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Antibiotic Potency of Extract from Streptomyces Isolated from Terrestrial Soil of Amirthi Forest, India

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Abstract

The aims of the present study were to isolate novel actinomycetes from the terrestrial soil and to evaluate their antibacterial potential. A total of 26 strains of actinomycetes were isolated and screened for antibacterial activity by the well diffusion method. Two of the isolates, VITAM-10 and VITAM-16, possessed a broad spectrum of antibacterial activity against the selected Gram positive and Gram negative ATCC bacterial strains. The isolate VITAM-16 produced a 22 mm zone of inhibition against *Proteus vulgaris* and VITAM-10 produced 17.6 mm zone of inhibition against *Bacillus cereus*. VITAM-16 exhibited potent antibacterial activity than VITAM-10. Hence, VITAM-16 was chosen for further studies. The isolate VITAM-16 was subjected to morphologic, cultural and biochemical characterizations and identified to be belonged to the genus *Streptomyces*. 16S rDNA nucleotide sequence of VITAM-16 showed 97 - 99 % sequences similarity with *Streptomyces* taxons. The ethyl acetate (EA) extract of VITAM-16 was analyzed by GC-MS, fumaric acid and propanoic acid were found to be the major compounds. The EA extract exhibited an MIC value from 0.06-0.90 mg/ml against tested bacterial pathogens. The results of the present study suggest that VITAM-16 can be explored further for identification of novel antibiotics.

Keywords: Terrestrial actinomycetes, Streptomyces, secondary metabolites, antibacterial activity

Introduction

Actinomycetes are a group of Gram positive filamentous bacteria possessing properties intermediate between fungi and bacteria. It has high G+C content and asexual spores. Most of the actinomycetes are non-motile and if it has motility, small flagellated spores are present. They are mostly considered as terrigenous bacteria because of their wide distribution and abundance in soil. It has a complex life cycle. It belongs to the phylum Actinobacteria. It consists of more than 30 families, 10 suborders and 160 genera.

Actinomycetes are an economically important and valuable source for new bioactive secondary metabolites. They are the most important and well accredited group of microorganisms present in both terrestrial and marine soil samples. Actinomycetes are extensively distributed in all ecosystems especially in soil, having the ability of produce numerous novel bioactive compounds and biomaterials by decomposing complex mixtures of polymers in the dead plant, animal and fungal materials [1]. The terrestrial ecosystems are enormously different from the marine environment, it is expected that terrestrial actinomycetes have different characteristics and therefore, might produce different types of bioactive compounds [2]. Terrestrial actinomycetes are a promising source for the exploration of new drugs against pathogenic micro-organisms [3]. Soil actinomycetes remain a tremendous resource for the isolation and identification of therapeutically important bioactive products which include antibiotics of high commercial value. The discovery of novel bioactive compounds from terrestrial actinomycetes isolated

from different types of soil samples has decreased and hence more studies are needed to isolate novel bioactive compounds from actinomycetes isolated from unexplored sites [3]. Soil microbes generally survive in crowded and competitive environmental conditions. The exploration of new soils and habitats for isolation of rare microorganisms capable of producing new bioactive compounds is continued [4,5].

Actinomycetes are diverse in nature, capable of producing secondary metabolites with unusual chemical structures and they have the capability of surviving in extreme environmental conditions [6-8]. Most of the antibiotics available in the market are obtained from *Streptomyces*. More than 30 compounds derived from *Streptomyces* are under clinical or preclinical trials for their anticancer activity [9]. It is estimated that approximately 2/3 of the naturally occurring antibiotics have been isolated from these organisms [10]. *Streptomyces*, soil-dwelling filamentous bacteria, are prolific producers of a wide range of antimicrobial agents [11]. They exhibit a unique metabolic diversity and enzymatic capabilities. The bioactive compounds extracted from actinomycete have been shown to possess antibacterial activities [12].

