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Original Article

Exploring the Diversity and Antimicrobial Potential of Actinomycetes Isolated From Forest Soil against *Pseudomonas aeruginosa*

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Abstract

Introduction: The actinobacteria are well-known for having the ability to produce bioactive secondary metabolites that effectively target bacteria, fungi, cancer cells, and the overactive immune system. Isolation and identification of novel bioactive compounds that selectively target multidrug-resistant *Pseudomonas aeruginosa* were made possible from actinobacteria inhabiting soil ecosystems.

Methods: The present investigation aims to isolate and characterize potent bioactive compounds from actinobacteria against MDR *P. aeruginosa*. The actinobacteria strains were isolated from forest soil samples using starch casein agar and their bioactive potential was determined against MDR *P. aeruginosa* strains by agar well diffusion method. The potent bioactive compounds produced by actinobacteria isolates against *P. aeruginosa* were identified using phenotypic characteristics and 16S rRNA gene sequencing. The ethyl acetate and n-butanol were used for the extraction of bioactive compounds. The column chromatography was used for the purification of fractions. The potent fraction having bioactivity was elucidated structurally using UPLC-UV-MS.

Results: The *Streptomyces tendae* and *Streptomyces albidoflavus* isolated from forest soil have the ability to produce bioactive compounds against MDR *P. aeruginosa*. *S. tendae* SI05 produced cyclic dipeptide L,L-Cyclo(leucylprolyl), whereas *S. albidoflavus* SI07 produced phthalate ester, diisobutyl phthalate, and non-proteinogenic peptide Norvaline.

Conclusion: The bioactive compounds obtained from *S. tendae* SI05 and *S. albidoflavus* SI07 were effective against MDR *P. aeruginosa* strains. Therefore, they can be used to overcome resistance mechanisms and target virulence factors of *P. aeruginosa* strains.

Keywords: Soil actinobacteria, MDR *Pseudomonas aeruginosa*, Bioactive metabolites

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Introduction

The Actinobacteria are a diverse group of gram-positive bacteria with high G+C content. The organisms from these groups play a significant role in the decomposition of soil organic matter, nutrient cycling, creation of healthy soil structure, and composition and bioremediation of recalcitrant compounds. In addition to these roles, Actinobacteria produce a variety of enzymes and enzyme inhibitors having industrial and pharmaceutical applications. Moreover, certain Actinobacteria also act as biocontrol agents suppressing plant pathogens and enhancing plant growth (1). Actinobacteria have also been reported as a rich source of antibiotics, including aureomycin, streptomycin, tetracycline, erythromycin, gentamycin, kanamycin, tobramycin, vancomycin, and rifamycin (2). The important actinobacterial genera having antibiotic-producing ability include *Saccharopolyspora*, *Amycolatopsis*, *Actinoplanes*, and *Streptomyces* (3). The species of the genus *Streptomyces* are important in the production of antifungal, antiviral, antitumor, anti-hypersensitive, immunosuppressive, and antibiotic compounds (4-8). The *Streptomyces* are responsible for the production of approximately 50% of therapeutically valuable antibiotics. The species of genus *Streptomyces* have a remarkable ability to produce bioactive secondary metabolites. This ability mainly lies in their large genomes that encode many biosynthetic gene clusters and their adaptability to diverse environmental conditions (9). The *Streptomyces*, natural inhabitants of soil, are also the most prolific species with the ability to produce a wide range of bioactive compounds, including nikkomycin-Z, 1H-indole-3-carboxylic acid, and phenolic esters (10). These *Streptomyces* species might have the potential to produce bioactive compounds with the ability to inhibit the growth of MDR *P. aeruginosa*. The compounds obtained from these organisms can act as potent bioactive

compounds. Therefore, *Streptomyces* species were isolated and used for the production of bioactive compounds against MDR *P. aeruginosa* in the present study.

Materials and Methods

Isolation of Actinobacteria

Samples were collected in sterile screw cap tubes from several locations of Painganga forest, Yavatmal (MH). The soil suspension was prepared using 1 g of fine soil suspended in 10 mL of distilled water and serially diluted up to 10^{-5}. A 100 µL suspension from 10^{-3} to 10^{-5} dilutions was spread on starch casein agar plates and incubated for 7 days at 30 °C. The phenotypic features of actinobacteria isolates were characterized using starch casein agar medium.

