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Metabolite Profiling and Anti-Multidrug-Resistant Activity of *Streptomyces anulatus* ACSAN21-05 Isolated from Indonesian Mangrove Rhizosphere

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Abstract

Streptomyces isolated from the rhizosphere of *Rhizopora apiculata* in Baros Mangrove Forest, Yogyakarta, Indonesia, has the potential to produce bioactive compounds with distinct structures and activities. This study aimed to screen *Streptomyces*-strains for bioactive compound production, identify the selected *Streptomyces* using a polyphasic approach, evaluate the antibacterial activity and minimum inhibitory concentration (MIC) of the bioactive compounds produced by the selected strain against multidrug-resistant (MDR) pathogens, and determine the bioactive compound profile. Based on a perpendicular streak method test, *Streptomyces* sp. ACSAN21-05 was selected as a strain producing bioactive compounds with a broad-spectrum antibacterial effect, inhibiting MDR *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa* with inhibition zones of 44, 43, 41 and 42 mm, respectively. Phenotypic and genotypic characterization using the 16S rDNA gene identified strain ACSAN21-05 as *S. anulatus*. The ethyl acetate extract of strain ACSAN21-05 exhibited MIC values of 12.5 and 25 µg/mL against MDR *E. coli* and MDR *S. aureus*, respectively, and 50 µg/mL against both MDR *P. aeruginosa* and MDR *B. subtilis*. Gas Chromatography-Mass Spectrometry (GC-MS) analysis identified 68 volatile bioactive compounds. Among them, 6 compounds, including eugenol, eucalyptol (1,8-cineole), diacetamide, n-tridecanoic acid, n-hexadecanoic acid, and pyrazoline, had antibacterial potential against MDR *E. coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. The *Streptomyces anulatus* ACSAN21-05 strain shows promise for development as a producer of novel antibiotics with potential health benefits.

Keywords: Broad-spectrum, *S. anulatus* ACSAN21-05, Polyphasic identification, MDR, Perpendicular streak, Volatile bioactive compounds, 16S rDNA

Introduction

The term multidrug-resistant (MDR) bacteria refer to bacterial strains resistant to at least 3 different classes of antibiotics [1]. The increasing resistance of pathogenic bacteria to various antibiotics has become a critical issue in the healthcare sector. The misuse and overuse of antibiotics, along with missed doses, are

primary factors contributing to antibiotic resistance [2]. This resistance enables sublethal bacteria to synthesize enzymes that degrade antibiotics, modify protein components targeted by antibiotics, and alter cell membrane permeability, thereby creating a barrier that prevents antibiotics from reaching their target cells.

The rising prevalence of MDR bacteria in infection cases has been reported in numerous countries. Researchers have identified MDR bacteria in samples collected from patients with urinary tract infections in hospitals [3,4]. Additionally, MDR bacteria have been detected in patients with solid organ transplant infections and wound infections [5-8].

The exploration of natural bioactive compounds is crucial in addressing the challenges posed by MDR pathogenic bacteria. *Streptomyces*, one of the largest genera in the phylum *Actinobacteria*, has significant potential as a producer of natural bioactive compounds [9]. This genus consists of Gram-positive filamentous bacteria characterized by a high G + C nitrogenous base content. The bioactive compounds produced by *Streptomyces* exhibit antibacterial activity distinct from conventional antibiotics, making them promising candidates for the development of new antibacterial agents to combat MDR pathogenic bacteria. Furthermore, *Streptomyces* is responsible for producing the highest number of antibacterial bioactive compounds, with nearly 60% of the antibacterial compounds sourced from microbes are obtained from *Streptomyces* [9,10].

The diversity of *Streptomyces* bioactive compounds is particularly intriguing due to their unique chemical structures and broad applications in medicine. Moreover, these bioactive compounds generally have fewer side effects, promoting extensive research into the identification of promising active molecules from *Streptomyces* in various environments. For instance, *Streptomyces* sp. Al-Dhabi-90, isolated from a marine environment, has been reported to produce 3-methylpyridazine, an antimicrobial agent effective against MDR bacteria [11]. Similarly, Maiti *et al.* [12] successfully isolated *Streptomyces* sp. from soil, which produces picolinamycin, a compound capable of inhibiting multiple MDR pathogenic bacteria. Furthermore, *Streptomyces* sp. PA5.6, isolated from forest soil, was also found to produce bioactive compounds such as benzebenzeneacetic acid, 4-hydroxy, and benzeneacetamide, all of which can inhibit the growth of various MDR bacterial strains [13]. *S. malachitospinus,* isolated from *Hopea ferrea* endophytes, has also demonstrated inhibitory effects against *S. mutans* [14].