The development of multidrug resistant pathogens emphasizes the need for new antibiotics from unexplored ecosystems for potential applications [13]. The terrestrial ecosystem and actinomycetes diversity of Amirthi forest, Vellore District, Tamil Nadu, India are not been fully explored. Hence, this study was planned to explore the Amirthi forest soil samples for potential actinomycetes capable of producing antimicrobial secondary metabolites against selected bacterial pathogens.

Materials and methods

Sample collection and isolation of actinomycetes

Soil samples were collected from pollution free area of 3 different locations in Amirthi forest, Vellore district, Tamil Nadu, India at the depth of 10 - 15 cm. Samples were kept in a sterile polythene cover storing in an air tight sealed container at ambient temperature and transported to the laboratory. All samples were kept at 4 °C until further use [14]. Soil samples were air-dried in laminar air flow for 3 days to suppress other bacterial growth. For the isolation, cultivation and maintaining of actinomycetes, actinomycetes isolation agar (AIA), starch casein agar (SCA) and International Streptomyces Project (ISP-1) were used. Soil samples were serially diluted up to 10^{-6} fold using sterile distilled water. About 0.1 ml of the aliquot was taken and spread over the medium and incubated at 30 °C for 7 - 28 days. All the isolates were individually maintained at 4 °C for further use and the selected isolate was allowed to grow in an ISP-1 broth to get a clear supernatant for antimicrobial activity.

Fermentation

The well grown actinomycetes isolates were used for the seed culture preparation by inoculating in 50 ml of ISP broth and kept in an incubator shaker at 30 °C. The 10 % inoculum was transferred to a production medium kept in the 1L Erlenmeyer flask containing ISP 1 broth and the production medium was incubated at 30 °C for 9 days in a rotary shaker at 110 rpm. After fermentation, the cell-free supernatant was collected by filtration through Whatman No.1 filter paper [15].

Extraction of secondary metabolites

The cellfree supernatant of fermented biomass was subjected to solvent extraction using ethyl acetate (EA) to recover secondary metabolites. EA was added to the filtrate in a ratio of 1:1(v/v) and incubated for 2 days at 30 °C in a rotary shaker at 150 rpm [16]. The extract was then concentrated in a rotary vacuum evaporator at 55 °C and the residue EA extract was collected and stored at room temperature for further analysis.

Test organisms

The bacterial strains used in the study are procured from American Type Culture Collection (ATCC). The test organisms include Gram positive bacteria, *Staphylococcus aureus* (ATCC 13565) and *Bacillus cereus* (ATCC 10987); and Gram negative bacteria, *Salmonella paratyphi* A (ATCC 9150), *Enterobacteriaceae* (ATCC 29915), *Pseudomonas aeruginosa* (ATCC 27853), *Shigella flexneri* (ATCC

12022), *Shigella boydii* (ATCC 9207), *Proteus vulgaris* (ATCC 6380) and *Escherichia coli* (ATCC 25922). All test organisms were cultured in Muller Hinton agar.

Determination of antimicrobial activity

Antibacterial activity was screened by the Kirby-Bauer method [17]. The lawn culture (24 h) of the target organisms was spread evenly on the Muller Hinton agar using a sterile swab. Agar diffusion was followed to estimate the antimicrobial activity. The plates were incubated for 24 h at 30 °C. The inhibition zones formed were visually detected and measured (mm) after 24 h. All experiments were done in triplicate.

Determination of minimum inhibitory concentration (MIC)

The MIC of EA extract was tested against different test organisms by the broth dilution method using nutrient broth. A loop of bacterial colonies was inoculated in a tube containing 2 ml of nutrient broth and incubated at 25 °C until the growth reaches a turbidity greater than that of a 0.5 McFarland standard. The lowest concentration of the extract that revealed no visible bacterial growth (no turbidity) was recorded as the MIC and the average of the 3 values was calculated [18].

Taxonomic characterization of the isolate

The actinomycetes isolate VITAM-16 was subjected to morphological, physiological, biochemical, cultural and molecular characterizations. Microscopic characterization was carried out by the Gram staining technique using a high power light microscope. Spore chain morphology along with aerial and substrate mycelium structure, color and pigmentation of the selected isolates were examined and compared with Bergey's Manual to identify the isolate at the species level.