Molecular Identification of Actinobacteria

The 16S rRNA sequencing of the isolated Actinobacteria was performed using universal forward primer 27F (5' AGAGTTTGATCMTGGCTCAG 3') and reverse primer 1492R (5' GGTTACCTTGTTA CGACTT 3'). The DNA was extracted using the NucleoSpin Microbial DNA kit. To verify purity, 1% agarose gel electrophoresis was used. The agarose gel was visualized using an ultraviolet (UV) transilluminator (Himedia). Using forward and reverse sequencing data, the BioEdit program produced a consensus sequence of the 16S rRNA gene. The NCBI GenBank database was used to perform BLAST analysis using the 16S rRNA gene sequence. The sequences were selected based on the maximum identity score and alignments were made with Clustal W, a multiple alignment software. Additionally, the distance matrix was created and the phylogenetic tree was constructed (11).

Production of Bioactive Compounds from S. tendae SI05 and S. albidoflavus SI07

The Actinobacteria isolates, *S. tendae* SI05 and *S. albidoflavus* SI07, were enriched with 50 mL of starch casein broth at room temperature for 3-4 days, respectively. After enrichment, the isolates were inoculated into 500 mL of starch casein broth. Then, the cultures were incubated in a rotatory shaker at 120 rpm for 7 days at 28 °C. After incubation, fermented broths were centrifuged for 15 minutes at 10000 rpm. After centrifugation, the supernatant was collected and the pellets were discarded. These supernatants were used for primary screening against 5 isolated strains of MDR *P. aeruginosa* (PA2, PA3, PA5, PA6, and PA7). The minimum inhibitory concentrations (MIC) of the P. aeruginosa strains used in this investigation have been determined.(Figure S1, Table S1)

The Mueller-Hinton agar plates were prepared and labeled, and active cultures of MDR *P. aeruginosa* strains were gently spread separately on MHA plates. The 6 mm diameter wells were formed using a sterile cork-borer. Afterwards, 100 µL of the supernatant of *Streptomyces* isolates obtained from forest soil added into respective labeled wells, as positive control, standard antibiotic streptomycin (100 µg/mL), and DMSO as negative control were added and the plates were kept at 4 °C for 10 minutes for diffusion. After diffusion, plates were kept for incubation at 37 °C for 48 hours (12).

Extraction and Screening of Potent Bioactive Compounds

The supernatants of *S. tendae* SI05 *and S. albidoflavus* SI07 were applied for extraction using the liquid-liquid extraction method in organic solvents, namely ethyl acetate and n-butanol. To do so, 250 mL of supernatant and ethyl acetate in a proportion of 1:1 was taken in a separating funnel and gentle mixing was done for 30 minutes. After proper shaking, the mixtures were allowed to settle, the aqueous layer was decanted, and the organic layer was collected in an evaporating dish and allowed to evaporate. After evaporation, crude extracts of bioactive secondary metabolites were collected and dissolved in DMSO. The same procedure was used with n-butanol for the extraction of bioactive secondary metabolites (13).

Secondary screening of crude extract obtained from *S. tendae* SI05 *and S. albidoflavus* SI07 using ethyl acetate and n-butanol was carried out. The activity was performed on Mueller-Hinton agar plates against 5 MDR *P. aeruginosa* strains. The plates were allowed to diffuse at 4 °C for 10 minutes. After diffusion, plates were incubated at 37 °C for 48 hours.

Characterization of Potent Bioactive Compounds

The characterization of fraction F6 from *S. tendae* SI05 and fractions F5 and F6 from *S. albidoflavus* SI07, which showed the maximum activity against MDR *P. aeruginosa*, was performed by Ultra Performance Liquid Chromatography-Ultra Violet-Mass Spectrometry (UPLC-UV-MS). The characterization was carried out at Venture Centre, NCL, Pune (MS), India.

Results

Phenotypic Characteristics

The actinomycetes isolates produced filamentous, dry, and gray colonies with aerial mycelium and other isolates produced dry powdery white colonies with hard consistency, both of which were Gram-positive filamentous bacteria.

