

Extraction and Characterization of Antibiotic Compounds produced by *Streptomyces* spp. VITGV01 against Selected Human Pathogens

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Abstract

Streptomyces are well known to produce a large number of diverse antibiotic compounds. New environments are being explored and some new *Streptomyces* strains have been discovered; the potential of the plant endophytic environments has been highly explored during the last decade. In the present study, an endophytic actinomycete, VITGV01, was isolated from a farm tomato plant collected from an agricultural field. These actinomycetes are cultured in ISP2 medium supplemented with nystatin (15µg/ml) and nalidixic acid (50µg/ml). Based on the 16S rRNA analysis and physiological tests, this isolate was found to be a member of the known genus *Streptomyces* spp. and suggesting that it might be a new spp. The isolated strain showed morphological and chemical characteristics of the genus *Streptomyces*. The strain VITGV01 produced different antibiotics on different media such as yeast extract, malt extract, glucose medium, which were active against some Gram-positive and negative bacteria [*Bacillus subtilis* (MTCC2756), *Staphylococcus aureus* (MTCC737), *Escherichia coli* – (MTCC1687), and *Klebsiella pneumoniae* (MTCC109)]. The primary and secondary screening was performed against these human pathogens. Maximum zone of inhibition was observed against *K. pneumoniae* (22 mm) and minimal zone of inhibition against *B. subtilis* (8 mm). Maximum biomass production was recorded in media containing 1% sucrose and 1% yeast extract cultured at pH 7.0 for 7 days at 30°C. FTIR analysis revealed amine, alkane (C = C) of the aromatic ring, secondary alcohol, and alkyl halide groups. The GC-MS data showed twenty compounds, of which fifteen are antimicrobial compounds.

1. Introduction

Antibiotics are chemical compounds used to prevent and treat bacterial infections. Antibiotic resistance occurs when bacteria change in response to the use of these drugs. Today, drug-resistant bacteria are a serious threat to people's health. Currently, at least 7,00,000 people die each year due to drug-resistant diseases, including 2,30,000 people who die from multidrug-resistant tuberculosis (WHO 2019). The discovery of new antibiotics that reduce bacterial drug resistance is essential for modern medicine. The World Health Organization (WHO) claims that Antimicrobial Resistance (AMR) presents a significant challenge to public health and the ecosystem. (Allcock et al. 2017) .

Among the antibiotic-producing microorganisms, the class actinomycetes represent a broad range of valuable and prominent sources of pharmaceutically active metabolites (Subramani and Sipkema 2019). Over 40 genera of filamentous Actinobacteria have been detected as endophytes in plants. Among the studied endophytes, *Streptomyces* species, which are isolated from a diverse plant species, are one greater group of interest because of their ability to produce antibiotics as well as other bioactive secondary metabolites (Yan et al. 2010). About 23,000 bioactive secondary metabolites were discovered from endophytic microorganisms, approximately 10,000 different substances were produced by these endophytic actinomycetes and 7,600 metabolites were found in the *Streptomyces* genus (Hong-Thao et al. 2016). Actinomycetes produce approximately 75% of the commercially used antibiotics (Sanghvi et al. 2014). The actinomycetes are one of the most attractive sources of new bioactive metabolites. However, the rate of discovery of new compounds has decreased since the available species have been studied extensively (Badji et al. 2007). Recently, rare actinomycetes are an important source of novel and useful antibiotics (Takahashi and Nakashima 2018). Several studies have been oriented towards the isolation of new uncommon actinomycetes from unexplored environments. Although numerous actinomycetes have been isolated and used as a producer of key drugs and biomedical agents, it is increasingly difficult to find novel compounds to identify a new organism.

To achieve this objective, we have isolate actinomycetes from tomato plants and the following parameters were studied such as morphological, antimicrobial, biochemical, phylogenetic characters, and optimization of growth for *Streptomyces* spp. VITGV01. Antimicrobial properties (human pathogens) of the secondary metabolites production and characterized on FTIR and GCMS.

2. Materials And Methods

2.1 Tomato plant sample collection:

Healthy tomato plants (*Solanum lycopersicum*) were collected from five different locations (Tirunelveli – 8.7815° N, 77.3942° E; Tuticorin 9.1727° N, 77.8715° E; Madurai – 9.9420° N, 77.9724° E; Dindigul – 10.4489° N, 77.9360° E and Theni – 10.0015° N, 77.6164° E) from the southern part of Tamil Nadu in India during August and September 2017. Plants were collected from the field at the fruiting stage. Three tomato plants were randomly collected from the corners and center of the field. Thus, a total of fifteen plants were collected in sterile polythene bags and passed directly to the laboratory for microbiological processing

2.2 Surface sterilization

Plant samples were washed in running tap water to remove all adherents. The plant samples were cut to 4–5 cm in length using a sterile surgical cutter. The cut segments were washed with sterile distilled water. Then with 70% ethanol (5 minutes), 90% ethanol (2 minutes), 1 % sodium hypochlorite (15 minutes) and 10 % sodium hydrogen carbonate (10 minutes). Sterile distilled water wash was done for every change in solution (5 minutes). Stems were cut in a cross direction and streaked on the ISP2 medium supplemented with 50 mg l⁻¹ nalidixic acid and nystatin 50 mg l⁻¹, incubated at 28°C for 7–10 days (Qin et al. 2009). Thus, actinomycetes alone were allowed to grow and separated.

The strains were purified and maintained in glycerol suspensions (30 % v/v) at -80⁰ C. Isolates were labeled serially as VITGV. Different strains were identified based on the morphological characteristics of colonies on the plate, areal hyphae on an agar plate, the morphology of spores, and pigment production (Williams et al. 1983).

2.3 Screening for effective strain

The preliminary antimicrobial screening was done by spot inoculation method (Shomura et al. 1979) using four test microorganisms. Gram-positive (*B. subtilis* (MTCC2756), *S. aureus* (MTCC737), and two Gram-negative strains (*E. coli* – (MTCC1687) and *K. pneumoniae* (MTCC109)). The diameter of inhibition zones was determined after 24 hrs. The experiment was performed in triplicate. The actinobacteria that showed promising results in the preliminary antimicrobial screening were subjected to secondary screening by the agar well diffusion method (Bauer et al. 1966). The above-specified microorganisms were used. Actinomycete antimicrobial extracts were recovered from the culture filtrate by solvent extraction using ethyl acetate (1:1, v/v). The diameters of the zone of inhibition were determined after 24 hours. Samples were assayed in triplicate.

2.4 Characterization of actinomycetes

Growth and morphology of VIT GV01 were observed on different media such as ISP2, Actinomycetes isolation agar, potato dextrose agar, Yeast peptone mannitol agar, Czapek's Dox agar (Nguyen and Kim 2015), Zobell marine agar (Subramani and Sipkema 2019), Yeast Peptone Dextrose agar, soybean casein digest medium, nutrient agar, Luria Bertani agar, Tryptophan agar, Christensen agar medium (Hirsch and Christensen 1983), starch agar, Soy flour Mannitol Agar.

Morphology of the strain was observed in a phase-contrast microscope (MT5210/MEIJI) and scanning electron microscopy was performed using Carl Zeiss – Evo 18).

The biochemical characterization was performed for temperatures (25, 30, 35, 40, and 45⁰C), utilization of different sugars as sole carbon sources such as sucrose, glucose, fructose, maltose and lactose, and pH (2, 4, 6, 7, 8 and 10) to find the growth optimum. Biochemical tests were performed as described by Bergey's manual ("Bergey, D.H. and Holt, J.G. (1994) for strain identification.

