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Antibacterial diketopiperazines from marine sponge-associated Actinobacteria against multidrug-resistant bacteria

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Abstract. *Larasati A, Budiarti S, Lestari Y. 2025. Antibacterial diketopiperazines from marine sponge-associated Actinobacteria against multidrug-resistant bacteria. Biodiversitas 26: 6480-6489.* The global rise of Multidrug-Resistant (MDR) bacterial infections has created an urgent demand for new antibacterial agents. Marine actinomycetes, particularly those associated with marine sponges, are considered a promising source of bioactive secondary metabolites. This study explored the antibacterial potential of 12 actinobacterial isolates obtained from the marine sponge *Callyspongia* sp., collected from the waters of Kepulauan Seribu, Indonesia. Among these, the isolate Cal24h exhibited the highest antibacterial activity. Based on previous 16S rRNA gene sequence analysis, this isolate was identified as *Streptomyces tendae*. Optimization of its culture conditions significantly enhanced inhibition zones against four MDR bacterial strains: 18.57±0.08 mm for Enteropathogenic *Escherichia coli* (EPEC) K1.1, 19.70±0.09 mm for *Pseudomonas aeruginosa*, 20.09±0.20 mm for *Bacillus subtilis*, and 20.54±0.31 mm for Methicillin-Resistant *Staphylococcus aureus* (MRSA). The Minimum Inhibitory Concentration (MIC) values of the ethyl acetate extract ranged from 62.5 to 125 µg/mL. Further analysis using GC-MS and LC-MS revealed the presence of diketopiperazine compounds, including Cyclo(Leu-Pro), Cyclo(L-Pro-L-Val), and Cyclo(Pro-Phe), all known for their antibacterial properties. Notably, this study is the first to report the use of a modified A1 medium to enhance antibacterial metabolite production in sponge-derived *Streptomyces*, representing a novel cultivation strategy. Cultivation in A1 medium yielded the highest extract quantity and exhibited the strongest antibacterial activity among all media tested. This study provides the first diketopiperazine production by *S. tendae* associated with the Indonesian sponge *Callyspongia* sp., thereby underscoring the untapped potential of sponge-derived actinobacteria as promising sources of antibacterial compounds against multidrug-resistant pathogens.

Keywords: Actinobacteria, antibacterial, antimicrobial resistance, MDR, optimization

INTRODUCTION

Antimicrobial Resistance (AMR) has emerged as one of the most critical global health threats. According to the World Health Organization (WHO 2022), bacterial AMR directly caused approximately 1.27 million deaths in 2019 and contributed to an additional 4.95 million deaths. Without effective interventions, drug-resistant infections are projected to surpass cancer as the leading cause of mortality by 2050 (van de Lagemaat et al. 2022; Tang et al. 2023). This escalating threat underscores the urgent need for new antibacterial agents, particularly those effective against Multidrug-Resistant (MDR) pathogens.

Marine environments have gained increasing attention as sources of structurally diverse and pharmacologically active natural products. Among marine organisms, sponges (Phylum Porifera) are prolific producers of secondary metabolites, with over 18000 unique compounds reported and more than 200 new metabolites discovered annually worldwide. These compounds account for approximately 23% of all approved marine-derived drugs (Hong et al. 2022). Between 2011 and 2020, over 2,600 new compounds were identified from marine sponges, spanning 20 taxonomic orders and nearly 700 species. Indonesian sponges alone have yielded 732 distinct metabolites between 1970 and 2019, reflecting their remarkable biochemical richness (Hanif et al. 2019; Chu et al. 2022; Mehbub et al. 2024).

Sponges are well-known reservoirs of microbial symbionts, particularly actinobacteria, which can comprise up to 60% of the sponge biomass and contribute significantly to their chemical diversity (Liang et al. 2023; Li et al. 2023). Many metabolites once attributed to sponges are now recognized as products of these symbiotic microbes, several of which exhibit potent antibacterial activity against MDR pathogens (Ahmed et al. 2022; Bahrami et al. 2022; Siro et al. 2023). Numerous promising compounds have been isolated from sponge-associated actinomycetes, such as chlocarbazomycin C, a chlorinated carbazole alkaloid from *Streptomyces diacarni* LHW51701, showing inhibitory effects against MRSA (Cheng et al. 2021). Furthermore, antimycin I from *Streptomyces* sp. NBU3104 displayed antifungal activity against *Candida albicans* (Li et al. 2022), while 6-lavandulyl-7-methoxy-5,2′,4′-trihydroxyflavanone from *Streptomyces* sp. G248 associated with *Halichondria panicea* inhibited *Mycobacterium tuberculosis* H37Rv (Cao et al. 2019). In addition, *Streptomyces tirandamycinicus* HNM0039 produces tirandamycins A and B with strong antibacterial properties (Huang et al. 2019). These findings highlight the pharmaceutical relevance of sponge-derived microorganisms, with some compounds already developed into approved drugs such as Viradabine® (antiviral), Cytosar®, and Halaven® (anticancer) (Romano et al. 2022).

Despite the chemical diversity of sponge-associated Actinobacteria and the growing number of bioactive compounds reported worldwide, research exploring their metabolite profiles and antibacterial potential remains limited in Indonesia. Although Indonesia was among the top three contributors to sponge-derived compound discoveries during 2001-2010, its output in the following decade declined compared to other countries such as China (Mehbub et al. 2024). This trend underscores the importance of further exploring sponge-derived microorganisms and their metabolites as potential sources of antibacterial agents.