Physiological and biochemical characteristics

After preliminary studies, the growth of the selected actinomycetes isolates on different mediums was observed ISP1, ISP2, ISP3, ISP4, ISP5, ISP6, ISP7, SCA and AIA for optimization. The ability of the isolate to utilize the different carbon source and different amino acids such as arabinose, raffinose, cellobiose, mannitol, inositol, and salicin was examined using the standard protocol [19]. Culture characteristics such as the color of the aerial mycelium, the color of the substrate mycelium and pigmentation of the selected isolate were recorded on various ISP mediums. Various biochemical tests including indole test, methyl red test, Voges-Proskauer test, citrate utilization, triple sugar ion test, melanin production, oxidase and urease test were also performed according to the international Streptomyces project to identify the species. The effect of salinity on growth was carried out by culturing the isolate at different NaCl concentrations ranging from 1 - 5 %.

Molecular taxonomy

The mycelium spores were scrapped and suspended in 500 µl of TE buffer and were vortexed vigorously. Cells were subjected to digestion by lysozyme (stock 10 mg/ml). Samples were then incubated for 1 h at 37 °C, followed by protease K (20 mg/ml) treatment. Then, 33.5 µl of 10 % SDS were added and incubated at 55 °C for 1h. Then, 5 M NaCl was added to CTAB and the CTAB-NaCl mixture was incubated at room temperature for 10 min, followed by centrifugation at 12000 rpm for 10 min. The supernatant was extracted by adding chloroform: iso-amyl alcohol (24:1) and centrifuged for 15 min at 12000 rpm. The 3 volume of absolute ethanol and 0.1 volume of 3 M sodium acetate was added to the supernatant, and then centrifuged for 15 min at 12000 rpm. The pellet was washed with 70 % ethanol before centrifuging for 10 min at 12000 rpm and finally, the pellet was air dried and dissolved in milli-Q water. The PCR conditions were adapted from Farris and Olson [20]. The 16S rRNA Universal primers (27F AGAGTTTGATCMTGGCTCAG, 1492R TACGGYTACCTTGTTACGACTT) gene fragment was amplified using an MJ Research Peltier Thermal Cycler. The PCR amplification was detected by 1 % agarose gel electrophoresis and was visualized by ultraviolet fluorescence. The fragmented DNA strands were subjected to 16S rDNA sequencing using a DNA sequencer (Applied Biosystems) and the sequence obtained was deposited in the GenBank (NCBI, USA, Accession number KJ184303). The similarity and

homology of the sequences were compared with the existing nucleotide sequences available in the data bank using BLAST search. The sequences were aligned and a phylogenetic tree was constructed by neighbor joining method. The bootstrap value of 100 was used for the tree construction.

Gas chromatography and mass spectroscopy (GC-MS)

A gas-chromatography coupled with mass spectroscopy (GC-MS) was used for identifying the molecular weight of the compounds. The extract was analyzed by using GC Clarus 500 (Perkin Elmer, Singapore) equipped with an Elite 5MS column (30×0.25 mm × 0.25 µM) composed of 5 % diphenyl and 95 % dimethylpolysiloxane). An electron ionization system with an ionizing energy of 70 eV was used. For GC-MS the GC oven was held for 2 min at 110 °C and then ramped from 110 to 280 °C at 10 °C. Total run time was 36 min, InjA auto was 250 °C at 2 µl volume and split 10:1. Helium was used as the carrier gas with a flow of 30 ml/min and the mass conditions included solvent delay 2 min, transfer and source temperature 150 °C and scan from 50 to 600 Da. The identification of the compound was based on 90 % similarity between the MS spectra of the unknown compound and reference compounds available in the MS spectra library of NIST (National Institute of Standards and Technology, US).