Molecular Identification of S. tendae SI05 and S. albidoflavus SI07

The molecular identification of the isolates was achieved by amplifying the DNA using the 16S rRNA gene primer, which appeared as distinct bands at around 700 bp. The obtained sequences were compared with other bacterial DNA sequences and analyzed using the NCBI online database (Figure 1).

In accordance with the sequencing data, the isolated strains belonged to the genus *Streptomyces*, the family *Streptomycetaceae,* and the phylum Actinobacteria. The isolates showed similarity with *S. tendae* strain MCCB 207 and *S. albidoflavus* strain CSEA02 based on the data analysis (Table 1). The sequences were uploaded to the

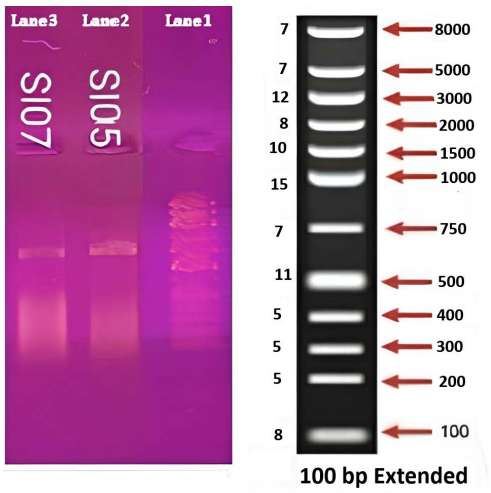

Figure 1. Lane 1: Molecular Marker DNA SiZerTM-100-1000; Lane 2: ~700 bp, Amplified Fragmented DNA of *S. tendae;* Lane 3: ~700 bp, Amplified Fragmented DNA of *S. albidoflavus* SI07

NCBI database and received GenBank accession numbers PV533967 and PV534072 for *S. tendae* SI05 and *S. albidoflavus* SI07, respectively.

The evolutionary relationships of *S. tendae* SI05 and *S. albidoflavus* SI07 strains and their other neighbors were shown in a neighbor-joining tree created using MEGA-11 software (Figures 2 and 3).

Antimicrobial Activity

The purified fraction F6 of *S. tendae* SI05 exhibited significant antimicrobial activity against 5 strains of *P. aeruginosa*, with zone of inhibition diameters of 16 mm, 18 mm, 16 mm, 12 mm, and 18 mm, respectively (Table 2), while purified fractions F5 and F6 of *S. albidoflavus* SI07 showed antimicrobial activity against 5 strains of *P. aeruginosa* with zone of inhibition diameters of 14 mm, 16 mm, 12 mm, 12 mm, and 16 mm for F5 and similarly fraction F6 of *S. albidoflavus* SI07 showed inhibition zone diameters of 18 mm, 16 mm, 17 mm, 15 mm, and 18 mm, respectively (Table 3).

Characterization of Potent Bioactive Compounds

The structural elucidation of potent bioactive fraction was performed using Ultra Performance Liquid Chromatography-Ultra Violet-Mass Spectrometry (UPLC-UV-MS). They were identified as L,L-Cyclo (leucylprolyl) from fraction F6 of *S. tendae* SI05 (Figure 4) and diisobutyl phthalate and norvaline from fractions F5 and F6 of *S. albidoflavus* SI07 (Figures 5 and 6).

Discussion

The two *Streptomyces* isolates were characterized using classical morphological methods and 16S rRNA analysis, followed by the evaluation of their antimicrobial potency against MDR *P. aeruginosa*. The *S. tendae* SI05 showed filamentous dry grey colonies with aerial mycelium, while *S. albidoflavus* formed dry powdery white colonies with hard consistency on starch casein agar. Microscopic observation confirmed that both isolates were Gram-positive and that they had features consistent with the *Streptomyces* genus (4). The 16S rRNA gene amplification through PCR yielded DNA fragments of 700 bp, and BLAST analysis results revealed that the identified isolates were *S. tendae* (MCCB 207) and *S. albidoflavus* (CSEA02). Similarly, *S. tendae* SI05 strain was also isolated from agro-waste soil using malt extract agar (ISP 2) (14). The assigned GenBank accession numbers were PV533967 and PV534072. Their phylogenetic trees were constructed using MEGA-11 software, and their taxonomic placements were confirmed within the *Streptomyces* clade (15).