Recent studies have demonstrated the remarkable antibacterial potential of Indonesian Actinobacteria, largely attributed to their diverse secondary metabolites. For instance, *Streptomyces koyangensis* SHP 9-3, isolated from the soil of Enggano Island, produced diketopiperazines such as cyclo(Pro-Val) and kuraramine with potent antibacterial activity, confirming that terrestrial Actinobacteria in Indonesia are capable of generating bioactive DKPs (Pahira et al. 2023). However, Actinobacteria isolates obtained from marine sediments of Pramuka Island in the Seribu Islands, while exhibiting notable antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, had not been chemically characterized (Setiawati et al. 2021). Taken together, these findings suggest that despite the promising bioactivities of Indonesian terrestrial and marine Actinobacteria, their metabolite diversity particularly the occurrence of diketopiperazines from marine sponge-associated Actinobacteria of the Seribu Islands, has not been investigated. To address this knowledge gap, the present study investigates the antibacterial activity and metabolite profiles of *Streptomyces tendae* Cal24h, a sponge-associated actinobacterial isolate from *Callyspongia* sp. in the Seribu Islands, through in vitro assays supported by GC-MS and LC-MS analyses.

MATERIALS AND METHODS

Materials

Twelve Actinobacteria isolates previously obtained from the marine sponge *Callyspongia* sp. were used in this study. All isolates are part of the microbial collection curated by Prof. Dr. Yulin Lestari at Institut Pertanian Bogor, Indonesia. The Actinobacteria used in this study originated from this established collection, which was developed from sponge samples obtained under institutional and national biodiversity regulations. Among these isolates, *Streptomyces tendae* Cal24h was identified as the most active strain based on antibacterial screening and was subsequently selected for further chemical characterization. The test bacteria included enteropathogenic *Escherichia coli* (EPEC) K1.1 obtained from the collection of Prof. Dr. dr. Sri Budiarti at Institut Pertanian Bogor, and *Pseudomonas aeruginosa*, *Bacillus subtilis*, and Methicillin-Resistant *Staphylococcus aureus* (MRSA) obtained from the Laboratory of Microbiology, Intitut Pertanian Bogor.

Bacteria subculture

Twelve actinobacteria isolates were cultivated on A1 agar containing (g/L): oatmeal (10), yeast extract (4), and peptone (2), and incubated at 150 rpm for 14 days. Test bacteria (EPEC K1.1, *P. aeruginosa*, *B. subtilis*, and MRSA) were grown in Mueller-Hinton (MH) broth at 37°C for 24 hours. For antibacterial assays, 100 µL of each bacterial suspension was transferred into 10 mL of fresh MH broth and incubated at 37°C, 100 rpm for 8 hours to reach a final cell density of approximately 10^8 CFU/mL.

Antibiotic sensitivity test

The antibiotic susceptibility of the test bacteria was evaluated using the Kirby-Bauer disc diffusion method on MH agar. Each bacterial strain was cultured in MH broth at 37°C for 24 hours, and 0.1 mL of the resulting culture was evenly spread onto MH agar plates. Commercial antibiotic discs containing ampicillin (10 µg), penicillin (10 µg), cefadroxil (30 µg), cefixime (5 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), streptomycin (10 µg), tetracycline (30 µg), and azithromycin (15 µg) were aseptically placed on the agar surface. The plates were incubated at 37°C for 18 hours.

After incubation, the diameters of inhibition zones were measured in millimeters. The results were interpreted according to the guidelines established by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (CLSI 2023; EUCAST 2024).

Colony-based screening for antibacterial activity of Actinobacteria

Antibacterial activity was evaluated using the agar plug diffusion method. Actinobacterial isolates were first grown on A1 agar plates for 14 days at room temperature. A 6 mm agar plug containing the colony was then cut and placed onto Mueller-Hinton Agar (MHA) plates previously inoculated with the test bacteria (~10^8 CFU/mL, equivalent to 0.5 McFarland). Plates were incubated at 37°C for 18-24 hours. Antibacterial activity was recorded qualitatively, based on the presence or absence of a clear inhibition zone around the plug. A negative control (sterile A1 agar plug without actinobacteria) was included (Ortlieb et al. 2021; Hossain 2024).

Supernatant-based screening for antibacterial activity of Actinobacteria

Supernatants from seven actinobacteria isolates that showed inhibition zones in the colony-based assay were used for further antibacterial testing. Each isolate was grown in 50 mL of A1 broth and incubated at 28°C for 14 days in a shaker incubator at 100 rpm. After incubation, 1.5 mL of each culture was centrifuged at 13,000 rpm for 30 minutes at 26°C to separate the supernatant from the cell biomass.

Table 1. Standard interpretative category and inhibition zone diameter (mm) of antibiotics to bacterial test

Standard antibiotic	EPEC K1.1 (-)			*Pseudomonas aeruginosa* (-)			*Bacillus subtilis* (+)			MRSA (+)		
	S	I	R	S	I	R	S	I	R	S	I	R
Ampicillin (10 µg)	≥17	14-16	≤13	≥17	14-16	≤13	≥29	1-28	≤20	>29	16-28	≤15
Penicillin (10 µg)	≥18	14-17	≤13	≥18	13-17	≤12	≥18	14-17	≤13	≥18	14-17	≤13
Cefadroxil (30 µg)	≥18	15-17	≤14	≥18	13-17	≤12	≥18	15-17	≤14	≥18	15-17	≤14
Cefixime (5 µg)	≥19	16-18	≤15	≥19	16-18	≤15	≥21	16-20	≤15	≥27	23-26	≤22
Chloram-phenicol (30 µg)	≥18	13-17	≤12	≥18	13-17	≤12	≥18	13-17	≤12	≥18	13-17	≤12
Nalidixic Acid (30 µg)	≥19	14-18	≤13	≥19	14-18	≤13	≥19	14-18	≤13	≥19	14-18	≤13
Streptomycin (10 µg)	≥17	14-16	≤13	≥17	14-16	≤13	≥29	21-28	≤20	≥25	13-24	≤12
Tetracyclin (30 µg)	≥15	12-14	≤11	≥15	12-14	≤11	≥15	12-14	≤11	≥16	11-15	≤10
Azythromycin (15 µg)	≥15	12-14	≤11	≥24	19-23	≤18	≥23	19-22	≤18	≥17	12-16	≤11

Note: R: Resistant, I: Intermediete, S: Susceptible with standard based on CLSI (2023) and EUCAST (2024)

The antibacterial activity of the supernatants was evaluated using the Kirby-Bauer disc diffusion method. Sterile paper discs (6 mm diameter) were loaded with 20 µL of each supernatant and placed onto MHA plates previously inoculated with test bacteria (~10^8 CFU/mL). The plates were incubated at 37 °C for 18 hours. Inhibition zones were then measured in millimeters. Chloramphenicol (30 µg) and 10% DMSO were used as positive and negative controls (Santos et al. 2022).