Results and discussion

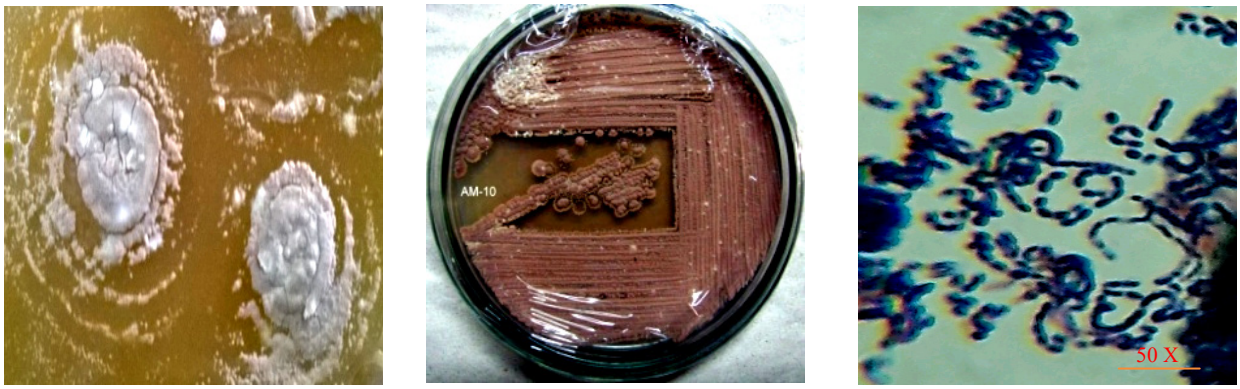
A total of 26 isolates were obtained from 3 samples collected from 3 different locations of Amirthi forest, Vellore district, Tamil Nadu, India using AIA and SCA medium. Based on the initial screening for antibacterial activity 2 isolates viz., (VITAM-10 and VITAM-16) were selected for further studies since they exhibited significant antibacterial activity. VITAM-16 produced different colors of aerial and substrate mycelium on the AIA and SCA medium. VITAM-16 showed a 20 mm zone of inhibition against Gram positive bacteria *Staphylococcus aureus* and 22 mm zone of inhibition against Gram negative bacteria *Proteus vulgaris.* The MIC of the ethyl acetate extract was found to be in the range of 0.064 to 0.9 mg/ml (**Table 1**) *Streptomyces* VITAM-16 showed the least MIC value of 0.06 mg/ml against *Staphylococcus aureus* and *Proteus vulgaris.* The antibacterial and antioxidant activity of actinomycetes isolated from Amirthi forest have already been reported [21,22]. As reported from several studies, actinomycetes are a major producer of secondary metabolites as well as many anti-bacterial compounds [23].

Table 1 Antibacterial activity of VITAM-10 and VITAM-16 against selected bacterial pathogens.

Bacterial pathogens	Zone of inhibition (mm) Cell free supernatant (100 µl)			
	VITAM-10	VITAM-16	Streptomycin (10 µg/disc)	MIC value (mg/ml)
Gram positive bacteria				
Staphylococcus aureus (ATCC 33591)	17 ± 0.81	20 ± 0.81	22 ± 0.23	0.064 ± 0.04
Bacillus cereus (ATCC 14579)	17.16 ± 0.62	19.6 ± 1.2	17 ± 0.5	0.066 ± 0.21
Gram negative bacteria				
Salmonella paratyphi A (ATCC 9150)	16.5 ± 0.5	18.1 ± 1.4	18 ± 0.87	0.3 ± 0.25
Enterobacteriaceae (ATCC 29915)	11.33 ± 0.47	17.33 ± 0.94	19 ± 0.45	0.15 ± 0.41
Pseudomonas aeruginosa (ATCC 27853)	17 ± 0.8	19.5 ± 1.08	16 ± 0.5	0.75 ± 0.43
Shigella flexneri (ATCC 12022)	13.17 ± 0.8	18.5 ± 1.2	20 ± 0.27	0.15 ± 0.32
Shigella boydii (ATCC 9207)	12.6 ± 0.9	17 ± 0.94	19 ± 0.5	0.3 ± 0.02
Proteus vulgaris (ATCC 6380)	11.6 ± 0.47	22 ± 1.63	15 ± 0.21	0.064 ± 0.08
Escherichia coli (ATCC 25922)	13 ± 0.8	17.3 ± 0.94	17 ± 0.32	0.075 ± 0.14