The antimicrobial potential of *S. tendae* SI05 and *S. albidoflavus* SI07 strains was evident from agar well diffusion assays against multidrug-resistant *P. aeruginosa* strains (PA2, PA3, PA5, PA6, and PA7). The bioactive fraction F6 of *S. tendae* SI05 inhibited the growth of *P. aeruginosa* with zone of inhibition ranging from 12 to 18 mm. Similarly, fractions F5 and F6 from *S. albidoflavus* SI07 exhibited zone of inhibition between 14 and 18 mm (16). These observations support the prominent inhibition of MDR *P. aeruginosa* strains by *S. tendae* SI05 and *S. albidoflavus* SI07. The yield optimization during production of bioactive compounds is crucial for lowering production costs, making expensive drugs more affordable for patients, and increasing profitability for manufacturers; however, yield optimization study was not performed in the present investigation because the major objective of the study was to obtain potent bioactive compounds against MDR *P. aeruginosa*. Ultra-Performance Liquid Chromatography coupled with UV and Mass Spectrometry (UPLC-UV-MS) elucidated the structure of bioactive metabolites. The dominant compound obtained from fraction F6 of *S. tendae* SI05 was identified to be L,L-Cyclo(leucylprolyl), a diketopiperazine. The diketopiperazine isolated from marine fungus *Penicillium chrysogenum* DXY-1 possesses quorum-sensing inhibitory and broad-spectrum antimicrobial properties (17). The fractions F5 and F6 of *S. albidoflavus* SI07 were identified as Diisobutyl phthalate and Norvaline. Diisobutyl phthalate, a phthalate ester known for membrane-disrupting antibacterial mechanisms, has been identified in earlier studies involving soil and marine *Streptomyces* isolates (18). Norvaline, a non-proteinogenic amino acid, has recently drawn interest for its ability to interfere with microbial metabolic pathways and protein synthesis, thereby exhibiting selective antimicrobial effects (19). In vivo toxicity studies of L,L-Cyclo (leucylprolyl), Diisobutyl Phthalate, and Norvaline provide essential data on the safety and potential risks, including toxicity, efficacy, bioavailability, and long-term health effects. However, in vivo toxicity study of L,L-Cyclo (leucylprolyl), Diisobutyl Phthalate, and Norvaline was not performed in the current study.

Conclusion

The *S. tendae* SI05 and *S. albidoflavus* SI07 strains producing novel bioactive compounds against

Table 1. 16S rRNA Sequence Alignments of the Isolates SI05 and SI07 with Available NCBI Database Sequences (BLAST-N)

Sample ID	Description	Max score	Total score	Query cover	E-Value	Per. Identity	Acc. Len	Accession number
SI05	*S. tendae* strain MCCB 207	771	771	100%	0	96.95%	1434	KF454842.1
SI07	*S. albidoflavus* strain CSEA02	789	789	100%	0	97.01%	1446	PV147350.1