Analysis of the 16S rRNA was done with the primers forward (5'-GAGTTTGATCC TGGCTCA-3') and reversed (5'-ACGGCTACCTTGTTACGACTT-3'). Amplified PCR product was sequenced and the nucleotide sequence was matched using the BLAST program. The phylogenetic tree was constructed using the neighbor-joining method (Fatima et al. 2019). The sequence of the isolate was submitted to GenBank (Accession ID: MN641011).

2.5 Metabolite production

Pure and active cultures of microbial strains selected from the secondary screening experiments were grown in ISP2 broth and incubated at 30°C for 7 days and cellular growth was confirmed by visible pellets, clumps, and aggregates in the flask. The culture broth was centrifuged and the supernatant was used to evaluate the antimicrobial activity against the above-mentioned test microorganisms. Antibiotic activities of the strains were compared with that of known commercially available erythromycin standard.

2.6 Extraction of the bioactive metabolites

The culture was grown in 1000 ml Erlenmeyer flasks containing 200 ml of ISP2 broth for 7 days as specified in metabolite production. The culture was centrifuged at 10,000 rpm for 20 min to separate the biomass. The secondary metabolite was recovered from the fermented ISP2 broth using a two-phase solvent extraction system with organic solvent (ethyl acetate). (Shetty et al. 2014). The mixtures of separating flask crude culture filtrate: solvent (1:1 ratio) were vigorously shaken for 10 min and in kept shaker from 18 to 24 hrs at 200 rpm. After 24 hrs this was left to settle for 30 minutes for separating flask kept stationary from 15 to 30 min. until the separation of aqueous and organic phases. Organic phases were collected and concentrated in a rotary evaporator (model RE100–Pro; SCILOGEX, LLC, CT, USA) at 54°C and 80 rpm. The dried crude extracts were then weighed, dissolved in 200µL of methanol, and finally stored at -20°C.

2.7 Screening of antibacterial activity

Concentrated crude ethyl acetate extract obtained from *Streptomyces* spp. VITGV01 was screened for their inhibitory activity against 4 known human pathogenic bacterial strains by agar Well Diffusion Assay (WDA) (Magaldi et al. 2004). The wells were prepared in the plate (already spread with the test organism) by using a sterile cork borer (6 mm in diameter) at 0.5 McFarland turbidity (1x10⁸ cells/mL). A volume of 25, 50, 75, and 100 µL of 1 mg/mL of crude extracts was carefully dispensed into each well and allowed to diffuse for 2 hrs and incubated at 37°C for 24 hrs. To validate the experiment, negative control (ethyl acetate) and positive control (1 mg/ml erythromycin) were also maintained, and each experiment was performed in triplicate. Diameters of inhibition zones were measured after 24 hrs. Tested pathogens such as *B. subtilis* (MTCC2756), *S. aureus* (MTCC737), *E. coli* – (MTCC1687), and *K. pneumoniae* (MTCC109).

2.8 FTIR and GCMS analysis of secondary metabolites

FTIR spectra were recorded on SHIMADZU FT- IR spectrophotometer, Model: IR Affinity in the range of 4000–400 cm⁻¹ (Sanghvi et al. 2014). GC-MS (GC trace ultra-version) was used to find out the chemical compounds. The peaks were identified with the library.

3. Result

The present study focuses on the isolation of potent antibiotic-producing endophytic actinomycetes. From two hundred and forty actinomycetes strains were isolated in five districts of Tamilnadu (Fig. 1), India. Among the isolated pure strains, VITGV01 was found to produce a wide spectrum of

antimicrobial activities against selected two Gram-positive and two Gram-negative bacteria. The isolated VITGV01 showed a clear zone of inhibition (Fig. 2) due to antimicrobial activity.

3.1 Biochemical characterization

The selected strains were subjected to various biochemical tests and the results are tabulated in Table 1, which supports that it belongs to actinomycetes.

Table 1
Various Biochemical Characteristics of VITGV01

S. No	Character	Result
1	Gram staining	Positive
2	Motility	Non-motile
3	Indole	Negative
4	Methyl Red (MR)	Negative
5	Voges Proskauer (VP)	Positive
6	Citrate utilization	Positive
6	Catalase	Positive
7	Lactose	Positive
8	Glucose	Positive
8.	Fructose	Positive
9.	Sucrose	Positive
10.	Galactose	Positive
11.	Starch	Positive
12	Mannitol	Positive
13	Spore chain	Positive
14	Shape and growth	Mycelial Spore chain
15	Temperature optimum	300 C
16	Temperature range for growth	250 C – 450 C
17	Range of pH for Growth	6–12
18	Urease	Positive
19	Oxidase	Negative
21	Aerial Mass color	Slight brown color
22	Soluble pigments	Negative
21	Casein	Positive
22	Gelatine	Negative
23	Alkaline phosphate	Positive
25	Anaerobic growth	Positive
26	Chitin	Positive
27	Ammonium chloride utilization	Positive
28	Nitrogen sulfate utilization	Positive
29	Aerial Hyphae	Positive
30	Diffusible pigment	Negative
31	Cellulose	Positive
32	Pectinase	Negative

3.2 Physiological Characters

Optimization parameters for growth of this Strain VITGV01 require pH 7, temperature 30⁰C and 1% yeast extract, 1% sucrose incubation duration 7 days were best suited (Fig. 3).

3.3 Morphological observation of VITGV63

The cultural characteristics and colony morphology were summarized in Table 2 and Fig. 4. These morphological observations revealed that VITGV01 was similar to *Streptomyces*. It showed well developed aerial and substrate mycelium and mycelial cluster showed continuous fragmented long and short chain-like structure (Fig. 5B). The scanning electron microscope image of (Fig. 5C) sample exhibited continuous filamentous spiral chains in a tubular shape. The continuous aerial hyphae differentiated into a small and smooth-surfaced spore. The center of the spore showed a cavity on the upper side of each spore (Fig. 5D).

Table 2
Colony Morphology of VITGV01 on Different Agar medium

S. No	Different Media	Size of the colony	Colour of the colony	Surface of the colony	Consistency / Texture of the colony	Overall Growth
A	ISP 2 Agar	Small 1–2 mm	Whitish Gray	Rough opaque	Brittle	Moderate
B	Actinomycetes Isolation Agar	Small 1–2 mm	Greyish white	Rough opaque	Viscid	Moderate
C	Potato Dextrose Agar	Pinpoint	Gray spot	Smooth	Viscid	Meagre
D	Yeast peptone Mannitol agar	Pinpoint	White ash gray	Rough & Transparent	Viscid	Meagre
E	Czapek Dox Agar	Large 2–3 mm	Gray	Smooth opaque	Dry	Strong growth
F	Zobell Marine Agar	Pinpoint	Yellowish white	Rough / Transparent	Friable	Meagre
G	Soybean casein digest agar	Large 2–3 mm	White	Rough opaque	Mucoid	Strong growth
H	Yeast Peptone Dextrose agar	Small 1–2 mm	Gray	Smooth opaque	Brittle	Moderate
I	Nutrient agar	Large 2–3 mm	White	Smooth opaque	Brittle	Strong growth
J	Luria Bertani Agar	Small 1–2 mm	White	Smooth opaque	Viscid	Moderate
K	Tryptophan agar	Large 2–3 mm	Yellowish	Butryous opaque	Viscid	Strong growth
L	Christensen agar	Small 1–2 mm	Ash Greyish	Smooth opaque	Friable	Moderate
M	Starch agar	Large 2–3 mm	White	Smooth opaque	Friable	Strong growth
N	Soy flour Mannitol Agar	Small 1–2 mm	Whitish ash Gray	Smooth	Jelly	Meagre

3.4 Genetic analysis of VITGV63

The 16S rRNA sequence of the strain VITGV01 (1040 nucleotide), to identify the 16S rRNA gene sequence with high similarity strain VITGV01 shared with 16S rRNA high degree of similarity with other types of genus *Streptomyces*. The phylogenetic tree of the strain VITGV01 forms a single separate

branch from other *Streptomyces* neighbors (Fig. 6). It shares 16S rRNA sequence to nearest neighbor is *Streptomyces atacamensis* (99.42 %), Phylogenetic tree builder uses sequences aligned with method System Software aligner.