Beyond marine and soil environments and plant endophytes, the rhizosphere of mangrove plants is another unique habitat for discovering bioactive compound-producing *Streptomyces*. In the mangrove ecosystem, *Streptomyces* can adapt to extreme conditions such as high salinity and low oxygen levels by producing bioactive compounds with potential applications in drug development [15]. The rhizosphere of the Baros Mangrove Forest in Yogyakarta, Indonesia, presents a particularly distinctive microenvironment for exploring various *Streptomyces* species capable of producing novel bioactive compounds [16]. Furthermore, the unique physicochemical conditions of the rhizosphere offer opportunities to discover *Streptomyces* species capable of producing novel bioactive compounds to effectively inhibit the growth of MDR bacteria [17,18]. Notably, *Rhizopora apiculata* exhibits the highest species diversity within the Baros Mangrove Forest, which spans 1.46 ha [16]. To date, no studies have been conducted on Streptomyces from the rhizosphere of *R. apiculata* in mangrove environments as potential producers of bioactive compounds that inhibit MDR bacteria. Therefore, this study aims to screen *Streptomyces* species from the rhizosphere of *R. apiculata* in the Baros Mangrove Forest, Yogyakarta, for their potential as new antibacterial agents against MDR pathogens. Additionally, the specific bioactive compounds produced by selected *Streptomyces* strains will be identified.

Materials and methods

Sample collection

Samples of the rhizosphere of R. apiculata were collected from the Baros Mangrove Forest in Yogyakarta, Indonesia (08°00’28.6 S”S110°16’ 59.4”E). The samples were placed in sterile plastic bags, securely sealed, and stored at 4 °C for further analysis.

Isolation of *Streptomyces* from the rhizosphere of *R. apiculata* in the Baros Mangrove Forest, Yogyakarta, Indonesia

For isolation, 5 g of rhizosphere samples were heated in an oven at 70 °C for 30 min. The samples were then serially diluted using 0.85% physiological saline to achieve a final concentration of 10^{-5}. A 100 µL aliquot of the diluted sample suspension was inoculated into starch casein nitrate agar (SCNA) medium using the pour plate method, with 100 µL of nystatin (100 µL/100 mL) added as an antifungal agent. Following an incubation period of 7 - 14 days at 30 °C, the resulting bacterial colonies were purified using the streak plate method on malt extract agar [19].

Screening of *Streptomyces* isolates for bioactive compound production

Bioactive compound production by *Streptomyces* isolates was assessed using the perpendicular streak method. Seven- day- old *Streptomyces* cultures were streaked centrally onto Mueller–Hinton agar plates and incubated at 30 °C for 5 days. The MDR Pathogenic test bacteria (*E. coli*, *P. aeruginosa*, *S. aureus* and *B. subtilis*) were then streaked perpendicularly to the *Streptomyces* growth and incubated at 30 °C for an additional 2 days. Antibacterial activity was indicated by clear zones of inhibition surrounding the test strains [20]. Isolates exhibiting the broadest spectrum and largest inhibition zones were selected for further polyphasic identification.

Polyphasic identification of the selected *Streptomyces* isolates

Selected *Streptomyces* isolates were identified through a polyphasic approach integrating phenotypic and genotypic analyses. Phenotypic characterization included assessments of cultural, morphological, biochemical, and physiological traits. Cultural properties, such as aerial and substrate mycelium coloration, growth type, and diffusible pigment production were examined on 6 media types after 7 days of incubation at 30 °C. The 6 media types used were Nutrient Agar (NA), Starch Casein Agar, Starch Casein Nitrate Agar (SCNA), Tryptone Yeast Extract (TYE), Malt Extract Agar (MEA), and Inorganic Salt Agar (ISA). Morphological traits, including colony morphology, hyphal branching, and spore characteristics (chain formation, shape, and surface ornamentation), were examined using scanning electron microscopy (SEM; JSM-6510 LA) [21-23].

Biochemical profiling involved assays for catalase activity, starch hydrolysis, milk coagulation, and peptonisation. The ability to utilize various carbon (e.g., glucose, fructose, lactose and sucrose) and nitrogen sources (e.g., L-arginine, yeast extract, peptone and ammonium salts) was also tested. Physiological responses were evaluated across a range of pH (4 -13), temperatures (16, 30 and 37 °C), and NaCl concentrations (0 - 9 % w/v) [23].

Genotypic identification was conducted via 16S rDNA gene sequencing. Genomic DNA was extracted using the Zymo-Research Quick-DNA Fungal/Bacterial Miniprep Kit. PCR amplification was performed with universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) using a Bio-Rad T100 Thermal Cycler [24]. The 25 µL PCR reaction mix contained 1 µL DNA template, 12.5 µL 2× MyTaq Red Mix, 1 µL of each primer (0.5 µM), and 9.5 µL ddH_2O. Thermal cycling conditions were: initial denaturation at 96 °C for 1 min; 30 cycles of denaturation at 96 °C for 45 s, annealing at 55.6 °C for 1 min, and extension at 72 °C for 2 min; followed by a final extension at 72 °C for 7 min. Amplified products were verified via 0.8 % agarose gel electrophoresis with SYBR Safe staining, purified with the Zymoclean™ Gel DNA Recovery Kit, and sequenced bidirectionally. Sequences were edited using GeneStudio and aligned using ClustalW. BLASTn analysis was performed by comparing the 16S rDNA gene sequence from strain ACSAN21-05 with the NCBI database (https://blast.ncbi.nlm.nih.gov/). Phylogenetic trees were constructed using the Maximum Likelihood method in MEGA11 with 1,000 bootstrap replicates.