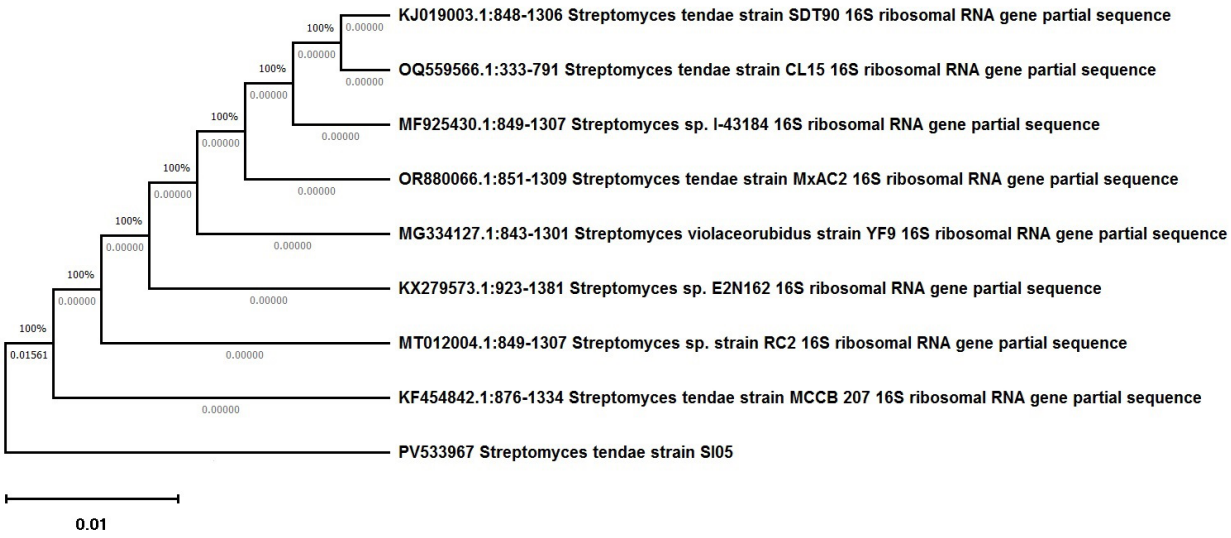

Figure 2. Phylogenetic Tree of Isolate *S. tendae* SI05

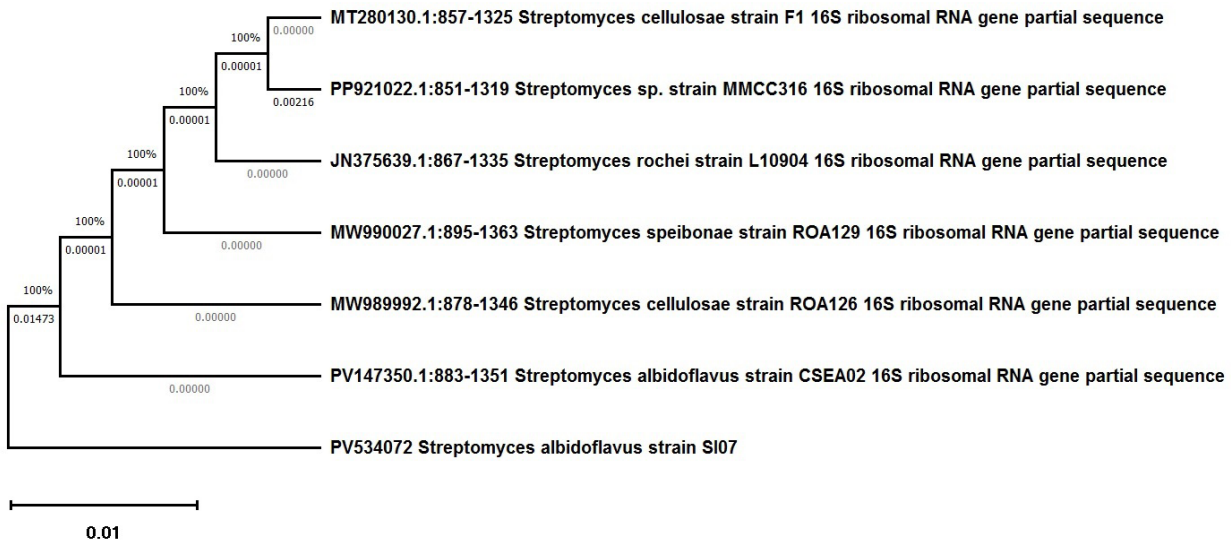

Figure 3. Phylogenetic Tree of Isolate *S. albidoflavus* SI07

multidrug-resistant *P. aeruginosa* strains are present in selected forest soil samples. The bioactive compounds were identified as L,L-Cyclo (leucylprolyl), Diisobutyl Phthalate, and Norvaline by Ultra-Performance Liquid Chromatography-Ultraviolet/Mass Spectrometry.

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Authors' Contribution

Conceptualization: Bhagvat Lad.
Data curation: Bhagvat Lad.
Formal analysis: Tukaram Kadam and Sanjay Chavan.
Funding acquisition: Bhagvat Lad and Sanjay Chavan.
Investigation: Bhagvat Lad.
Methodology: Bhagvat Lad.