3.5 Extraction of bioactive compounds

The crude extract exhibited antimicrobial properties proved by the Well diffusion method (Table 3) for secondary screening. Effective zone of inhibition was also recorded against *B. subtilis*, *S. aureus*, *K. pneumoniae*, and *E. coli*. (Fig. 7). The zone of inhibition was observed between the range from 8 to 22 mm. No zone of inhibition was observed in negative control with ethyl acetate. A maximum inhibition zone of 22 mm was observed against *K. pneumoniae*, which was also to positive control as erythromycin (22 mm).

Table 3
Zone of inhibition (mm) in secondary screening of crude extracts produced from VITGV01 by using well diffusion

VITGV01	Zone of inhibition (µg/ml)															
	Gram Positive								Gram Negative							
	B. subtilis				S. aureus				K. pneumoniae				E. coli			
	25	50	75	100	25	50	75	100	25	50	75	100	25	50	75	100
Crude Extract	8	11	12	8	16	18	19	22	15	18	20	22	15	17	18	20
Positive control (Erythromycin)	10	11	13	15	14	15	17	18	10	14	16	24	10	11	12	13
Negative control (Ethyl Acetate)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

3.6 FTIR analysis

The FTIR spectrum of the antimicrobial compounds were compared with Library. It also showed characteristic band corresponding to 21 peaks 3406.29, 3298.28, 2958.80, 2926.01, 2854.65, 1707.00, 1647.21, 1537.27, 1454.33, 1413.82, 1334.74, 1274.95, 1244.09, 1091.71, 1008.77, 894.97, 767.67, 740.67, 607.58, 489.92, 422.41 (Fig. 8).

3.7 GCMS analysis

Streptomyces VITGV01 extract was characterized with GCMS (Fig. 9). Based on the retention time, peak area, molecular weight, and molecular formula, twenty major chemical compounds were identified by comparing mass spectra with the library as shown in Table 4. The major identified compounds were 1. Isophorone; 2. 1H-Indene, 1-methylene; 3. 2-Trifluoromethylbenzoic acid; 4. Acetic acid, 2-phenyl ethyl ester; 5. 2-Methoxy-4-vinyl phenol; 6. Phenol, 2,4-bis(1,1-dimethyl ethyl)-; 7. Semustine; 8. Diethyl Phthalate; 9. Hexadecanoic acid, ethyl ester; 10. n-Hexadecanoic acid; 11. 2,4-Diamino-6-nitroquinazoline; 12. 3,5-dimethoxy phenol; 13. Tetradecyl trifluoroacetate; 14. 2,5-Dimethoxyaniline; 15. Octadecanoic acid, ethyl ester; 16. 4-Nitro-3-picoline-N-oxide; 17. Pyrrolo[1,2-a]pyrazine-1,4-dione hexahydro-3-(2-methyl propyl)-; 18. 2-hydroxy-3,5-trimethyl-2-cyclohexenone; 19. Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-; and 20. 2(3h)-oxazolethione 4,5-diphenyl-2-cyclohexenone are present in the sample. Their predicted chemical structures were reported in Fig. 10. Among all the twenty compounds 4-Nitro-3-picoline-N-oxide and Phenol, 2,4-bis(1,1-dimethyl ethyl)- showed the highest peak area compared to other chemical compounds in the extract of *Streptomyces* spp. VITGV01.

Table 4
Major Chemical Compounds identified by GC–MS for *Streptomyces* spp. VITGV01.

S. No	Chemical Compound	RT	Molecular weight	Molecular Formula	Area %	Nature of The Compound	Activity	Spectrum of action	Reference
1	Isophorone	8.585	138.21	C ₉ H ₁₄ O	1.68	Cyclohexane	Antimicrobial	Broad	(Kiran et al., 2013)
2	1H-Indene, 1-methylene-	9.558	128.17	C ₁₀ H ₈	4.85	Methylene	Antimicrobial	Broad	(Osuntokun and Cristina, 2019)
3	2-Trifluoromethylbenzoic acid,	10.313	190.12	C ₈ H ₅ F ₃ O ₂	0.89	Ester	Antimicrobial	Broad	(Krátký and Vinšová, 2012)
4	Acetic acid, 2-phenylethyl ester	10.464	164.20	C ₁₀ H ₁₂ O ₂	0.67	Ester	Antimicrobial	Broad	(Al-Dhabi et al., 2016)
5	2-Methoxy-4-vinylphenol	11.487	150.17	C ₉ H ₁₀ O ₂	2.09	Phenolic	Antimicrobial	Broad	(Rubab et al., 2020)
6	Phenol, 2,4-bis(1,1-dimethylethyl)-	13.165	278.5	C ₁₇ H ₃₀ O	22.35	Phenolic	Antimicrobial	Narrow	(Padmavathi et al., 2014)
7	Semustine	13.727	247.72	C ₁₀ H ₁₈ ClN ₃ O ₂	0.78	Methyl	Anticancer	Antitumor	(Agarwal et al., 2015)
8	Diethyl Phthalate	15.220	222.24	C ₁₂ H ₁₄ O ₄	1.92	Ethyl ester	antimicrobial	Broad	(Premjanu N and Jaynthy C, 2014)
9	Hexadecanoic acid, ethyl ester	17.821	284.4	C ₁₈ H ₃₆ O ₂	0.82	Stearic acid	antioxidant	-	(Kim et al., 2020)
10	n-Hexadecanoic acid	17.913	256.4	C ₁₆ H ₃₂ O ₂	12.92	carboxylic	antibacterial	Narrow	(Abubakar and Majinda, 2016)
11	2,4-Diamino-6-nitroquinazoline	18.349	205.1	C ₈ H ₇ N ₅ O ₂	1.27	amine	antimicrobial	Broad	(Mallikarjuna Reddy et al., 2015)
12	3,5-dimethoxyphenol	18.937	154.1	C ₈ H ₁₀ O ₃	1.61	phenol	Antimicrobial	Broad	(Laddha and Biyani, 2019)
13	Tetradecyl trifluoroacetate	19.130	310.1	C ₁₆ H ₂₉ F ₃ O ₂	0.66	Ester	Antimycobacterial	Narrow spectrum	(Nimbeshaho et al., 2020)
14	2,5-Dimethoxyaniline	19.297	153.18	C ₈ H ₁₁ NO ₂	3.02	Benzene	Antimicrobial	Broad	(Nordin et al., 2017)
15	Octadecanoic acid, ethyl ester	19.675	312.5	C ₂₀ H ₄₀ O ₂	0.63	Ester	Antioxidant	-	(Kim et al., 2020)
16	4-Nitro-3-picoline-N-oxide	19.817	289.8	C ₁₄ H ₂₄ ClNO ₃	26.16	pyridine	Antibacterial	Broad	(Karayannis et al., 1974)
17	Pyrrolo[1,2-a]pyrazine-1,4-dione hexahydro-3-(2-methylpropyl)-	19.943	210.2	C ₁₁ H ₁₈ N ₂ O ₂	10.36	pyrazine	Antibacterial and Antioxidant	Broad	(Putra and Karim, 2020)
18	2-hydroxy-3,5,5-trimethyl-2-cyclohexenone	20.153	154.21	C ₉ H ₁₄ O ₂	0.85	Cyclohexane	Antimicrobial	Broad	(Nguyen et al., 2019)
19	Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	24.557	244.2	C ₁₄ H ₁₆ N ₂ O ₂	1.72	pyrazine	Antioxidant	-	(Ser et al., 2015)
20	4,5-Diphenyloxazole-2(3H)-thione	26.444	253.3	C ₁₅ H ₁₁ NOS	4.75	Oxazole	antimicrobial	Broad	(Kakkar and Narasimhan, 2019)

4. Discussion

Actinomycetes play a major role in combating various diseases. Earlier exploration has shown actinomycetes having excellent potential to control the growth of pathogens (Ayoubi et al. 2018). In our study, a total of 240 endophytic actinomycetes were isolated and screened for their antimicrobial

activities, out of which 77 actinomycetes showed the antimicrobial property. A similar report of recognizing 619 endophytic actinomycetes was reported by Tan et al., (2006) out of this 8 alone exhibited antagonistic property.