Values are mean ± S.D (n = 3)

The isolate VITAM-16 was medium to large sized (3 mm), powdery with irregular margins, pinkish white mycelium **Figure 1(a)** and produced pink color reverse side of colonies. The isolate initially produced smooth white colonies and later turned into a dark pink color on AIA medium **Figure 1(b)**. VITAM-16 is a gram positive coil shaped micro-organism possessing a spiral type of spore morphology, **Figure 1(c)**. The mycelial and cellular morphology of actinomycetes isolates was identified as reported earlier [24]. Other morphological and cultural characteristics of the different medium are given in **Table 2**. Culturing characteristics revealed that the isolate showed excellent growth on AIA agar, ISP-1 and nutrient agar. The aerial and substrate mycelium exhibited different colors on different media. The physiological and biochemical characteristics of the isolate are given in **Table 3**. Among the 10 different carbon sources, maximum growth was seen in the lactose contained medium. The potential isolate utilized NaCl (1 - 2 %), showed excellent growth and aerial mycelium formation at pH 7 and temperature of 28 °C. The characteristics of the isolate were compared with the reference strain *Streptomyces fraidaie* **Table 4**.



(a) (b) (c)

Figure 1 *Streptomyces* VITAM-16 isolate characters (a) Aerial mycelium on AIA media, (b) Single colony of *Streptomyces* sp. VITAM-16 on SCA media, (c) Morphology of the isolate under the light microscope (X 100).

Table 2 Cultural and morphological characteristics of the *Streptomyces* isolate VITAM-16.

Medium	Growth pattern	Aerial mycelium
ISP 1	Abundant	White pink
ISP 2	Poor	White
ISP 3	Poor	White
ISP4	Moderate	Light brown
ISP 5	Good	Grayish brown
ISP 6	Good	White
ISP 7	Moderate	Grayish brown
AIA	Abundant	Pinkish brown
SCA	Good	Light pink
Nutrient agar	Abundant	Grayish brown

Table 3 Biochemical characterization of the isolate VITAM-16.

Properties	**VITAM16**
Gram strain	+
Motility	-
Morphological characteristics	
Aerial mycelium	Pink white
Substrate mycelium	Red
Colony color	Brown
Production of pigments	-
Reverse side pigment	-
Spore chain	Spiral
Spore surface	Smooth
Biochemical characteristics	
Indole production	-
Methyl red	+
Citrate utilization	-
Nitrate reduction	+
Urease	+
Oxidase	+
Voges Proskauer	-
Melanin production	-
Triple sugar iron	-
Carbon source utilization	
Arabinose	++
Sucrose	+++
Mannitol	+
Dextrose	+
Cellobiose	+
Inositol	+++
Maltose	++
Glucose	+
Lactose	+++
Salicin	++
Rhamnose	+++
Physiological characteristics	
Optimum Temperature for growth	30 °C
Optimum pH	7
Growth in the presence of NaCl	
1 %	+++
2 %	-
3 %	-
4 %	-
5 %	-

+ Positive; - Negative; Good +++; Fair ++; + Poor.

Table 4 Characteristics of *Streptomyces* sp. VITAM-16 *Streptomyces fraidaie*.

Characteristics	**Isolate VITAM-16**	***Streptomyces fraidaie***
Gram strain	+	+
Mobility	-	-
Endospore staining	-	-
Spores	Smooth	Smooth
Spore chain	Spiral	Spiral
Colony color	Brown	Brown
Aerial mycelium	Pink white	White
Substrate mycelium	Red	Red
Production of pigments	-	Red
Reverse side pigment	-	Yellow red
Melanin production	-	+
Optimum Temperature for growth	30 °C	30 °C
NaCl %	1 %	3 %
Sucrose	-	-
Mannitol	-	-
Rhamnose	+	+