Table 2. Antipseudomonal Activity of Column Chromatography Fractions of Ethyl Acetate Extract of *S. tendae* SI05 against MDR *P. aeruginosa*

Fraction of ethyl acetate extract from isolate *S. tendae* SI05	*P. aeruginosa* PA2	*P. aeruginosa* PA3	*P. aeruginosa* PA5	*P. aeruginosa* PA6	*P. aeruginosa* PA7
	Zone of inhibition (mm)				
F1	-	-	-	-	-
F2	-	-	-	-	-
F3	-	12	-	10	10
F4	14	14	-	12	-
F5	10	15	14	12	-
F6	16	18	16	12	18
Streptomycin	28	28	28	27	26

Table 3. Antipseudomonal Activity of Column Chromatography Fractions of Ethyl Acetate Extract of *S. albidoflavus* SI07 against MDR *P. aeruginosa*

Fraction of ethyl acetate extract from Isolate *S. albidoflavus* SI07	*P. aeruginosa* PA2	*P. aeruginosa* PA3	*P. aeruginosa* PA5	*P. aeruginosa* PA6	*P. aeruginosa* PA7
	Zone of inhibition (mm)				
F1	-	-	10	10	15
F2	-	-	-	-	-
F3	-	-	-	-	-
F4	16	12	-	-	12
F5	14	16	12	12	16
F6	18	17	16	15	18
Streptomycin	18	14	16	18	19