The size of the inhibition zone is the first criterion for screening antimicrobial activity on pathogens, in the present study, there was a good number of microbes (77/240) which shows a good zone of inhibition (Fig. 3). A total of 44 (18%) isolates inhibited the growth of both Gram-positive and Gram-negative pathogen, while 18 isolates (8%) inhibited Gram-negative and 15 isolates (6%) inhibited Gram-positive bacteria. Thus seventy-seven (32%) isolates were selected to explore their potentials. Strain VITGV01 showed the highest antimicrobial activity against all four pathogens.

Morphological characteristics such as aerobic, powdery, flat appearance, aerial and substrate mycelia with different colors in different media and colony size suggested that the isolates are affiliated to genus *Streptomyces*. The above comparison with the description given in Bergey's manual of systematic bacteriology indicates a major resemblance of this strain with *Streptomyces* species. This study coincides with reports of Al-Ansari et al., (2019) SEM results showed hyphae's were compartmentalized and spores chain-like and continuation (Fig. 5). The mature spore's surface was smooth and unique with a small concave depression; this morphology was the same as that of actinobacteria AFD7 strains (Fatima et al. 2019)

The nature and concentration of sugars such as sucrose, maltose, fructose, and glucose are crucial for the growth, nutrient consumption, and production of secondary metabolites. And are important for signal transduction, gene regulation, and development. The utilization rate of carbon and nitrogen sources by VITGV01 seems to be unique. Out of five different carbon sources, the highest growth was obtained with 1 % sucrose. On the other hand, glucose recorded the lowest growth rate. Similarly, El-Hadi et al., (2019) reported lactose for maximum and mannitol for minimal growth in *Streptomyces greseoplanus*. This study support that according to carbon sources, growth in *Streptomyces* spp. is strongly influenced.

Nitrogen source is required for the synthesis of cells and energy sources. Also, biomass yield and cell morphology are strongly influenced by the nitrogen source. Similar results have been reported by (Al-Ansari et al. 2020) in their studies associated with *Streptomyces greseoplanus*. In another study, by Wang et al., (2010) nitrogen sources from yeast extract supported the greatest mycelium biosynthesis in *Saccharothrix yanglingensis* sp. nov.

Microorganisms are capable of growing over a wide pH range. Moreover, pH is the third important factor that influences microbial growth. In this way, responses to environmental signals are transmitted through global transcription factors that mediate environmental signals like carbon (CreA) (Janus et al. 2008), nitrogen (AreA) (Tudzynski et al. 1999), and pH (PacC) (Tilburn et al. 1995), translating environmental cues into Secondary Metabolites and concomitant physiological responses. The enhancement or inhibition of the secondary metabolite is influenced by a change in pH and can also affect the growth of microorganisms (Kamjam et al. 2019). In this experiment, we report the optimum pH for maximum growth is pH 7, and the lowest growth was observed in pH 2. This is very similar to the study by *Streptomyces polaris* sp by Kamjam et al., (2019).

An important criterion for the potency assessment is the time course for growth plateau formation and continuous growth was observed till the 14th day. A similar report was documented by Yagüe et al., (2012) in *Streptomyces coelicolor* for growth optimization. Another factor is the incubation temperature, which affects the microbial growth, metabolite production, rate of the chemical reaction, which in turn affects the rate of metabolite production. In the present study, the maximum activity was recorded at 30°C. By increasing the incubation temperature, a gradual decrease in growth was observed. This is a negative impact on this organism; this could be due to the thermolabile nature of extracellular enzymes.

Studies have suggested that to identify actinomycetes details of biochemical, morphological, and phylogenetical characterization are necessary. The biochemical characterization proved that it belongs to actinomycetes (Table 2). The 16s rRNA study and phylogenetic tree result again proved that this strain clustered within the genus *Streptomyces* and exhibited close phylogenetic affinity and high sequence similarity to *Streptomyces atacamensis* (99.42 %), *Streptomyces mangrovi* (99.33 %), *Streptomyces chitinivorans* (99.23 %), and *Streptomyces atacamensis* (99.04%), This is the first study to identify this *Streptomyces* spp. (Gen Bank Accession - MN641011). A similar type of conformation for *Streptomyces* was reported by Duddu & Guntuku, 2016).

Isolation of antibacterial compounds from the endophytic environment is in advance interest in recent times to isolate novel bioactive actinomycetes. Musa et al.,(2020) isolated fifty-four endophytic actinomycetes, which showed antagonistic activities. During secondary screening with crude extracts, a wide range of inhibition zone against tested bacterial strains were observed. Similar findings were reported earlier by Gebreyohannes et al., (2013) in actinomycetes spp. In general, the antibacterial activity of crude extracts has fluctuated widely. The results demonstrate that a Gram-positive and Gram-negative bacterium both were highly susceptible to the tested crude extracts. This result was in agreement with the report of Sanghvi et al., (2014) who reported it with *Streptomyces werraensis*.

VITGV01 crude extract showed excellent activity on Gram-positive and Gram-negative bacteria, suggest that it could inhibit DNA synthesis, RNA synthesis, cell wall synthesis, or protein synthesis (Kohanski et al. 2010). There was a marked difference between the crude extracts and pure antibacterial drug (Erythromycin). Some of the crude extracts showed a higher or equivalent zone of inhibition compared to the standard drug erythromycin, which shows excellent activity against Gram-positive organisms and modest activity against Gram-negative bacteria. Rex et al. (2001) reported that a significant difference was normally presented in crude (unpurified) extracts compared with a pure drug that was already in clinical use.. The MIC varied among the tested isolates against all the tested pathogen. These results were similar to the report of Núñez-Montero et al., (2019). Therefore, crude extracts could be a potent source for antibiotic production, which leads to the development of novel drugs for the treatment of infectious diseases. The report of antibacterial activities of actinomycetes isolated from Tomato endophytes of agrofield Madurai in Tamilnadu was first-hand information as per our knowledge.

Infrared spectra showed a primary amine (3406.06 cm^{-1} , 3298.28 cm^{-1}), alkane groups ($2958.80\text{--}2854.65\text{ cm}^{-1}$), (C = C) of aromatic ring ($1537.27\text{--}1413.82\text{ cm}^{-1}$), Amine (C-N) ($1334.74\text{--}1091.71$) and secondary alcohol (1008.77 , $894.97\text{--}489.92$) Alkyl Halide (C-Cl) (740.67 , 607.58). A similar result was reported by Mathur et al (2015) for *Streptomyces* spp. and Badji et al (2007) in *Nonomuraea* sp. NM94. Some differences were also noted with imine functional groups as per the report of Shangvi et al (2014) in *Streptomyces werranesis*. (Badji et al. 2007; Kumar et al. 2014a; Mathur et al. 2015; Jung et al. 2018).