Extract-based screening for antibacterial activity of Actinobacteria

Selected actinobacteria isolates from the supernatant assay were cultured in 1,000 mL A1 broth (inoculated with 10% seed culture) and incubated at 28°C, 100 rpm for 14 days. The cultures were extracted twice with ethyl acetate (1:1, v/v), and the combined organic phase was concentrated using a rotary evaporator at 40°C to yield crude extracts.

Each extract (1 mg) was dissolved in 10 mL of 10% DMSO to prepare a 100 ppm stock solution. Serial dilutions (250-1000 ppm) were prepared for testing. Antibacterial activity was assessed using the Kirby-Bauer disc diffusion method by applying 20 µL of each dilution onto sterile paper discs (6 mm) placed on MHA plates seeded with test bacteria (~10^8 CFU/mL). After 18 hours of incubation at 37°C, inhibition zones were measured in millimeters. Chloramphenicol (30 µg) and 10% DMSO served as positive and negative controls (Tornberg-Belanger et al. 2022).

Optimization of growth media

The selected bacterial strain was initially cultured in various media, including ISP 1, ISP 2, ISP 4, Molasses, SCN, and A1 culture medium (composition detailed in supplementary information). Next, the cultures were incubated at room temperature for 14 days in a shaker incubator at 150 rpm. The optimal growth medium was then identified based on dry extract mass yield and antibacterial activity after the incubation period (Larasati et al. 2021).

Determination of MIC and MBC

A 96-well plate was prepared for broth microdilution, with serial dilution series from 250 µg/mL to 1.95 µg/mL. The plates were then incubated for 18 hours at 37°C. Optical density was measured at 625 nm to assess bacterial growth. The MIC was determined as the lowest concentration at which bacterial growth (turbidity) was no longer visibly detected. Next, to determine the MBC (minimum bactericidal concentration), 100 µL samples from wells exhibiting no growth at the MIC were plated onto MHA plates and incubated for 18 hours at 37°C. The MBC was then determined as the lowest concentration that prevented any visible colony formation on the MHA plates (Seukep et al. 2025).

Identification of Actinobacteria compounds with GC-MS

The potential Actinobacteria extracts were analyzed using GC-MS to identify their chemical constituents. The analysis was performed on an SH-Rxi-5Sil MS column (30 m × 0.25 mm × 0.25 µm) using helium as the carrier gas at a constant flow rate of 0.9 mL/min. A 1 µL aliquot of the ethyl acetate extract was injected in split mode. The oven temperature was initially set at 50°C, then increased at a rate of 15°C per minute to a final temperature of 280°C and held for 10 minutes. Compound detection was conducted for 30 minutes, and identification was achieved by comparing the mass spectra with those in the GC-MS W10N14 library database. Compounds were identified based on mass spectral similarity indices (≥90%) with reference spectra in the library (Aziz et al. 2025).

Identification of Actinobacteria compounds with LC-MS

The potential Actinobacteria extracts were analyzed using a 5 µL sample injected into an Ultra-Performance Liquid Chromatography (UPLC) system (ACQUITY UPLC® H-Class, Waters, USA) coupled with a quadrupole time-of-flight mass spectrometer (Xevo G2-S QTof, Waters, USA). Separation was achieved on a C18 column (2.1 mm × 100 mm, 1.8 µm) maintained at 50°C. The mobile phase consisted of solvent A (distilled water with 5 mM formic acid) and solvent B (acetonitrile with 0.05% formic acid), delivered at a flow rate of 0.2 mL/min under a 23-minute gradient program. Electrospray Ionization (ESI) was operated in positive ion mode over a mass range of 50-1200 m/z. Mass spectra were acquired using the QTof analyzer, and compound identification was performed by comparing the MS data with reference spectra available in the ChemSpider database (de Athayde et al. 2024).

RESULTS AND DISCUSSION

Antibiotic sensitivity test

From antibiotic sensitivity testing (Table 1, Figure 1), MRSA and *Bacillus subtilis* (Gram-positive) were more susceptible to the antibiotics tested, while EPEC K1.1 and *Pseudomonas aeruginosa* (Gram-negative) exhibited greater resistance. The disc diffusion test revealed consistent resistance across all Multidrug-Resistant (MDR) bacteria to all β-lactam antibiotics (ampicillin, penicillin, cefadroxil, and cefixime) and macrolide (azithromycin). Among the tested antibiotics, amphenicol antibiotic (chloramphenicol) exhibited the highest efficacy, showing susceptibility activity in three out of four strains (*P. aeruginosa*, *B. subtilis*, MRSA, and intermediate activity against EPEC K1.1.

Screening of Actinobacteria for the production of antibacterial compounds

Colony-based screening

Figure 2 shows twelve actinobacteria screened, and seven isolates inhibited at least one of the MDR bacteria. The presence of a clear zone (black squares) indicates successful inhibition, while the absence of a clear zone (white squares) indicates no inhibition (Figure 2). Based on their activity and spectrum, seven actinobacteria isolates coded as XGS, Dbi28t, Crc21t, Cal24h, IVNF 1-1, TBL24SP, and HVB were selected for further testing.