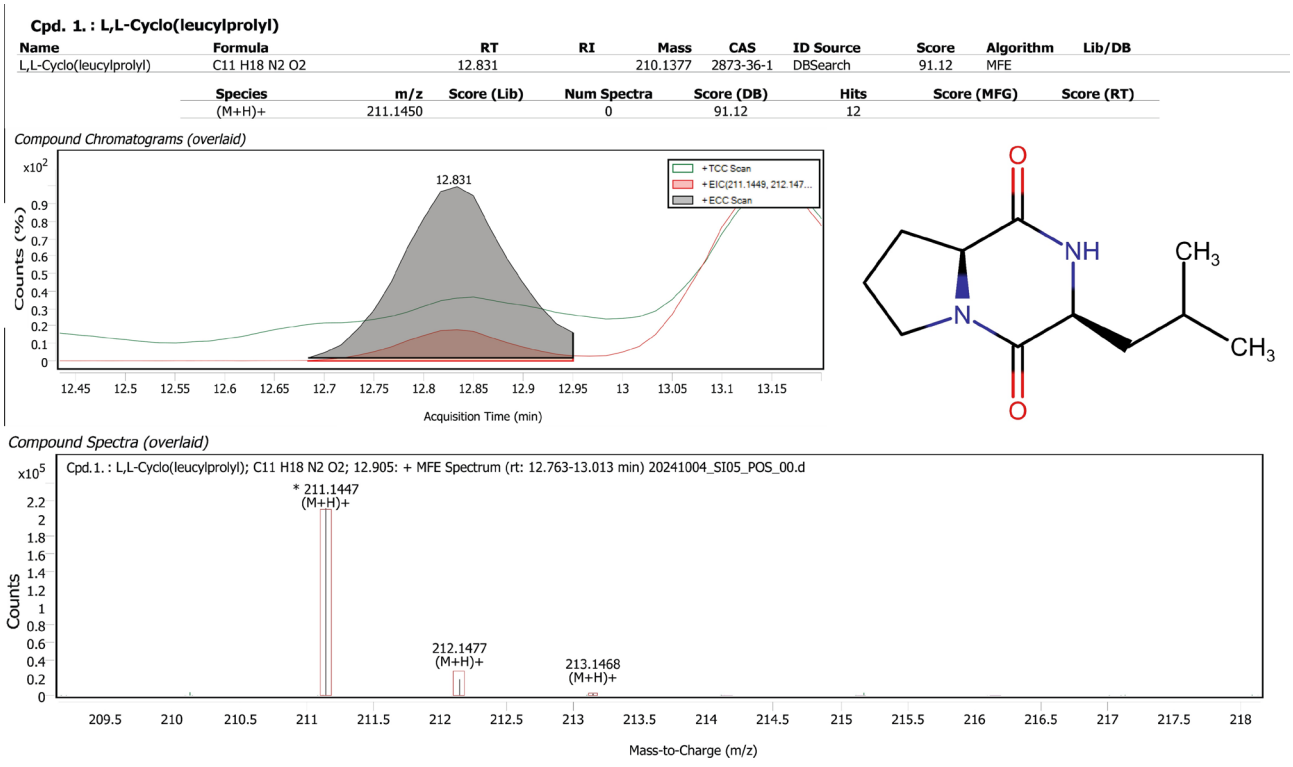

Figure 4. Structural Elucidation of Fraction F6 from Ethyl Acetate Extract of *Streptomyces Tendae* SI05 by UPLC-UV-MS

Cpd. 1. : Norvaline

Name	Formula	RT	RI	Mass	CAS	ID Source	Score	Algorithm	Lib/DB
Norvaline	C5 H11 N O2	2.784		117.0795	760-78-1	DBSearch	96.71	MFE	

Species	m/z	Score (Lib)	Num Spectra	Score (DB)	Hits	Score (MFG)	Score (RT)
(M+H)+	118.0867		0	96.71	23		

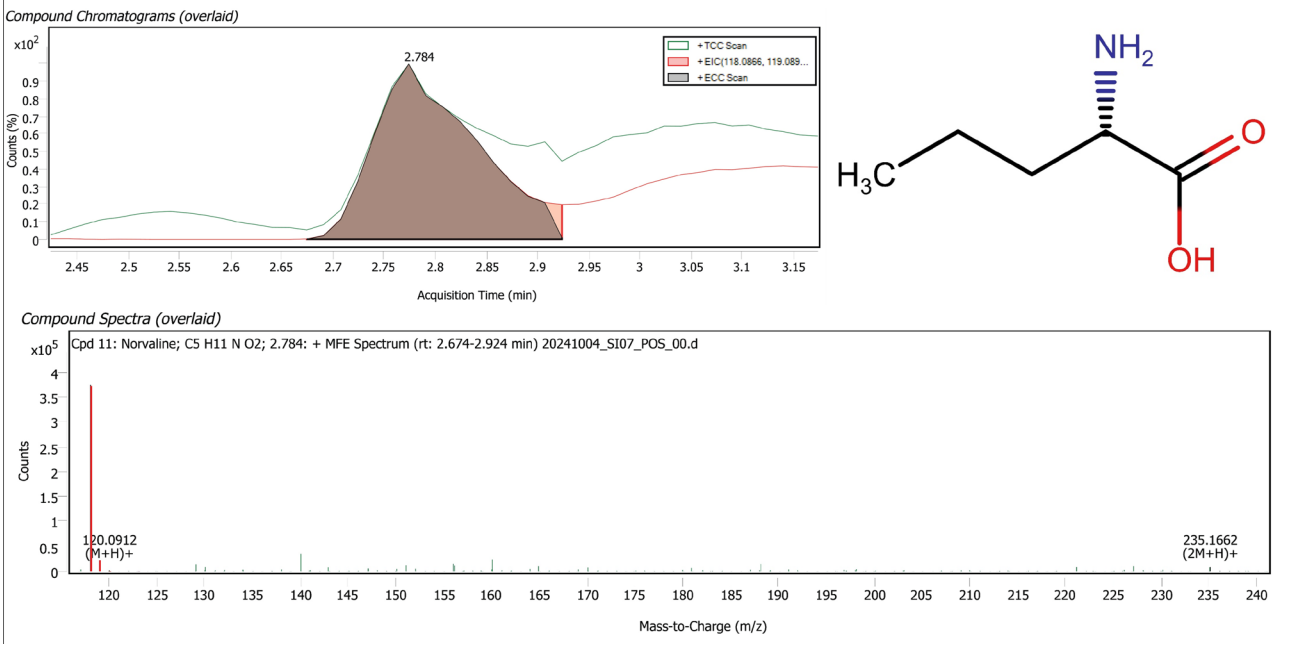

Figure 5. Structural Elucidation of Fraction F5 from Ethyl Acetate Extract of *Streptomyces Albidoflavus* SI07 by UPLC-UV-MS