GC-MS analysis has contributed significantly to the bioprospecting of natural products isolated from *Streptomyces* bacteria (Schöller et al. 2002; Selvakumar et al. 2015; Ser et al. 2015a). There are many reports for the GC-MS-based chemical analysis of actinobacterial secondary metabolite. In this study, GC-MS analysis shows twenty major chemical compounds with different retention times. The identified compounds were in the class of methylene, methyl, ethyl ester, stearic acid, carboxylic, amine, phenol, benzene, ester, pyridine, pyrazine, cyclohexane, pyrazine, and oxazole in nature. The two major highest peaks (area %) of 26.16 and 22.35 were obtained for 4-Nitro-3-picoline-N-oxide and Phenol, 2,4-bis(1,1-dimethyl ethyl)- with the retention time of 26.16 and 13.165 min respectively, and the lowest peak (area %) of 0.63 was obtained for Octadecanoic acid, ethyl ester with the retention time of 19.675 min. Among the detected compounds by GC MS analysis, compound 4-Nitro-3-picoline-N-oxide is pyridine in nature (PubChem) which is most frequently detected in microbial extract of *Streptomyces avermitilis* NIC B62, demonstrating antibacterial and antifungal activities (Lysenkova et al. 2010). The compound, Phenol, 2,4-bis(1,1-dimethyl ethyl)- is a phenolic compound which is previously one study reported by Tan et al., (2019) from *Streptomyces* sp. MUM265 is a potential source of the antioxidant and anti colon-cancer agent. Most of the other compounds detected by GC-MS in a few *Streptomyces* sp. VITGV01 extract has been reported previously in *Streptomyces* spp. extracts, such as n-Hexadecanoic acid (El-Naggar et al. 2017), Pyrrolo [1 2-a]pyrazine-1 4-dione hexahydro-3-(2-methyl propyl)- (Ser et al. 2015b), 1H-Indene, 1-methylene- group (Wang et al. 2013), 2-Methoxy-4-vinyl phenol (Naureen et al. 2017), 2,5-Dimethoxyaniline (Nain-Perez et al. 2016; Nepali et al. 2016), Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- (Sanjenbam and Kannabiran 2016), 3,5-dimethoxy phenol (Murray et al. 2020), Diethyl Phthalate (Kapanen et al. 2007), Acetic acid, 2-phenyl ethyl ester (Al-Dhabi et al. 2019). These compounds could be the key contributors to the potential antimicrobial activity of *Streptomyces* sp. VITGV01. These known compounds detected in GCMS are well-known ability to inhibit the growth of the four tested pathogens. However, to date, there is no report on Isophorone, 2-Trifluoromethylbenzoic acid, Semustine, 2,4-Diamino-6-nitro quinazoline, and 4,5-Diphenyloxazole-2(3H)-thione, from any other *Streptomyces* spp. These major compounds 4-Nitro-3-picoline-N-oxide, Phenol, 2,4-bis(1,1-dimethyl ethyl)-, n-Hexadecanoic acid, and Pyrrolo[1 2-a]pyrazine-1 4-dione hexahydro-3-(2-methyl propyl)- comes under phenolic, carboxylic, pyridine, and pyrazine group. These compounds together constitute 71.79% of the total compounds present in the extract of *Streptomyces* sp. VITGV01. Kumar et al., (2014b) reported strong antimicrobial activity of phenolic compounds (Phenol, 2,4-bis(1,1-dimethyl ethyl)), from *Streptomyces* sp. SCA7 strain strongly inhibited the growth of Gram-positive and negative organisms. Kumari et al., (2019) reported that the antimicrobial effect of different carboxylic, pyridine, and pyrrolidine group of compounds on antibacterial activity. The above report suggests the inhibitory effect on both Gram-positive and negative properties of *Streptomyces* sp. VITGV01 extract.

Conclusion

Plants are a rich source of endophytes. The identification of novel *Streptomyces* strains may lead to new drug discovery. In this study, 240 endophytic actinomycetes were primarily screened and among these 77 isolates showed antimicrobial activity against selected human pathogens. Based on the biochemical characterization, and 16s rRNA studies, it can be a new strain. Our study encourages the exploration of diverse ecosystems for the isolation of new species for novel bioactive compounds. As there is an alarming increase in multi-drug resistance in pathogens, exploration of new bioactive compounds helps to combat the multi-drug resistance.

Declarations

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Authors' contributions: All the authors contributed equally

Informed consent statement: Not applicable

Ethical statement: Not applicable

Data availability statement: Data supporting the results are given in tables.

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Figures

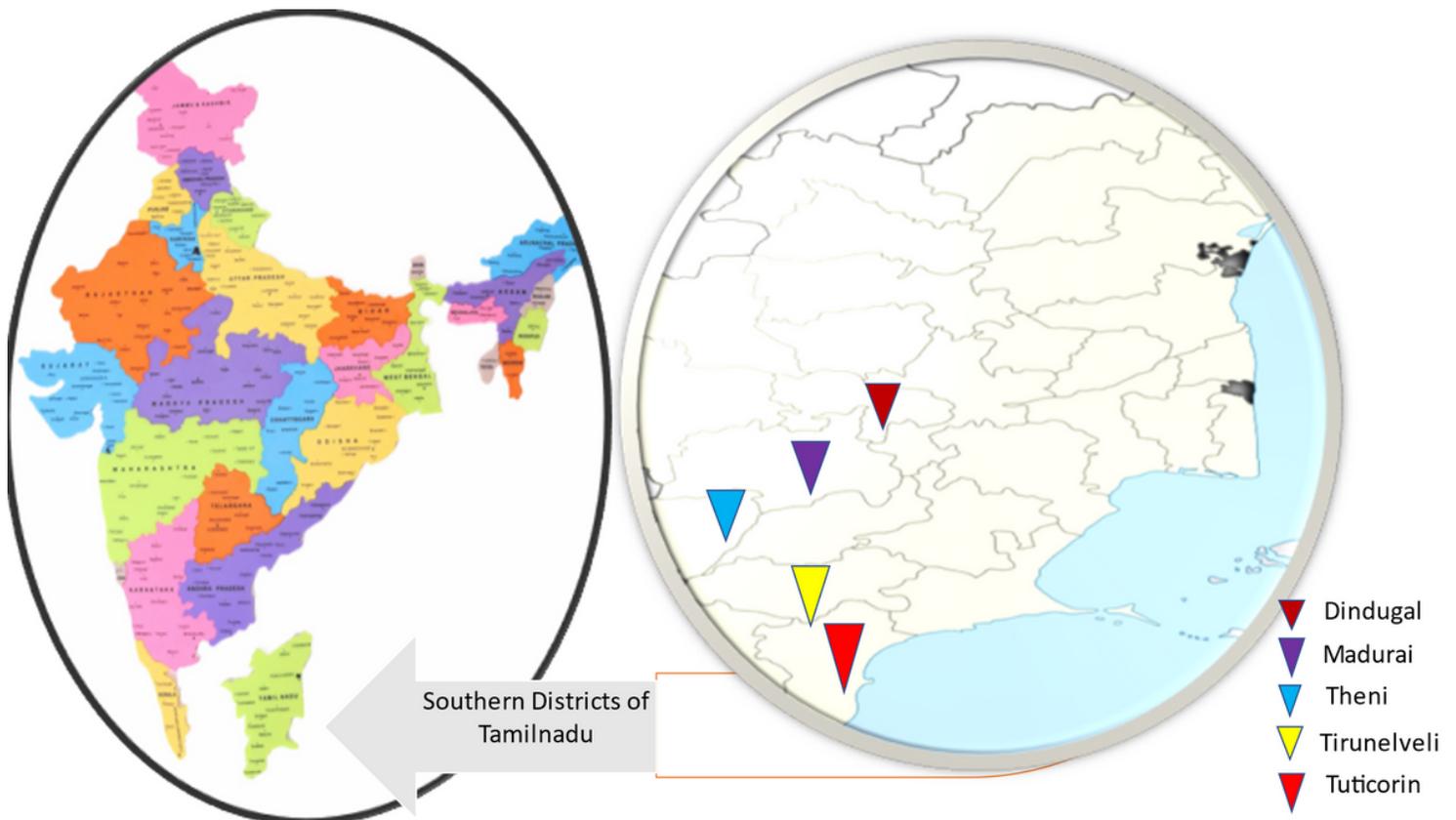


Figure 1
Map showing the different districts of samples were a collection. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.