Table 2. Inhibition zone of antibiotic sensitivity test

Standard antibiotic	EPEC K1.1 (-)		*P. aeruginosa* (-)		*B. subtilis* (+)		MRSA (+)	
	Inhibition zone (mm)	Sensitivity	Inhibition zone (mm)	Sensitivity	Inhibition zone (mm)	Sensitivity	Inhibition zone (mm)	Sensitivity
Ampicillin (10 µg)	0	R	15	R	8	R	8	R
Penicillin (10 µg)	0	R	0	R	7,5	R	8	R
Cefadroxil (30 µg)	0	R	15	R	8	R	10.25	R
Cefixime (5 µg)	0	R	0	R	11	R	15.61	R
Azythromycin (15 µg)	10	R	0	R	12,7	R	12.42	R
Chloramphenicol (30 µg)	16,6	I	22	S	22	S	24.15	S
Nalidixic Acid (30 µg)	13,8	I	12	R	18,5	I	20	S
Streptomycin (10 µg)	12,9	I	15	I	10	R	12	R
Tetracyclin (30 µg)	0	R	10	R	17,43	S	17.21	S

Note: R: Resistant, I: Intermediete, S: Susceptible based on CLSI (2023) and EUCAST (2024)

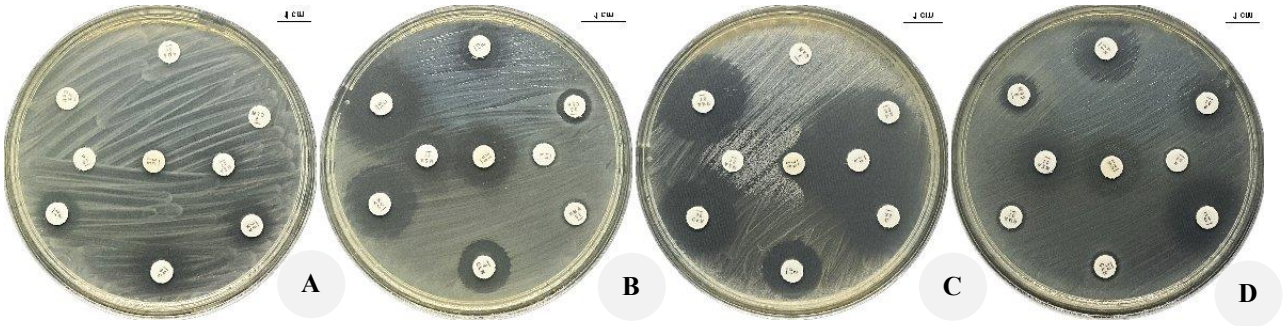

Figure 1. Antibiotic sensitivity test against MDR. A. EPEC K1-1, B. *Pseudomonas aeruginosa*, C. *Bacillus subtilis*, D. MRSA

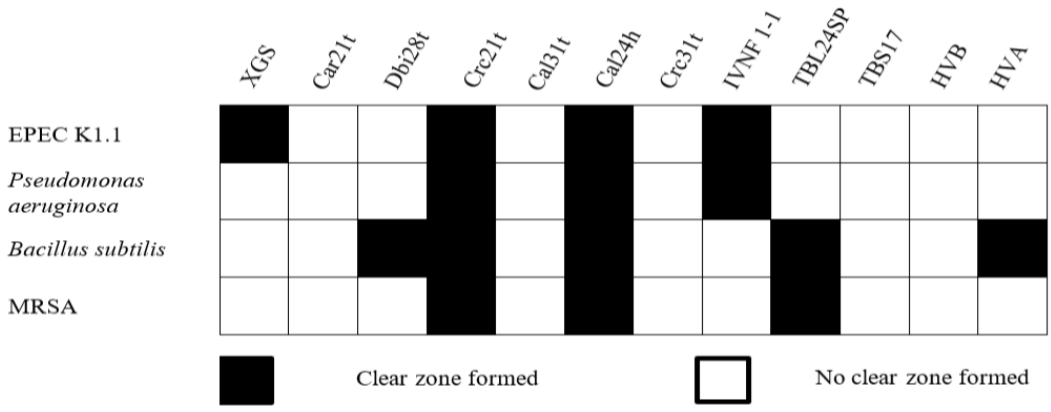

Figure 2. Antibacterial activity of Actinobacteria colonies against MDR bacteria

Supernatant-based screening

Table 3 summarizes the antibacterial activity of culture supernatants from seven actinobacteria isolates against four Multidrug-Resistant (MDR) bacterial strains. The supernatants of Crc21t and Cal24h inhibited the growth of all tested bacteria, with inhibition zones ranging from 7.3 to 9.4 mm. TBL24SP showed inhibitory activity against all strains except *P. aeruginosa*, while IVNF1-1 inhibited all strains except MRSA. The supernatant of XGS was active only against EPEC K1.1, and HVA showed limited activity against *B. subtilis* (8.0±0.0 mm). Dbi28t exhibited no observable inhibition against any of the test strains. Based on the extent and consistency of the inhibitory effects across the four test organisms, Crc21t, Cal24h, IVNF1-1, and TBL24SP were selected for further screening using ethyl acetate extracts.

Extract-based screening

The ethyl acetate extracts of four Actinobacteria isolates exhibited varying antibacterial activity against MDR bacteria (Table 3). Among them, Cal24h showed the highest inhibition across all test strains, with inhibition zones ranging from 18.57±0.08 mm to 20.54±0.31 mm. Its activity was statistically comparable to the positive control, chloramphenicol (30 µg). IVNF1-1 also demonstrated strong inhibition, particularly against *P. aeruginosa* and MRSA. In contrast, TBL24SP and Crc21t showed moderate activity, while 10% DMSO (negative control) produced no inhibition.