Production and extraction of bioactive compounds

Streptomyces sp. ACSAN21-05 was initially cultured on MEA at 30 °C for 7 days, and 1 g of the resulting cell pellet was inoculated into 100 mL starch casein broth. The culture was incubated for 10 days at 30 °C with continuous agitation at 120 rpm in a water bath shaker. Following incubation, the culture was centrifuged at 4,000 rpm for 35 min (Gemmy PLC

Series 3), and the supernatant containing secreted bioactive compounds was collected. Extraction was performed with ethyl acetate at a 1:1 (v/v) ratio, followed by overnight incubation at 30 °C with shaking at 120 rpm. The organic phase was separated and concentrated using a rotary evaporator at 45 °C until a paste-like residue was obtained. The crude extract was further dried at 55 °C and stored for subsequent bioactivity assays [25].

Antimicrobial activity testing and Minimum Inhibitory Concentration (MIC) of bioactive compound extracts

The antimicrobial activity of crude extracts from *Streptomyces* sp. ACSAN21-05 was assessed using the well diffusion method [11]. The MDR strains of *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. subtilis* were inoculated into nutrient agar (NA) plates using the pour plate technique. Bacterial suspensions were standardized to 0.5 McFarland turbidity (~10^8 CFU/mL). Wells were created using a sterile cork borer, and 50 µg/mL of the crude extract was added to each well. Plates were incubated at 37 °C for 24 h, and inhibition zones were measured to evaluate antibacterial activity.

Minimum inhibitory concentration (MIC) values were determined using the broth microdilution method in 96-well microplates [11]. The crude extract was dissolved in 1.5% dimethyl sulfoxide (DMSO) to prepare 4 concentrations: 6.25, 12.5, 25 and 50 µg/mL. Test strains were cultured in nutrient broth to 0.5 McFarland density and combined with the extract in equal volumes (100 µL each) per well. Plates were incubated at 37 °C for 24 h. Bacterial growth was quantified by measuring optical density at 600 nm using a spectrophotometer. Azithromycin (50 µg/mL) served as a positive control.

Profiles of bioactive compounds of *Streptomyces* sp. ACSAN21-05

The chemical composition of the volatile bioactive compounds produced by *Streptomyces* sp. ACSAN21-05 was analyzed using gas chromatography- mass spectrometry (GC-MS; GCMS-QP2010S, Shimadzu). A 2 µL sample of the ethyl acetate extract was injected into an EC-5 capillary column. The oven temperature was programmed to increase from 60 to 305 °C, with the injector set at 300 °C. Helium was used as the carrier gas, maintaining a constant flow rate of 2 mL/min. The mass spectrometry scan began at 5.20 min and ended at 80 min. Compound identification was carried out by comparing the obtained mass spectra to entries in available reference MS databases [26].

Data analysis

All experiments were performed in triplicate, and results are reported as mean ± standard deviation. Statistical significance of differences in inhibition zone diameters and MIC values was evaluated using 1-way analysis of variance (ANOVA), followed by Duncan's multiple range test. Analyses were conducted using SPSS software (version 25.0), with significance defined at $p < 0.05$.

Results and discussion

Streptomyces isolates producing bioactive compounds

A total of 15 *Streptomyces* isolates were obtained from the rhizosphere of *R. apiculata* in the Baros Mangrove Forest, Yogyakarta, Indonesia (**Table 1**). Five of these isolates demonstrated the ability to produce bioactive compounds, as evidenced by the formation of clear inhibition zones in the screening test. The mean diameters of the inhibition zones varied among isolates. Notably, *Streptomyces* ACSAN21-05 exhibited the most significant bioactive compound production, effectively inhibiting both Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus* and *B. subtilis*) pathogenic bacteria. The inhibition zones for these strains exceeded 40 mm in diameter, suggesting that the bioactive compounds produced by *Streptomyces* ACSAN21-05 possess broad-spectrum antimicrobial activity against both Gram-negative and Gram-positive pathogens.

Table 1 *Streptomyces* isolates producing bioactive compounds using the perpendicular streak method.

No.	*Streptomyces* isolate	Diameter of inhibition zone (mm)				Inhibition spectrum
		MDR Gram-negative bacteria		MDR Gram-positive bacteria		
		EC	PA	SA	BS	
1.	ACSAN1-02	-	-	-	-	-
2.	ACSAN1-03	-	-	-	-	-
3.	ACSAN1-11	42	48	33	40	Broad spectrum
4.	ACSAN2-01	-	-	-	-	-
5.	ACSAN2-03	-	-	-	-	-
6.	ACSAN2-05	-	-	-	14,5	Narrow spectrum
7.	ACSAN3-02	-	-	-	-	-
8.	ACSAN3-04	-	-	-	-	-
9.	ACSAN21-05	41	42	44	43	Broad spectrum
10.	ACSAN3-08	-	-	-	-	-
11.	ACSAN5-02	-	-	-	-	-
12.	ACSAN5-05	-	-	-	-	-
13.	ACSAN28-01	31,5	9	26	15	Broad spectrum
14.	ACSAN29-01	15,5	-	15	-	Broad spectrum
15.	ACSAN31-01	-	-	-	-	-

Note: EC: *E. coli*, PA: *P. aeruginosa*, SA: *S. aureus*, BS: *B. subtilis*

Polyphasic identification of selected *Streptomyces* isolates

The identification of the selected *Streptomyces* sp. ACSAN21-05 isolate was performed through a polyphasic approach, integrating both phenotypic and genotypic analyses. Phenotypic characterization, based on cultural traits, is summarized in **Table 2** and **Figure 1**. The strain demonstrated vigorous growth across 5 distinct media, with the formation of both aerial and substrate mycelia in varying colours, including gray, white-gray, and light gray. Notably, the isolate produced a distinctive golden-yellow pigment when cultured on TYE and MEA, highlighting its potential for secondary metabolite production.

