023), and findings from this study contribute directly to these global research priorities.

Overall, this study demonstrates that tropical karst ecosystems in Indonesia represent an untapped and high-potential biological niche for sourcing novel Actinomycetes with strong antibacterial activity. The combination of ecological uniqueness, morphological and physiological diversity, and the detection of an alkaloid-type metabolite provides compelling evidence that this environment supports distinct evolutionary and biosynthetic trajectories.

CONCLUSIONS

This study identified 42 Actinomycetes isolates from rhizosphere and tropical karst environments in South Sulawesi, with four isolates showing notable antibacterial activity. KMR 1E2 and SDR 2a exhibited the strongest inhibition against *Staphylococcus aureus* and were selected for metabolite extraction. Ethyl acetate extracts, particularly from KMR 1E2, demonstrated clear antibacterial activity supported by TLC, bioautography, and MIC analyses. These findings indicate that Indonesian karst and rhizosphere ecosystems represent valuable sources of bioactive Actinomycetes with potential for developing new antibacterial compounds.

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