Determination of MIC and MBC of Cal24h extract

The MIC and MBC values of Cal24h ethyl acetate extract were evaluated against four Multidrug-Resistant (MDR) bacterial strains (Table 5, Figure 4). The MIC values were 62.5 µg/mL for *B. subtilis* and MRSA, and 125 µg/mL for EPEC K1.1 and *P. aeruginosa*. All MBC values were >250 µg/mL, indicating that the extract was not bactericidal at the tested concentrations. Chloramphenicol (30 µg), used as a positive control, showed MIC and MBC values below 0.23 µg/mL across all strains.

Visual inspection of the 96-well plate (Figure 4) supported the MIC data, with visible inhibition of bacterial growth at the corresponding concentrations for each strain.

Optimization of growth media

Table 6 and Figure 5 revealed that ISP 1 and Starch Casein Nitrate (SCN) media produced minimal inhibition zones and yielded the lowest dry weight of extracts at 6.00 g, indicating no antibacterial activity. ISP 4 media demonstrated moderate antibacterial activity, especially against Gram-positive bacteria. Both molasses and ISP 2 media exhibited moderate inhibition zones but were less effective compared to ISP 4 and A1 media. Notably, A1 medium was the most efficient in producing antibacterial metabolites, yielding the highest crude extract weight of 2.88 mg and displaying the strongest antibacterial activity with inhibition zones against EPEC K1.1 (18.75 mm), *P. aeruginosa* (19.75 mm), *B. subtilis* (20.34 mm), and MRSA (20.54 mm)

Table 3. Antibacterial activity of Actinobacteria supernatant against MDR bacteria

Isolate code	Average of inhibition zone diameter±standard deviation (mm)			
	EPEC K1.1	***Pseudomonas aeruginosa***	***Bacillus subtilis***	**MRSA**
XGS	10±0.0^{c}	nd	nd	nd
Dbi28t	nd	nd	nd	nd
Crc21t	7.37±0.21^{d}	8.1±0.1^{c}	7.27±0.12^{d}	8.23±0.15^{d}
Cal24h	9.33±0.05^{b}	9.43±0.32^{b}	8.23±0.06^{c}	9.03±0.06^{c}
IVNF1-1	9.23±0.25^{b}	8.13±0.23^{c}	9.36±0.25^{b}	nd
TBL24SP	9.01±0.10^{c}	nd	8.1±0.30^{c}	9.37±0.06^{b}
HVA	nd	nd	8.0±0.0^{c}	nd
Chloramphenicol 30 µg	17.0±0,0^{a}	19.0±0.0^{a}	23.16±0.21^{a}	21.23±0.21^{a}
DMSO 10%	nd	nd	nd	nd

Note: The values presented above represent the means and standard deviations from three replicates. Statistical analyses were conducted using a one-way ANOVA, followed by the Duncan test to assess significance ($p<0.05$). Values with different superscript letters indicate statistically significant differences. *nd: not determined

Table 4. Antibacterial activity of Actinobacteria extract against MDR bacteria

Isolat code	Average of inhibition zone diameter±standard deviation (mm)			
	EPEC K1.1	***Pseudomonas aeruginosa***	***Bacillus subtilis***	**MRSA**
TBL24SP	14.25±0.13^{e}	13.50±0.03^{e}	16.29±0.04^{c}	17.59±0.01^{d}
Cal24h	18.57±0.08^{b}	19.70±0.09^{b}	20.09±0.20^{b}	20.54±0.31^{b}
IVNF1-1	16.55±0.04^{d}	19.31±0.04^{c}	11.08±0.08^{e}	13.39±0.02^{e}
Crc21t	16.81±0.27^{c}	16.83±0.04^{d}	16.04±0.05^{d}	17.66±0.03^{c}
Chloramphenicol 30 µg	20.47±0.06^{a}	21.0±0.0^{a}	25.04±0,04^{a}	25.01±0.02^{a}
DMSO 10%	nd	nd	nd	nd

Note: The values presented above represent the means and standard deviations from three replicates. Statistical analyses were conducted using a one-way ANOVA, followed by the Duncan test to assess significance ($p<0.05$). Values with different superscript letters indicate statistically significant differences. *nd: not determined

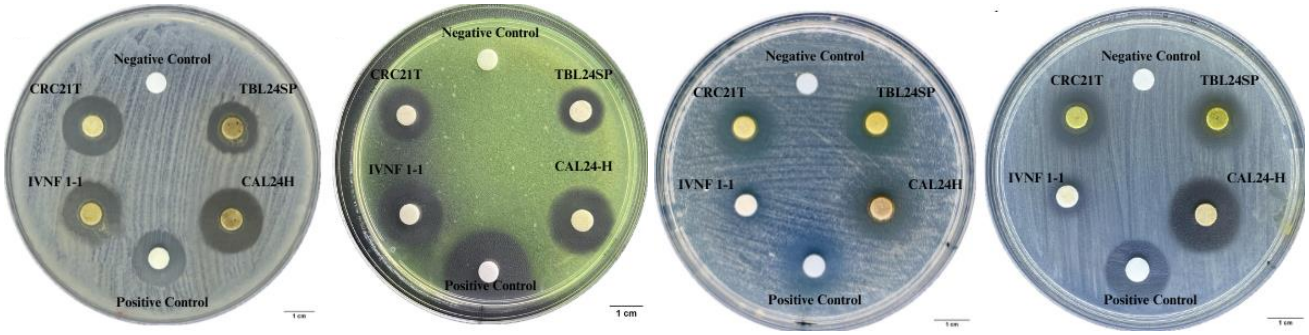

Figure 3. Inhibition zone activity of Actinobacteria against MDR. A. EPEC K1-1, B. *Pseudomonas aeruginosa*, C. *Bacillus Subtilis*, D. MRSA