Table 2 Cultural characteristics of *Streptomyces* sp. ACSAN21-05 on 5 different media.

Agar medium	Growth	Aerial mycelium	Substrate mycelium	Diffusion pigmen
NA	Good	Light gray	Cream	-
SCNA	Good	Gray	Brownish yellow	-
TYE	Good	Gray	White yellow	Golden yellow
MEA	Good	Gray	Brown	Golden yellow
ISA	Good	White gray	Cream yellow	-

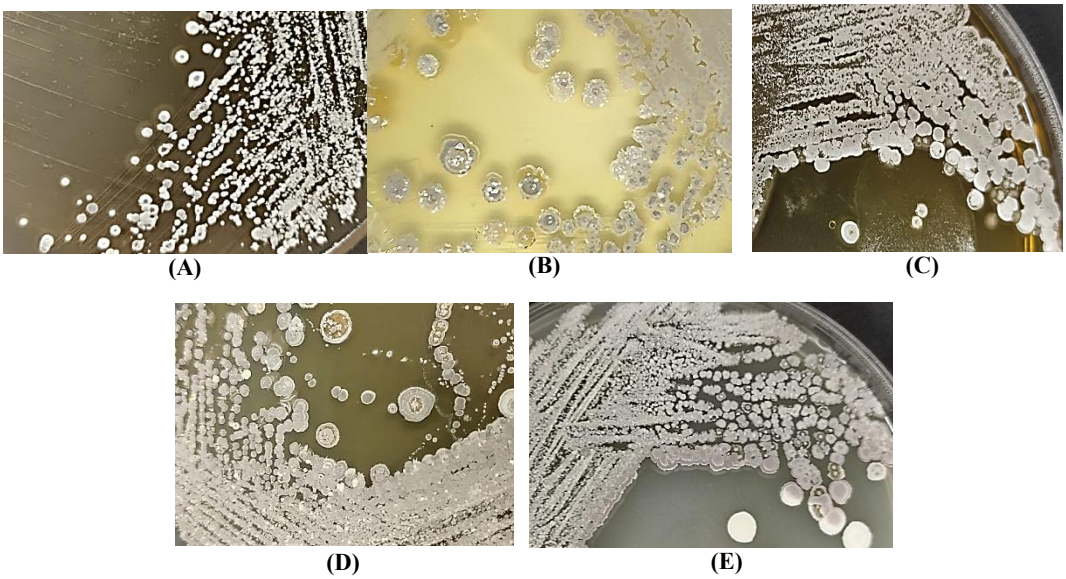
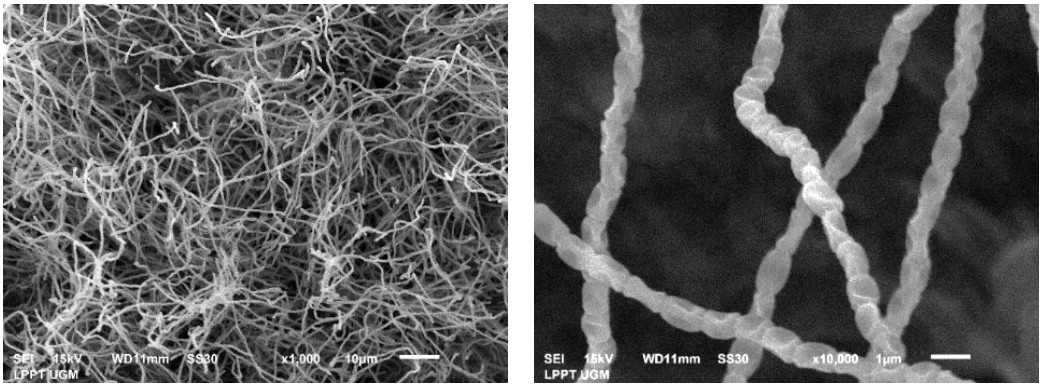


Figure 1 Growth of *Streptomyces* sp. ACSAN21-05 on various media (A) NA; (B) SCNA; (C) TYE; (D) MEA; and (E) ISA.

The morphological features of *Streptomyces* sp. ACSAN21-05 are summarized in **Table 3**. Colonies of this strain were round, measuring 6 mm in diameter, with flat edges, convex elevation, and powdery surfaces due to the production of spore-like structures. Gram staining revealed that the cells were Gram-positive. The spore chain type was classified as polysporous, with each chain containing over 50 spores, a typical characteristic of the *Streptomyces* genus. The spores exhibited irregular, rugose surface ornamentation, indicating the presence of surface wrinkles, and were rod-shaped with a distinct central notch. Scanning electron microscopy (SEM) further confirmed the presence of recti flexible aerial hyphae, displaying straight or flexible spore chains, some of which were arranged in fascicles (**Figure 2**).