The PCR amplification of the genomic DNA with forward and reverse primers specific to 16S rDNA resulted in the formation of 1235 bp amplicon. The 16S rDNA nucleotide sequence of the isolate VITAM-16 was blasted through the GenBank database. A phylogenetic tree was constructed with the boot strap values of 100, **Figure 2**. The 16S rDNA sequence of the isolate showed 99 % similarity with *Streptomyces fradiae* (NR 043485.1), 98 % *Streptomyces coeruleoprunus*, (NR 041176.1) and 98 % *Streptomyces somaliensis strain* (NR 025292.1). The 16S rDNA nucleotide sequence (1235 nucleotides) of the *Streptomyces* sp. VITAM-16 has been deposited in the GenBank (NCBI, USA) under the accession number KJ184303. The neighbour-joining tree based on 16S rDNA gene sequences showed that the isolate VITAM-16 occupies a comparable phylogenetic position within the radiation including representatives of the *Streptomyces* family. The isolate VITAM-16 was identified by morphological, physiological, biochemical and cultural characterization. The molecular taxonomy and phylogeny revealed that the isolate belonged to the genus *Streptomyces* and is designated as *Streptomyces* sp. VITAM-16.

The EA extract was subjected to GC-MS. The chromatogram obtained was matched with reference compounds available in the MS spectra library, NIST (National Institute of Standards and Technology, USA) **Figure 3**. Fumaric acid and propanoic acid are the major compounds found in the EA extract. The other compounds identified through NIST library matching were P-(Methylthio)benzyl alcohol, 2-Acetyl-1,3-cyclohexanedione, 2,5-piperazinedione, 3-benzyl-6-isopropyl-,1,2-benzenedicarboxylic acid, mono(2-ethylhexyl)ester and 4'-Methyl-α-(2-oxopyrrolidino)hexanophene **Table 5**. Fumaric acid and propanoic acid are thought to be the major compounds responsible for antibacterial activity. Fumaric acid, is an organic acid and has been reported to possess antimicrobial activity against Gram positive and Gram negative pathogens and is used in the food industry [25-27]. European Union (EU) certifies that propanoic acid can be used as a preservative for both animal feed and food for human consumption. It was reported to be very effective in controlling *Salmonella* and other pathogens [28]. A large number of actinomycetes species with novel properties have been discovered but still, a huge amount of marine as well as terrestrial actinomycetes species remain untapped, which can be further exploited for the discovery of many potent metabolites with commercial, biomedical, environmental as well as industrial applications [29].

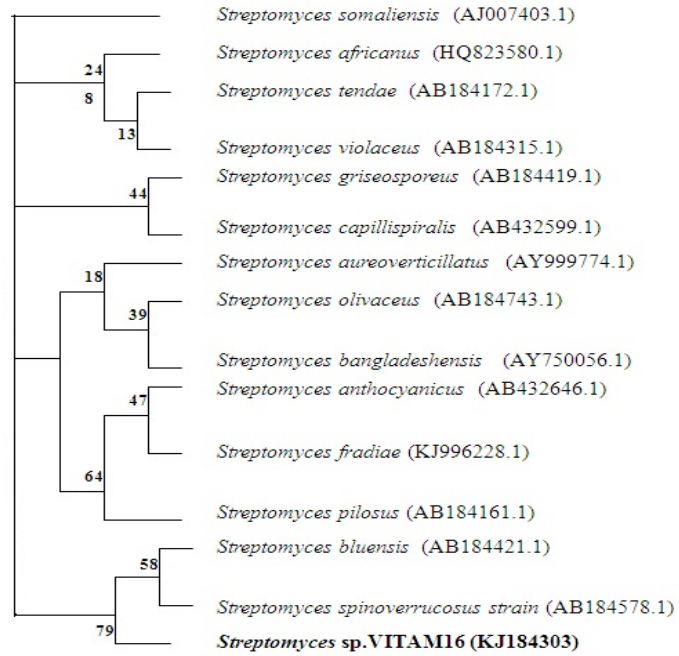


Figure 2 The phylogram showing the relationships between *Streptomyces* sp. VITAM-16 and other *Streptomyces* spp. based on 16S rRNA gene sequence.