Table 6. Optimization of culture media for potential actinobacteria Cal24h

Media	Dry extract mass (mg)	Average of inhibition zone diameter±standard deviation (mm)			
		EPEC K1.1	*Pseudomonas aeruginosa*	*Bacillus subtilis*	MRSA
International *Streptomyces* Project 1	0.73±0.15e	6.0±0.0^{e}	6.0±0.0^{e}	6.0±0.0^{f}	6.0±0.0^{f}
International *Streptomyces* Project 2	1.38±0.03c	9.22±0.01^{d}	9.33±0.05^{c}	10.56±0.05^{b}	11.57±0.05^{b}
International *Streptomyces* Project 4	1.17±0.04d	12.93±0.04^{b}	10.51±0.09^{b}	9.24±0.03^{c}	10.84±0.04^{c}
Molase	1.83±0.02^{b}	9.22±0.01^{d}	8.14±0.03^{d}	6.77±0.02^{e}	6.0±0.0^{e}
Starch Caseine Nitrate	1.23±0.04^{d}	6.0±0.0^{e}	6.0±0.0^{e}	8.03±0.06^{d}	6.91±0.04^{d}
A1	2.88±0.04^{a}	16.75±0.02^{a}	17.75±0.04^{a}	19.93±0.03^{a}	19.34±0.04^{a}

Note: The values presented above represent the means and standard deviations from three replicates. Statistical analyses were conducted using a one-way ANOVA, followed by the Duncan test to assess significance ($p<0.05$). Values with different superscript letters indicate statistically significant differences

Table 5. MIC and MBC Value of Cal24h Extract

MDR bacteria	MIC value (µg/mL)	MBC value (µg/mL)	Positive control (Chloramphenicol)
Gram-negative bacteria			
EPEC K1-1	125	>250	<0.23
Pseudomonas aeruginosa	125	>250	<0.23
Gram-positive bacteria			
Bacillus subtilis	62.5	>250	<0.23
MRSA	62.5	>250	<0.23

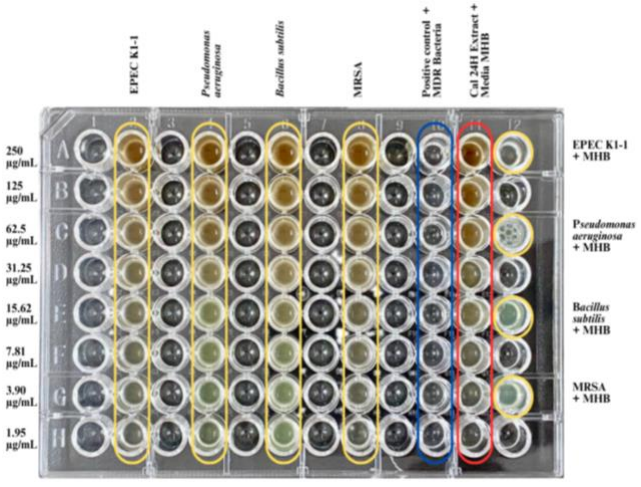

Figure 4. Minimum inhibitory concentration test of Cal24h extract

Identification and profiling of bioactive compounds from Cal24h extract

GC-MS-based profiling of bioactive compounds from Cal24h extract

Table 7 presents the results of Gas Chromatography Mass Spectrometry (GC-MS) analysis of the Cal24h extract. Several bioactive compounds were identified, including Bis (2-ethylhexyl) phthalate (DEHP), Cyclo (Leu-Pro), Cyclo (L-Pro-L-Val), Cyclo (L-Leu-L-Leu), Cyclo (Pro-Phe), and *n*-pentadecanol.

LC-MS-based profiling of bioactive compounds from Cal24h extract

Based on Liquid Chromatography-Mass Spectrometry (LC-MS) analysis, several bioactive compounds were identified in the Cal24h extract (Figure 6, Table 8). These include Cyclo (Leu-Pro), Cyclo (L-Pro-L-Val), Cyclo (Pro-Tyr), Cyclo (Pro-Phe), Ghanamycin B, Fistupyrone, Lemonomycin, and Surugapyrone A.

The LC-MS chromatogram, along with the annotated chemical structures of the detected compounds, is presented in Figure 6. Detailed information on the identified compounds, including their molecular formulas, retention times, peak areas, biological activities, and references, is summarized in Table 7.

Discussion

The increasing global burden of Multidrug-Resistant (MDR) infections has intensified the search for alternative antibacterial agents derived from natural sources. Among these, marine sponge-associated actinobacteria are considered a promising but underexplored source of novel bioactive

compounds. In this study, the antibacterial potential of 12 actinobacterial isolates was evaluated using a three-stage screening approach consisting of colony-based, supernatant-based, and extract-based assays against both Gram-positive and Gram-negative MDR strains.

Among the twelve actinobacterial isolates initially screened, seven exhibited antibacterial activity at the colony level. However, only four isolates, namely Cal24h, Crc21t, IVNF1-1, and TBL24SP, maintained consistent inhibitory effects in both supernatant and extract-based assays. Cal24h, previously identified as *S. tendae* (Retnowati et al. 2019), demonstrated the highest and most consistent antibacterial activity, with inhibition zones ranging from 18.57 to 20.54 mm and MIC values between 62.5 and 125 µg/mL against Enteropathogenic *E. coli* (EPEC) K1.1, *P. aeruginosa*, *B. subtilis*, and Methicillin-Resistant *S. aureus* (MRSA).