Cpd. 2. : Diisobutyl phthalate

Name	Formula	RT	RI	Mass	CAS	ID Source	Score	Algorithm	Lib/DB
Diisobutyl phthalate	C16 H22 O4	26.533		278.1524	84-69-5	DBSearch	96.19	MFE	

Species	m/z	Score (Lib)	Num Spectra	Score (DB)	Hits	Score (MFG)	Score (RT)
(M+H)+	279.1597		0	96.19	4		

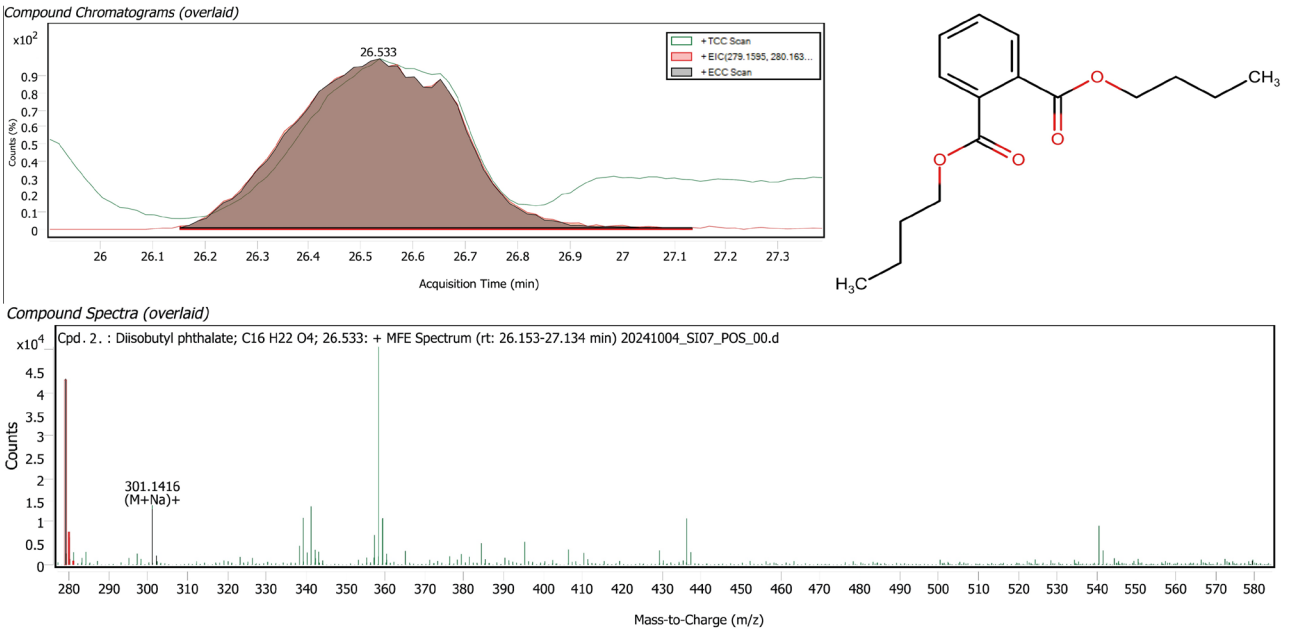

Figure 6. Structural Elucidation of Fraction F6 from Ethyl Acetate Extract of *Streptomyces Albidoflavus* SI07 by UPLC-UV-MS

Project administration: Tukaram Kadam.
Resources: Bhagvat Lad.
Software: Bhagvat Lad.
Supervision: Tukaram Kadam.
Validation: Tukaram Kadam.
Visualization: Bhagvat Lad and Sanjay Chavan.
Writing–original draft: Bhagvat Lad.
Writing–review & editing: Sanjay Chavan and Tukaram Kadam.

Competing Interests

The authors declare that they do not have any conflict of interests.

Ethical Approval

Ethical approval was not required for this study, as no human participants or personal data were involved.

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Supplementary File 1

Minimum inhibitory concentration data of *Ps. aeruginosa* strains involved in study are provided in the supplementary file. The supplementary file contains Table S1 and Figure S1.

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