Figure 2

Dual plate assays showing antimicrobial activity by VITGV01

Optimization of Secondary Metabolite Production

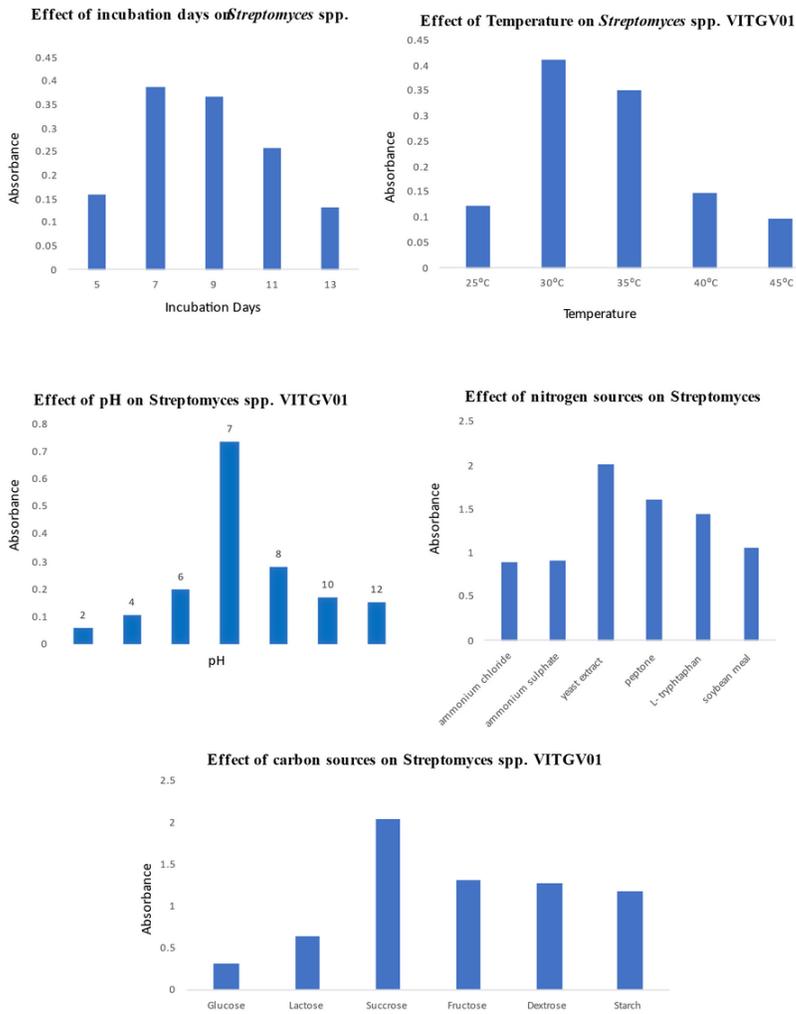


Figure 3

Showing optimization of growth parameters of VITGV01.

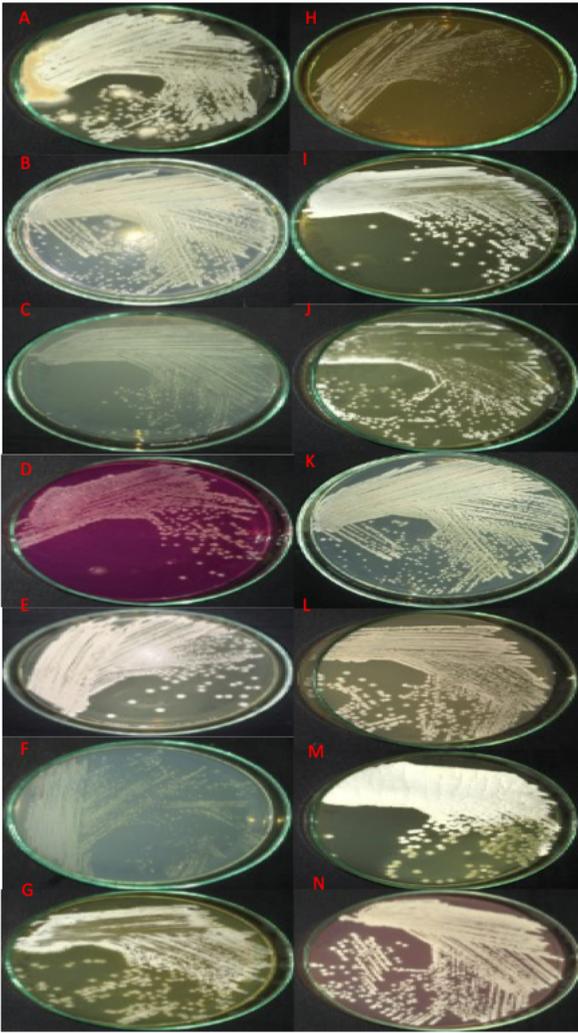


Figure 4

Colony Morphology of VITGV63 on Different agar medium: A- ISP2, B - Actinomycetes isolation agar, C - Potato Dextrose agar, D - Yeast Peptone Mannitol, E- Czapek Dox agar, F- Zobell Marine agar G - Soybean Casein digest agar, H – Yeast Peptone Dextrose agar I - Nutrient agar, J - Luria Bertani agar, K - Tryptophan agar, L - Christenson agar, M - Starch agar and N- Soy flour Mannitol.

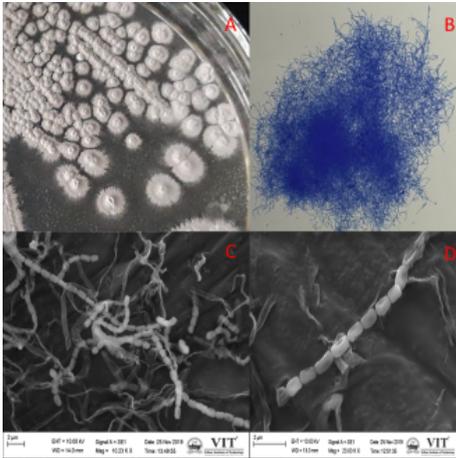


Figure 5

Images of Streptomyces spp. VITGV01 in A. Petri plate, B. Hyphal growth in phase contrast microscope. C. SEM image of Hyphae D. SEM image of Spore

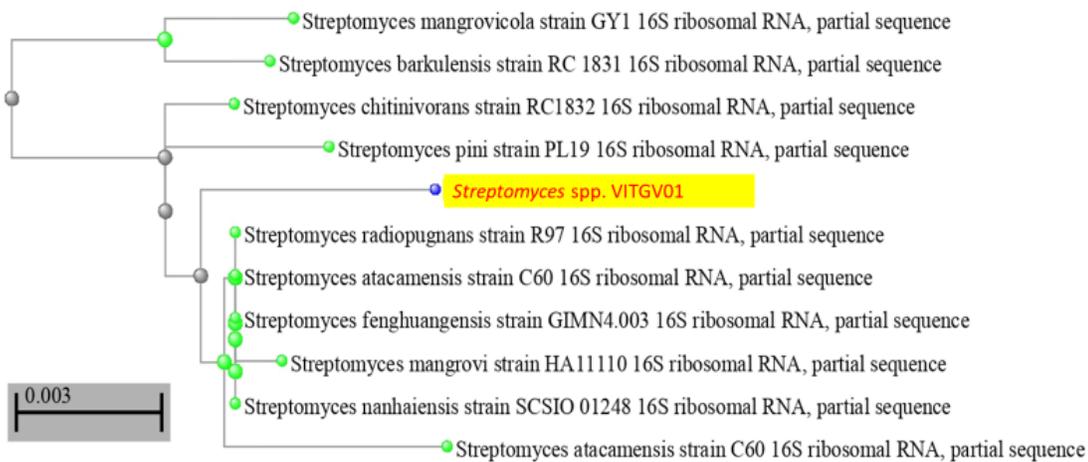


Figure 6

Neighbour- Joining tree based on 16S rRNA of Streptomyces spp. VITGV01 strain (The scale bar corresponds to 0.003 substitutions per nucleotide position)