Table 3 Morphological characteristics of *Streptomyces* ACSAN21-05.

Morphological characters	Observations	Results
Colony	Shape	Circular
	Diameter size	5 mm
	Margin	Entire
	Elevation	Convex
	Surface	Powdery
Cell	Gram reaction	Positive
	Shape	Rod
Spore	Spore chain	Polysporus
	Surface ornament	Irregular rugose
Aerial hyphae	Branching type	Rectiflexibiles



(A) **(B)**

Figure 2 Morphology of the aerial mycelium of *Streptomyces* sp. ACSAN21-05. (A) 1,000× magnification; (B) 10,000× magnification.

The phenotypic characterization of *Streptomyces* sp. ACSAN21-05, based on its biochemical and physiological properties, is detailed in **Table 4**. Biochemical analysis revealed a positive catalase reaction, starch hydrolysis, milk coagulation (positive coagulase), and the conversion of peptone into casein (positive peptonization). The strain demonstrated the ability to utilize various carbon sources, including dextrose, fructose, galactose, glucose, maltose, mannitol, xylose, lactose, sorbitol, and sucrose. It also grew on several nitrogen sources, such as yeast extract, peptone, L-arginine, L-tyrosine, KNO_3, $(NH_4)_2SO_4$, and (NH_4) H_2PO_4. Physiologically, *Streptomyces* sp. ACSAN21-05 exhibited growth across a pH range of 4 - 8, temperatures from 30 to 37 °C, and NaCl concentrations of 0 - 9%. These phenotypic traits were consistent with the profile described in *Bergey's Manual of Systematic Bacteriology*, confirming its identification as *Streptomyces anulatus* ACSAN21-05 [27].

BLASTn analysis of the 16S rDNA gene sequence from *Streptomyces sp*. ACSAN21-05, compared with the NCBI database, revealed a 98.78% identity match with *S. anulatus* (**Table 5**). Phylogenetic analysis further supported this finding, classifying *Streptomyces* sp. ACSAN21-05 as *S. anulatus* (**Figure 3**). Thus, the polyphasic identification approach confirmed the consistency between phenotypic and genotypic data, solidifying the identification of *Streptomyces* sp. ACSAN21-05 as *S. anulatus*.

Antimicrobial activity of the crude extract of bioactive compounds from *Streptomyces anulatus* ACSAN21-05

The crude extract of bioactive compounds from *Streptomyces anulatus* ACSAN21-05 demonstrated significant antimicrobial activity ($p < 0.05$) against 4 multidrug-resistant (MDR) pathogenic bacteria, as assessed by the inhibition zone formation (**Table 6**). The extract exhibited the strongest inhibitory effect against MDR *E. coli*, followed by MDR *S. aureus*, MDR *P. aeruginosa*, and MDR *B. subtilis*, with inhibition zone diameters of 17, 16, 15 and 14 mm, respectively. These results surpass those reported by Djebbah *et al.* [28], where *S. anulatus* ACSAN21-05 produced bioactive compounds with larger inhibition zones and stronger antimicrobial activity against the tested MDR pathogens.

Minimum Inhibitory Concentration (MIC) of bioactive compound extract of *Streptomyces anulatus* ACSAN21-05

The Minimum Inhibitory Concentration (MIC) results for the crude bioactive compound extract from *Streptomyces sp.* ACSAN21-05, compared to streptomycin as a standard antibiotic, are shown in **Table 7**. MDR *E. coli* exhibited the lowest MIC of 12.5 µg/mL, followed by MDR *S. aureus* at 25 µg/mL. The MIC values for both MDR *P. aeruginosa* and MDR *B. subtilis* were 50 µg/mL. Notably, the MIC values of the bioactive compounds from *Streptomyces sp.* ACSAN21-05 were lower than those of streptomycin

against several pathogens. These findings suggest that the bioactive compounds produced by *Streptomyces sp. ACSAN21-05* hold potential as novel antimicrobial agents.

Table 4 Biochemical and physiological characteristics of *Streptomyces* sp. ACSAN21-05.

Biochemical characteristics Biokimiawi		**Physiological characteristics**	
Characters	**Results**	**Characters**	**Results**
Catalase	+	pH tolerance	
Starch hydrolysis	+	4	+++
Coagulase	+	5	+++
Milk peptonization	+	6	+++
Carbon sources		7	+++
Dextrose	+++	8	+++
Fructose	+++	Temperature tolerance (°C)	
Galactose	+++	16	-
Glucose	+++	30	+++
Lactose	++	37	+++
Maltose	+++	NaCl tolerance (%)	
Mannitol	+++	0	+++
Sorbitol	++	1	+++
Sucrose	+	2	+++
Xylose	+++	3	+++
Nitrogen sources		4	+++
L-arginine	++	5	+++
L-tyrosine	++	6	+++
Yeast extract	+++	7	++
Peptone	+++	8	++
KNO_3	++	9	+
$(NH_4)_2SO_4$	++		
$(NH_4)\ H_2PO_4$	++		

Note: (+) indicates growth/positive reaction; (++) indicates moderate growth; (+++) indicates optimal growth; (-) indicates no growth/negative reaction.