These results are consistent with reports of marine-derived *Streptomyces* species exhibiting broad-spectrum antibacterial activity. Against *E. coli* EPEC K1.1, the Plantaricin compound from *Lactobacillus plantarum* S34 produced an inhibition zone of 6±0.82 mm (Ahaddin et al. 2021). In contrast, for *P. aeruginosa*, *Streptomyces rajshahiensis* and *Streptomyces* SAE4034 exhibited inhibition zones of only 8-10 mm (Ryandini et al. 2018; Djebbah et al. 2021), whereas isolates B1 and B2 generated up to 14 mm (Risan et al. 2022). Against MRSA, *Streptomyces* sp. JS20 and *S. gancidicus* VITBKA3 demonstrated inhibition zones of approximately 18 mm (Bhakyashree and Kannabiran 2020; Somasundaram et al. 2024). Similarly, against *B. subtilis*, *Micromonospora terminaliae* MKP30, *Streptomyces cellulosae* SL2-2-R9, and *Streptomyces* sp. VITGV01 displayed inhibition zones of 12-14 mm (Malisorn et al. 2020; Sari et al. 2021; Veilumuthu and Christopher 2022), while *Streptomyces* isolates from Surabaya mangroves reached 17.3 mm (Retnowati et al. 2023).

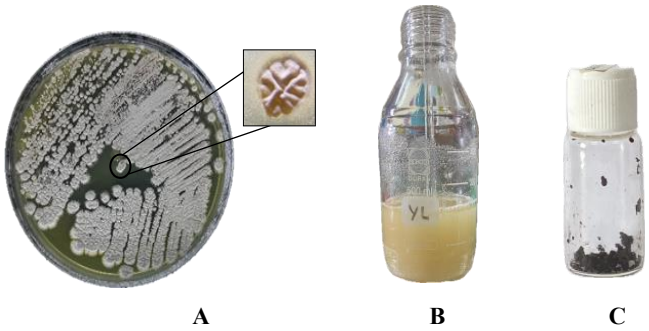

Figure 5. Cal24h on A1 medium. A. A1 agar, B. A1 Broth, C. A1 extract

Table 7. Bioactive compounds identified from Cal24h extract by GC-MS analysis

Compound name	Formula	Retention time	Peak area %	Activity	References
Bis (2-ethylhexyl) phthalate (DEHP)	$C_{24}H_{38}O_4$	46.190	20.61	Antibacterial, antifungal	Al-Bari et al. (2005)
Cyclo (Leu-Pro)	$C_{11}H_{18}N_2O_2$	35.211	2.86	Antibacterial	Alshaibani et al. (2017)
Cyclo (L-pro-L-val)	$C_{10}H_{16}N_2O_2$	33.23	2.22	Antibacterial	Castaldi et al. (2022)
Cyclo (L-leu-L-leu)	$C_{12}H_{22}N_2O_2$	40.006	1.13	Antibacterial, antifungal	Jamal et al. (2017)
Cyclo (Pro-Phe)	$C_{14}H_{16}N_2O_2$	43.665	0.45	Antibacterial, antifungal	Kumar et al. (2014)
n-pentadecanol	$C_{15}H_{32}O$	28.494	0.32	Antibacterial	Chatterjee et al. (2018)

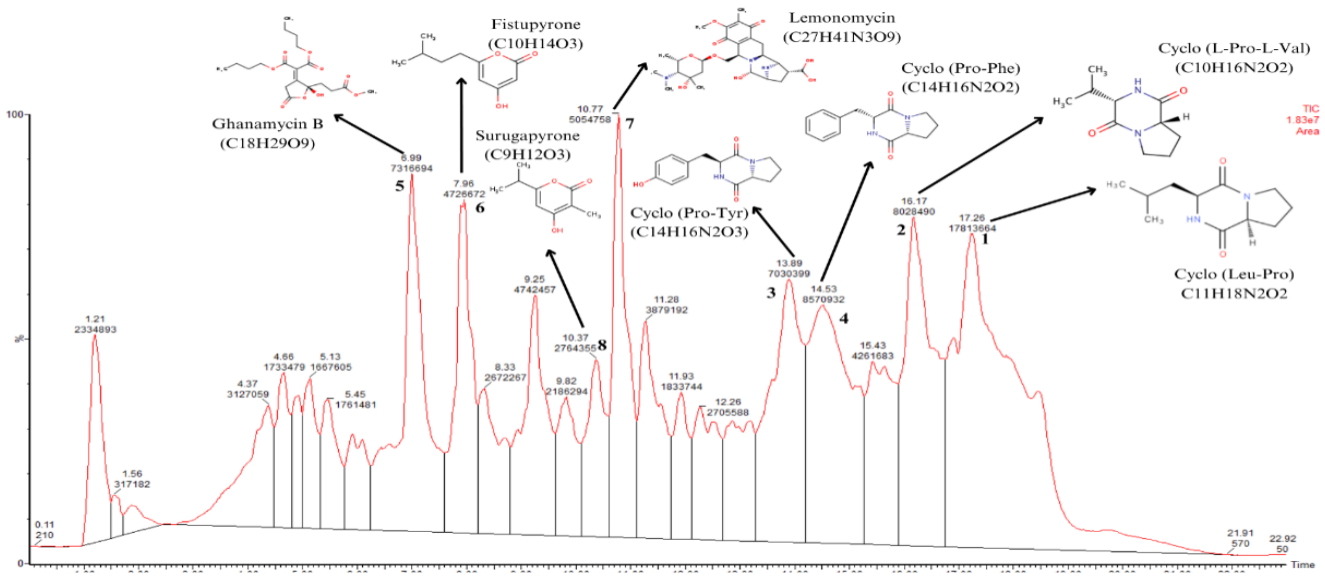

Figure 6. LC-MS chromatogram and structure annotation of bioactive compounds identified from Cal24h extract