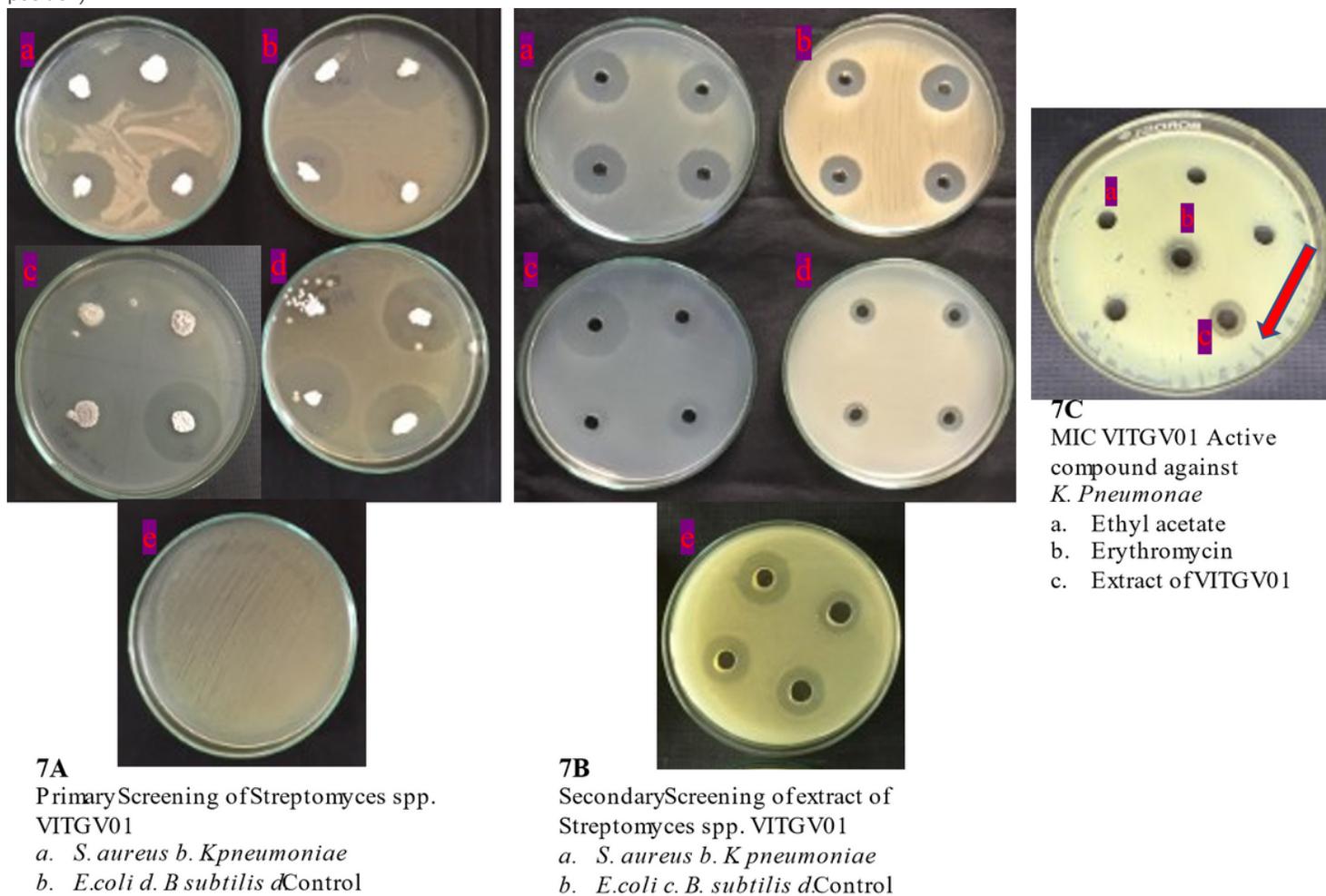
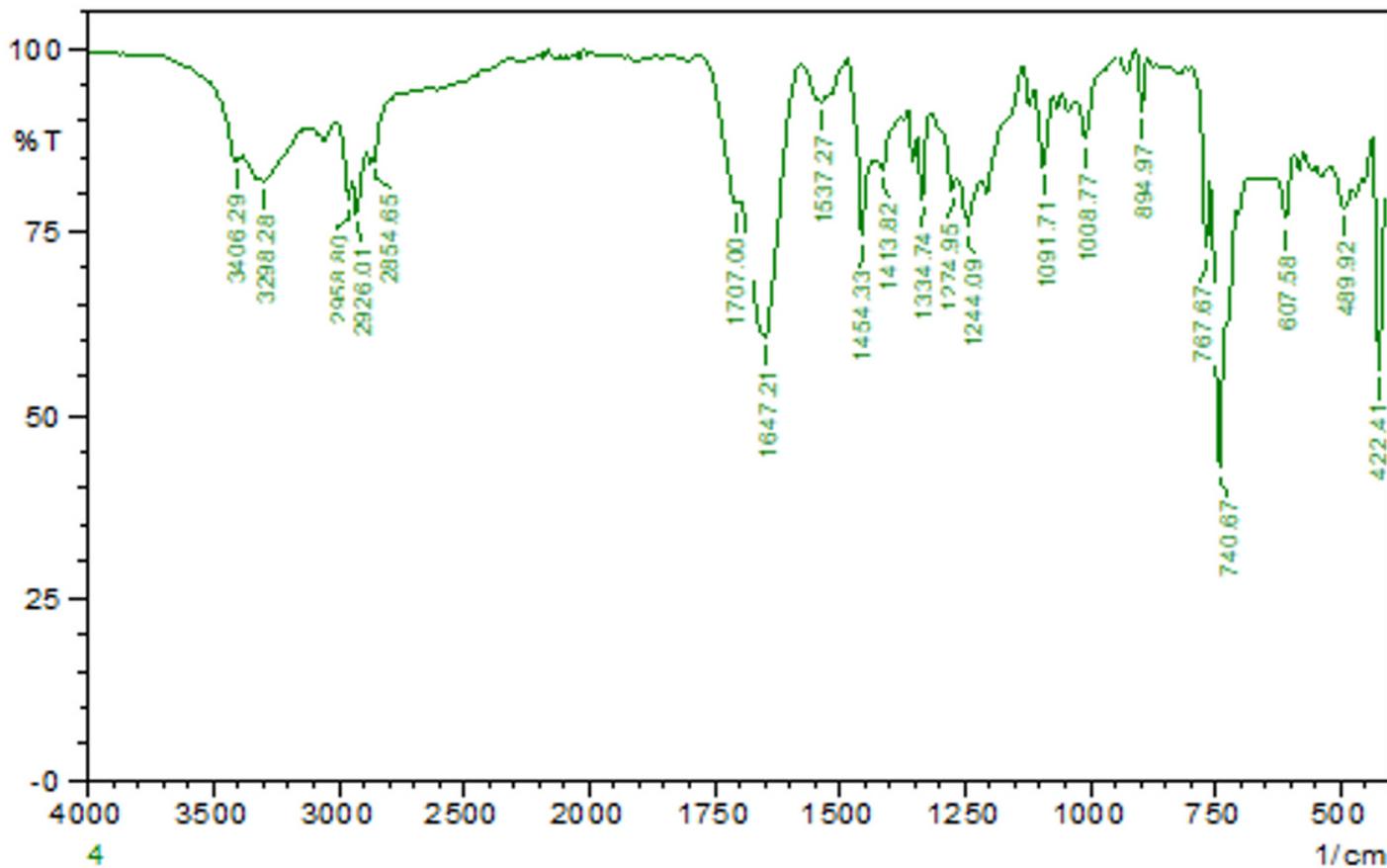