Table 5 BLASTn results of the 16S rDNA gene sequence of *Streptomyces* sp. ACSAN21-05.

Strain	**Species homolog**	**Identity**	**Accession number**
ACSAN21-05	*Streptomyces anulatus* strain NRBC 12853	98.78%	AB269712.1
	Unculture bacterium clone C26 13	91.06%	KC229742.1
	Unculture bacterium clone C21 59	90.59%	KC229375.1
	Unculture bacterium clone C8 63	90.59%	KC228418.1
	Unculture bacterium clone C18 40	90.42%	KC229199.1
	Unculture bacterium clone C14 19	90.42%	KC228864.1
	Unculture bacterium clone C27 78	90.26%	KC229877.1
	Unculture bacterium clone C25 17	90.26%	KC229668.1

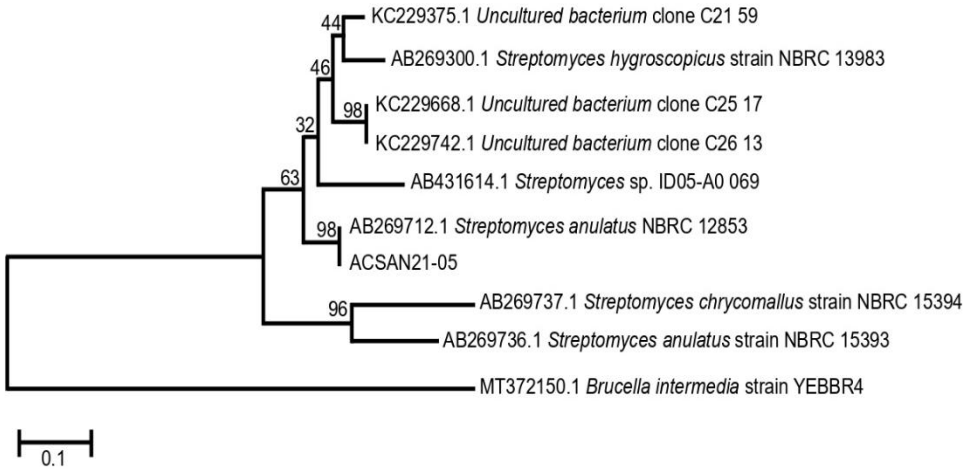


Figure 3 Phylogenetic tree of *Streptomyces* sp. ACSAN21-05 based on the 16S rDNA gene sequence, constructed using the Neighbor-Joining method with 1,000 bootstrap replicates

Table 6 Antibacterial activity of the crude extract of bioactive compounds from *Streptomyces anulatus* ACSAN21-05 against 4 MDR bacterial pathogens.

MDR Bacterial Pathogen	Zone of inhibition (mm)
E. coli	17 ± 0.05
P. aeruginosa	15 ± 0.02
S. aureus	16 ± 0.04
B. subtilis	14 ± 0.02

Table 7 Minimum inhibitory concentration (MIC) of crude ethyl acetate extract of bioactive compounds from *Streptomyces anulatus* ACSAN21-05.

MDR Bacterial Pathogen	MIC (µg/mL)	
	Crude extract	Streptomycin
E. coli	12.5 ± 0.01	25 ± 0.01
P. aeruginosa	50 ± 0.05	50 ± 0.02
S. aureus	25 ± 0.03	50 ± 0.01
B. subtilis	50 ± 0.02	50 ± 0.03

Metabolite profile of the broth extract from *Streptomyces anulatus* ACSAN21-05

GC-MS analysis of the crude extract from ethyl acetate extraction revealed 68 peaks, representing compounds in the extract at varying concentrations (**Figure 4**). The numbers on the peaks correspond to their respective retention times in minutes. Mass spectrometry (MS) analysis identified the structures and characteristics of the compounds eluted at different retention times. Among the 68 volatile compounds detected, 22 exhibited antibacterial activity, as summarized in **Table 8**.