Table 8. Bioactive compounds identified from Cal24h extract by LC-MS analysis

Compound name	Formula	Time retention	Peak area %	Activity	References
Cyclo (Leu-Pro)	$C_{11}H_{18}N_2O_2$	17.23	16.71%	Antibacterial	Saadouli et al. (2020)
Cyclo (L-Pro-L-Val)	$C_{10}H_{16}N_2O_2$	16.17	12.03%	Antibacterial, antifungal	amal et al. (2017)
Cyclo (L-Pro-L-Tyr)	$C_{14}H_{16}N_2O_3$	13.89	9.69%	Antibacterial	Rahman et al. (2019)
Cyclo (Pro-Phe)	$C_{14}H_{16}N_2O_2$	14.51	8.64%	Antibacterial	Yang et al. (2021)
Ghanamycin B	$C_{18}H_{29}O_9$	7.01	7.6%	Antibacterial	Xu et al. (2017)
Fistupyrone	$C_{10}H_{14}O_3$	9.23	4.73%	Antifungal	Čihák et al. (2017)
Lemonomycin	$C_{27}H_{41}N_3O_9$	11.25	3.9%	Antibacterial, antitumor	Tao et al. (2022)
Surugapyrone A	$C_9H_{12}O_3$	10.35	2.61%	Antioxdant	Sugiyama et al. (2010)

Compared with these studies, the Cal24h extract inhibited all tested MDR strains with zones reaching up to 20 mm, suggesting that it exhibits relatively broader and stronger antibacterial activity than previously reported marine or terrestrial Actinobacteria. This enhanced activity may be associated with the combined effects of several secondary metabolites detected in the extract, particularly diketopiperazines, which have been reported to interfere with bacterial membrane integrity and cell wall synthesis (Alshaibani et al. 2017; Yang et al. 2021). Such results also support the view that sponge-associated *Streptomyces* can exhibit distinct metabolic capabilities, possibly influenced by ecological adaptation and the presence of specialized biosynthetic gene clusters that increase metabolite diversity (Ahmed et al. 2022; Liang et al. 2023).

Among the tested fermentation media, A1 medium produced the highest extract yield (2.88 mg) and exhibited the strongest antibacterial activity against all MDR strains. The A1 formulation represents an innovative adaptation of conventional culture media, designed to provide balanced nutrient conditions for marine actinobacteria. Its composition, combining oatmeal as a carbon source with yeast extract and peptone as nitrogen sources, offers a nutrient profile that supplies both simple sugars and amino acid precursors essential for the biosynthesis of peptide-based secondary metabolites such as diketopiperazines. In *Streptomyces*, nutrient abundance, particularly amino acid availability, has been shown to activate Nonribosomal Peptide Synthetase (NRPS) pathways that promote the formation of diketopiperazine and other small cyclic peptides (Challis and Hopwood 2003; Borthwick 2012). In contrast, other media such as ISP1, SCN, and Molasses contained simpler or less balanced nutrient profiles, which likely limited metabolite biosynthesis and resulted in weaker antibacterial activity. Therefore, the results indicate that A1 medium not only enhances biomass accumulation but also creates favorable metabolic conditions for secondary metabolite production in the sponge-associated *S. tendae* Cal24h.

The MIC values of the Cal24h extract ranged from 62.5 µg/mL for *B. subtilis* and MRSA to 125 µg/mL for EPEC K1.1 and *P. aeruginosa*, whereas the MBC values exceeded 250 µg/mL for all tested bacteria. These results indicate that the extract acts primarily as a bacteriostatic agent under the tested conditions. Similar MIC ranges have been reported for crude extracts of marine *Streptomyces*, typically between 60 and 200 µg/mL (Gunes et al. 2016; Viswapriya and Saravana Kumari 2022). The lower MIC values observed against Gram-positive bacteria are consistent with differences in cell wall structure, since the outer membrane of Gram-negative bacteria restricts the penetration of hydrophobic compounds (Mary et al. 2021). Although less potent than the positive control chloramphenicol (MIC<0.23 µg/mL), the Cal24h extract demonstrates promising inhibitory activity for a natural product mixture, supporting its potential as a lead for antibacterial compound discovery.

GC-MS and LC-MS analyses of the Cal24h extract confirmed the presence of several diketopiperazine derivatives, including cyclo(L-Pro-L-Val), cyclo(Pro-Phe), and cyclo(Leu-Pro). These cyclic dipeptides are small, hydrophobic molecules known to disrupt bacterial membrane stability and inhibit peptidoglycan synthesis (Alshaibani et al. 2017; Yang et al. 2021). The detection of multiple DKPs in the same extract suggests possible additive or synergistic effects that contribute to the broad-spectrum antibacterial activity observed in Cal24h. Among these compounds, cyclo(L-Pro-L-Val) and cyclo(Pro-Phe) have previously been reported from terrestrial *Streptomyces* isolates, but their occurrence in sponge-associated *Streptomyces* from the Seribu Islands represents the first record from this region. This finding expands current knowledge of Indonesia's marine microbial chemical diversity and supports the ecological premise that sponge symbiosis can stimulate the expression of unique biosynthetic pathways in actinobacteria.

Overall, the combination of antibacterial screening, culture optimization, and metabolite profiling demonstrates that nutrient-driven modulation of secondary metabolism in sponge-associated *Streptomyces* can lead to the production of diketopiperazines with significant activity against multidrug-resistant pathogens. These findings highlight the potential of Indonesian marine actinobacteria as a valuable source of bioactive compounds for further pharmacological exploration.

In conclusion, this study demonstrated that the sponge-associated actinobacterium *S. tendae* Cal24h exhibits strong antibacterial activity against Multidrug-Resistant (MDR) pathogens, with inhibition zones up to 20 mm and MIC values ranging from 62.5 to 125 µg/mL. Optimization of culture conditions revealed that A1 medium enhanced metabolite production, supporting the biosynthesis of peptide-based secondary metabolites. Chemical profiling by GC-MS and LC-MS confirmed the presence of diketopiperazine derivatives, which are likely responsible

for the observed antibacterial effects. These findings provide new insight into the chemical diversity and antimicrobial potential of Indonesia's marine actinobacteria, particularly those associated with sponges in the Seribu Islands. Further studies focusing on the purification, structural characterization, and mechanism of action of these compounds are needed to explore their potential as natural antibacterial agents.

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