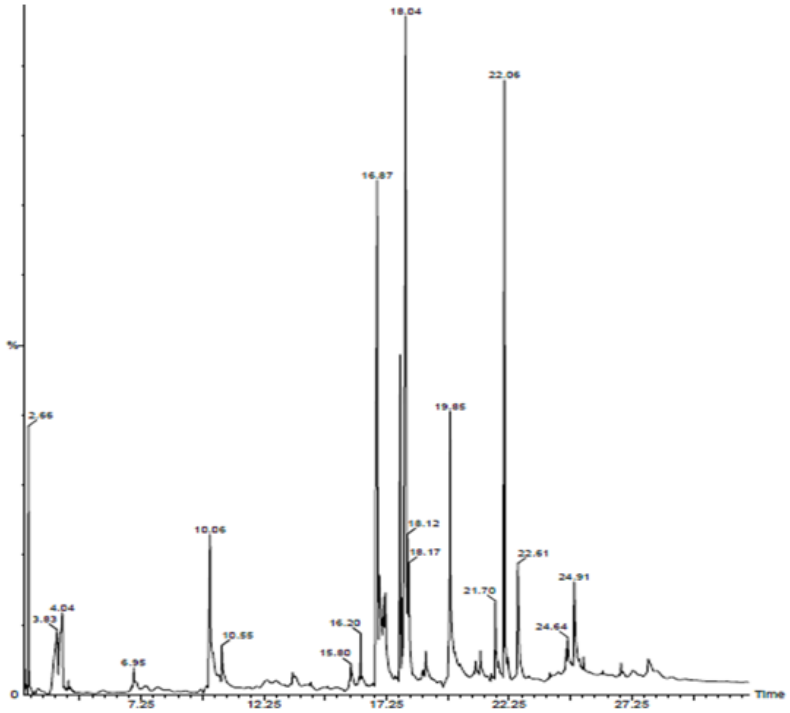
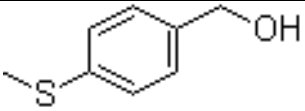
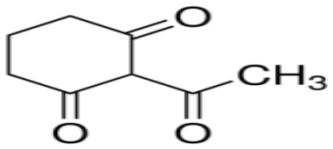
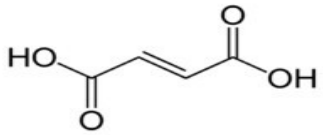
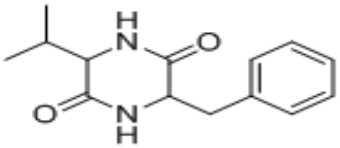
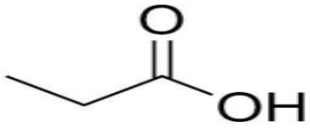
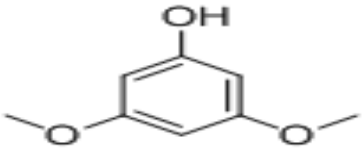
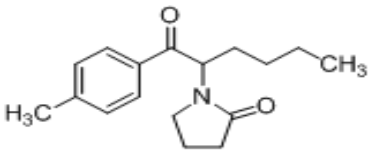


Figure 3 GC-MS spectra of the extract of *Streptomyces* sp. VITAM-16.

Table 5 Secondary metabolites of *Streptomyces* VITAM-16 identification using GC-MS.

Retention time	Compound name	Structure
10.06	P-(Methylthio)benzyl alcohol	
16.87	2-Acetyl-1,3-cyclohexanedione	
18.040	Fumaric acid	
21.70	2,5-piperazinedione, 3-benzyl-6-isopropyl-	
22.06	Propanoic acid	
22.61	1,2-benzenedicarboxylic acid, mono(2-ethylhexyl)ester	
24.91	4'-Methyl-α-(2-oxopyrrolidino)hexanophene	

Conclusions

The strain has shown significant antibacterial activity against human pathogens such as bacillus and staphylococcus species. It also produced 2 antibacterial compounds fumaric acid and propanoic acid, and these compounds can be developed as potential antibacterial agents after extensive *in vitro*, *in vivo* and toxicity studies. The other biological activities of these 2 compounds can also be studied to utilize these compounds for other applications.

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