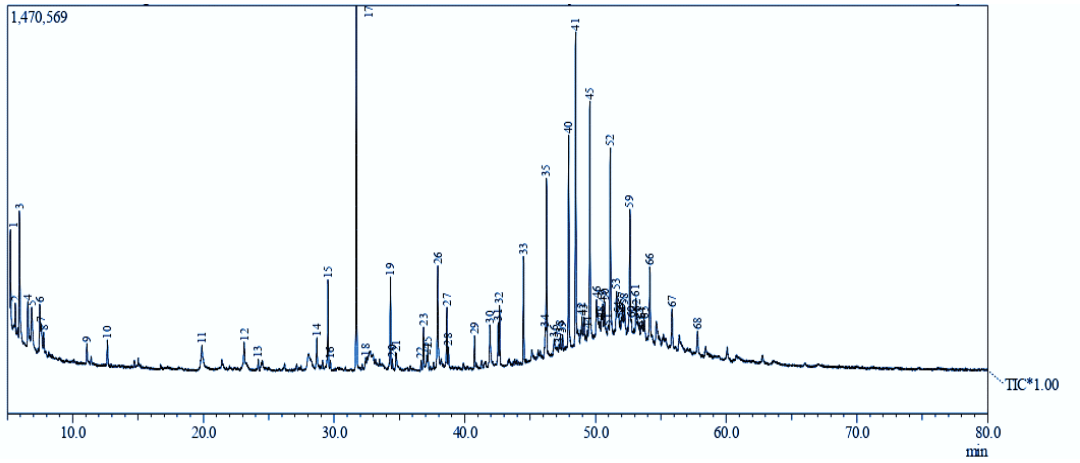


Figure 4 GC-MS chromatogram of the ethyl acetate extract from *Streptomyces anulatus* ACSAN21-05.

Table 8 Metabolite profile of the of the ethyl acetate extract from *Streptomyces anulatus* ACSAN21-05.

No	Compound name	RT	Area (%)	Chemical Formula	Molecular weight	Biological activity
1	1,3,5-Cycloheptatriene	5.24	1.23	C_7H_8	92	Antibacterial activity [29]
2	Cyclooctane	5.614	0.39	C_8H_{16}	112	Antibacterial activity [30]
3	Diacetamide	5.93	3.94	$C_4H_7NO_2$	101	Antibacterial activity [31,32]
4	Eucalyptol (1,8-Cineole)	12.661	0.72	$C_{10}H_{18}O$	154	Antibacterial activity [33]
5	Eugenol	23.121	0.71	$C_{10}H_{12}O_2$	164	Antibacterial activity [34-36]
6	1-Hexadecene	24.201	0.25	$C_{16}H_{32}$	224	Antibacterial activity [37]
7	n-Tridecanoic acid	28.692	0.95	$C_{13}H_{26}O_2$	214	Antibacterial activity [32]
8	1-Tetradecene	29.541	2.26	$C_{14}H_{28}$	196	Antibacterial activity [38]
9	2-Pyrazoline	31.71	9.2	$C_{16}H_{16}N_2$	236	Antibacterial activity [39,40]
10	n-Hexadecanoic acid	37.936	3.3	$C_{16}H_{32}O_2$	256	Antibacterial activity [41-43]
11	Tetradecanoic acid	41.929	1.24	$C_{14}H_{28}O_2$	228	Antibacterial activity [44]
12	4-Tetradecanol	42.566	0.9	$C_{14}H_{30}O$	214	Antibacterial activity [45]
13	Heptadecane	42.669	1.35	$C_{17}H_{36}$	240	Antibacterial activity [46]
14	Tetracosane	46.259	4.28	$C_{24}H_{50}$	338	Antibacterial activity [47]
15	Nonadecane	47.952	5.45	$C_{19}H_{40}$	268	Antibacterial activity [48]
16	Octadecane	49.573	6.63	$C_{18}H_{38}$	254	Antibacterial activity [48]
17	Tetradecane, 5-methyl-	50.35	0.72	$C_{15}H_{32}$	212	Antibacterial activity [48]
18	n-1-Eicosanol	50.958	0.28	$C_{20}H_{42}O$	298	Antibacterial activity [49]
19	Pentatriacontane	51.135	5.22	$C_{35}H_{72}$	492	Antibacterial activity [50]
20	Heneicosane	51.618	1.82	$C_{21}H_{44}$	296	Antibacterial activity [51]
21	Dotriacontane	52.078	0.71	$C_{32}H_{66}$	450	Antibacterial activity [47]
22	Pyrrolidine	53.158	0.2	$C_8H_{15}NO$	141	Antibacterial activity [52]

Among the 22 identified compounds, 6-diacetamide, eucalyptol (1,8-cineole), eugenol, n-tridecanoic acid, 2-pyrazoline, and n-hexadecanoic acid (as shown in **Figure 5**) - were individually tested and all demonstrated antibacterial activity against various pathogenic strains [31-36,39-43]. Nithya *et al.* [31] reported that diacetamide, an amide compound, inhibited the growth of several pathogens, including *E. coli*, *S. aureus*, *P. vulgaris*, *P. aeruginosa*, *K. pneumoniae*, and *E. faecalis*. Diacetamide exerts its antibacterial effects by inhibiting enzymes involved in proton pumps, leading to the accumulation of hydrogen ions and a decrease in intracellular pH. Additionally, diacetamide binds to enzymes through steric

interactions with amino acid residues at their active sites, causing competitive or non-competitive inhibition and reducing catalytic efficiency. It also interacts with P2 receptors in *E. coli*, forming hydrogen bonds with the carbonyl oxygen in the hydrophobic protein of the bacterial cell membrane.