Figure 7

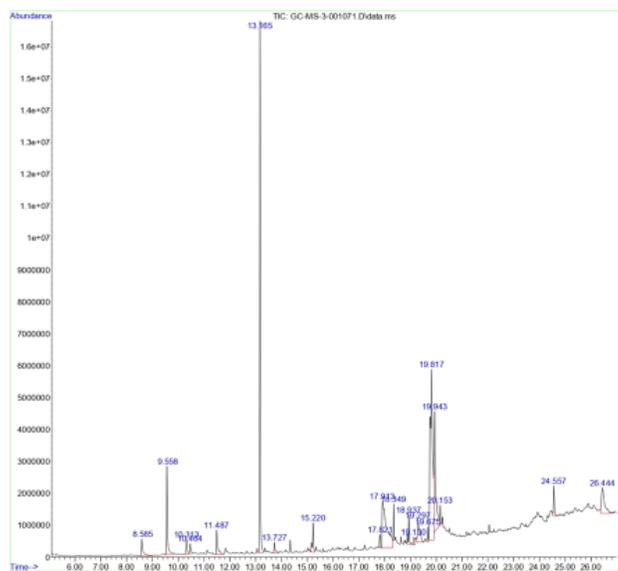
A. Performance of crude extract for primary screening appearance of clear zone showing antimicrobial activity. 7B. Secondary screening of crude extract showing clear zone in well diffusion method. 7C. Minimal inhibitory concentration zone forming at 10ug/ml shown with a pointer



FTIR spectrum of antimicrobial agent produced by *Streptomyces* VITGV01 (mg/ml con)

Figure 8

FT-IR Spectra of the partially purified compound showing structural



DMFU-27-01-21-M.M.Fil Jan 29 12:16:08 2021

Page: 1

Figure 9

GC MS Chromatogram of *Streptomyces* spp. VITGV01 –extract showing the presence of a secondary metabolite.

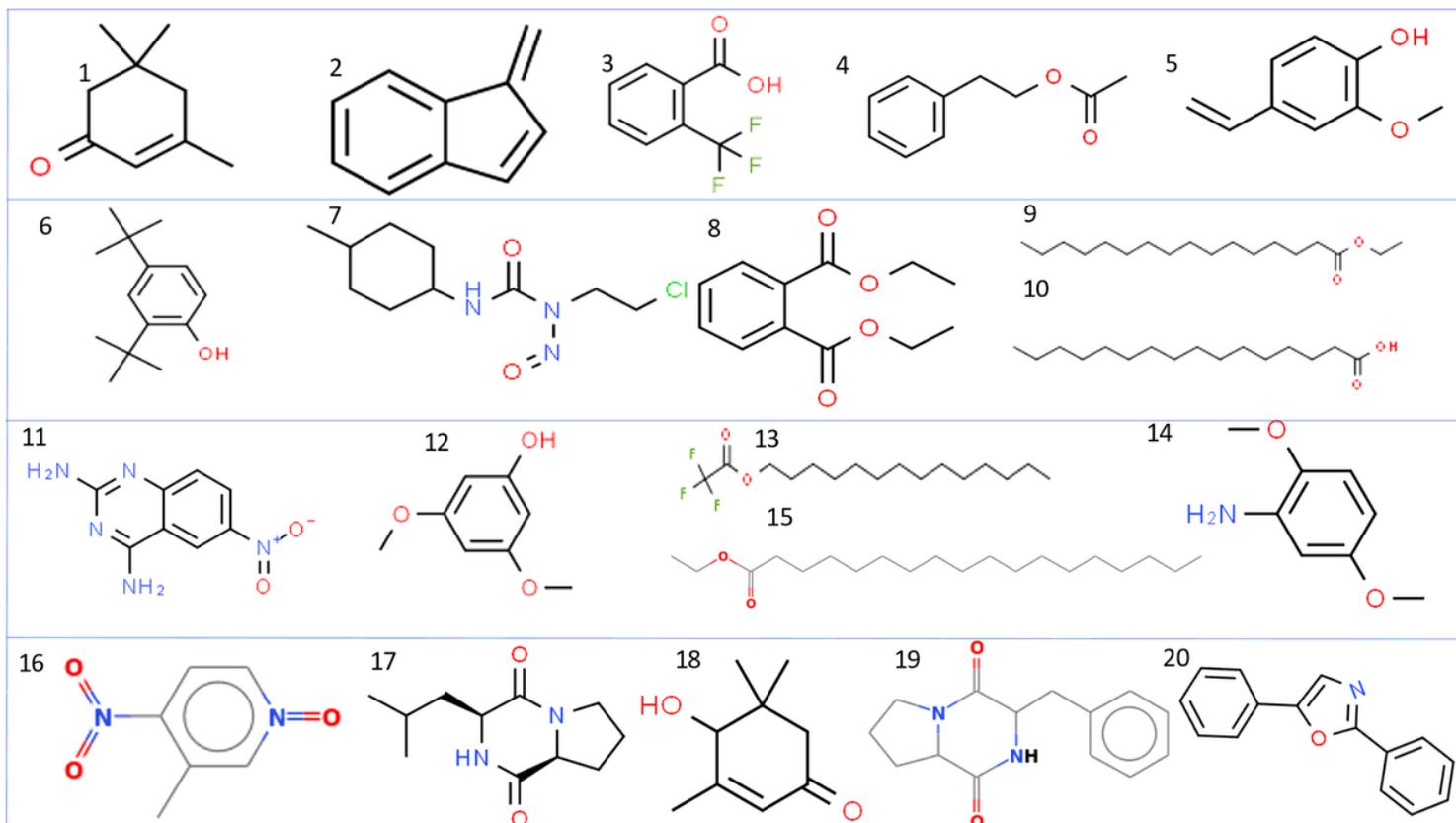


Figure 10

Active compounds from the GC MS library of *Streptomyces* spp. VITGV01. 1. Isophorone; 2. 1H-Indene, 1-methylene-; 3. 2-Trifluoromethylbenzoic acid; 4. Acetic acid, 2-phenylethyl ester; 5. 2-Methoxy-4-vinylphenol; 6. Phenol, 2,4-bis(1,1-dimethylethyl)-; 7. Semustine; 8. Diethyl Phthalate; 9. Hexadecanoic acid, ethyl ester; 10. n-Hexadecanoic acid; 11. 2,4-Diamino-6-nitroquinazoline; 12. 3,5-dimethoxyphenol; 13. Tetradecyl trifluoroacetate; 14. 2,5-Dimethoxyaniline; 15. Octadecanoic acid, ethyl ester; 16. 4-Nitro-3-picoline-N-oxide; 17. Pyrrolo[1,2-a]pyrazine-1,4-dione hexahydro-3-(2-methylpropyl)-; 18. 2-hydroxy-3,5,5-trimethyl-2-cyclohexenone; 19. Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-; and 20. 2(3h)-oxazolethione 4,5-diphenyl-2-cyclohexenone