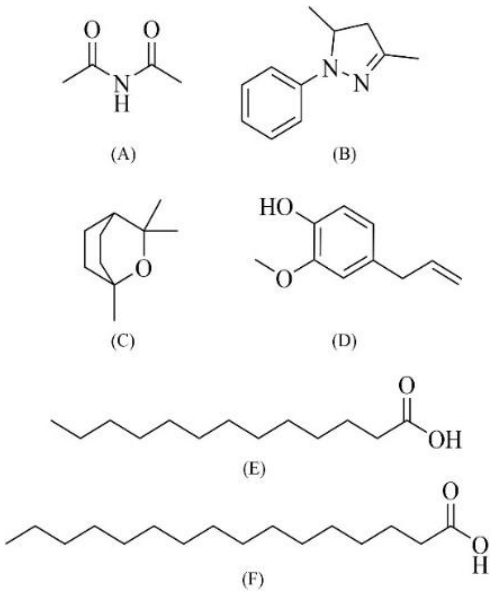


Figure 5 Volatile compounds with antibacterial activity found in the extract of *Streptomyces* sp. ACSAN21-05. (A) diacetamide; (B) 2-pyrazoline; (C) eucalyptol; (D) eugenol; (E) n-tridecanoic acid; (F) n-hexadecanoic acid

Eucalyptol (1,8-cineole), a terpenoid compound produced by *Streptomyces sp.* ACSAN21-05, has been shown to inhibit quorum sensing (QS) and virulence gene expression in *E. coli* O101. Wang *et al.* [33] reported that eucalyptol reduced luxS gene expression by 65 %, suggesting its potential to disrupt biofilm formation and pathogenicity in *E. coli* O101.

This study also identified eugenol, a phenolic compound produced by *Streptomyces sp.* ACSAN21-05, which has been shown to inhibit *MRSA* [34]. Jayapal *et al.* [35] reported that eugenol inhibited the growth of *Klebsiella pneumoniae*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and MRSA, with MIC values of 4.17, 33.32, 16.6, 0.96 and 66.64 mg/mL, respectively. Furthermore, Kong *et al.* [36] found that eugenol increased membrane permeability, causing significant damage in MDR *P. aeruginosa* and *K. pneumoniae*. Eugenol exerts its antibacterial effects by inducing protein denaturation, interacting with phospholipids in bacterial membranes, and disrupting ion and ATP transport, thus altering the bacterial fatty acid profile. Additionally, it damages the cell membrane of *Legionella pneumophila*, leading to cytoplasmic leakage and bacterial cell death [35].

Other bioactive compounds in the crude extract of *Streptomyces sp.* ACSAN21-05 include n-tridecanoic acid and n-hexadecenoic acid, both fatty acids. N-tridecanoic acid inhibits growth and biofilm formation in *E. coli* [32], while n-hexadecenoic acid shows inhibitory effects against *E. coli*, *K. pneumoniae*, and *P. aeruginosa* [41-43]. Pyrazoline, an azole compound produced by *Streptomyces sp.* ACSAN21-05, contains a 5-membered heterocyclic ring with 2 nitrogen atoms. It has been shown to inhibit the growth of *E. coli* and several MDR pathogens, including *S. aureus* (MRSA), vancomycin-resistant *E. faecalis* (VRE), carbapenem-resistant *K. pneumoniae* (CRKP), and extended-spectrum beta-lactamase-producing *E. coli* (ESBL-E. coli) [39,40]. This discovery, related to natural synthesis in *Streptomyces* cells, holds promise for future antibiotic development.

These findings suggest that the bioactive compounds produced by *Streptomyces sp.* ACSAN21-05 offer significant potential for developing new antibiotics to combat the growing issue of bacterial resistance to existing treatments.

Conclusions

This study highlights the significant potential of *Streptomyces* from mangrove ecosystems as a source of bioactive compounds with antimicrobial properties. We successfully isolated fifteen *Streptomyces* strains from the rhizosphere of *Rhizophora apiculata* in the Baros Mangrove Forest, Yogyakarta, Indonesia, and evaluated their antimicrobial activities. Among these, *Streptomyces* sp. ACSAN21-05 emerged as the most promising isolate, exhibiting strong and broad-spectrum inhibitory effects against several clinically relevant MDR pathogens, including *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa*. These potent activities underscore its potential as a valuable source of novel therapeutic agents. In addition, comprehensive polyphasic taxonomic analysis identified the isolate as *Streptomyces anulatus* ACSAN21-05, providing a robust taxonomic framework for further investigation. Chemical profiling of the ethyl acetate extract via GC-MS revealed 68 distinct compounds, including 6\ with previously reported antimicrobial activity. This supports the observed bioactivity and suggests that both known and potentially novel compounds may contribute to the antimicrobial effects.

Overall, *Streptomyces anulatus* ACSAN21-05 represents a promising microbial resource for the discovery and development of new antimicrobial agents, particularly in the context of escalating antibiotic resistance. Furthermore, this study reinforces the ecological and pharmaceutical importance of mangrove-associated microbes, a largely untapped reservoir of chemically diverse secondary metabolites. Future research should prioritize the purification and structural characterization of individual bioactive compounds, elucidation of their mechanisms of action, and *in vivo* efficacy studies. Additionally, genome mining and metabolic engineering approaches may enhance production yields and facilitate the identification of novel biosynthetic gene clusters, thereby accelerating the discovery of next-generation antibiotics from this compelling strain.

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Declaration of Generative AI in Scientific Writing

The authors did not use generative AI tools in the preparation of this manuscript, including not using AI in any content generation or data interpretation. The authors are solely responsible for the content and conclusions of this work.

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