Contents lists available at NCBI

The American Journal of Science and Medical Research

Journal homepage: https://ajsmrjournal.com/

Research Article

Spectral analysis of antibiotic Staurosporine and its Antimicrobial Activity

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https://dx.doi.org/10.5281/zenodo.20476256

Received: 21 April 2026
Revised 25 May 2026
Published on 31 May 2026

ISSN: 2377-6196
Published by AIRA

Keywords: *Staurosporine, antimicrobial, Champavatii, BHPL-KSKU5, 16s rDNA*

ABSTRACT

In the present investigation, twenty-eight soil samples were collected from coalmine locations in Telangana, India. The isolates underwent purification and identification through their culture characteristics on various media, including oatmeal agar, glycerol asparagine agar, yeast extract-malt extract agar, inorganic salt starch agar, and starch casein agar. Additionally, the supernatants of all isolates were evaluated for their antimicrobial and antifungal properties. The biochemical and microscopic analyses of the isolated strains suggest that the potential isolates belong to the Streptomyces genus. Among all the strains, BHPL-KSKU5 exhibited the most significant antibacterial and antifungal activities. Molecular characterization of the 16s rDNA gene sequence of BHPL-KSKU5 and the resulting phylogenetic tree indicated a close relationship with the Streptomyces champavatii strain. This isolate has been deposited in the NCBI gene bank under the accession number MH553077. Furthermore, spectral analyses, including GC-MS, FTIR, and NMR (1H and C13), revealed that the antimicrobial compound is antibiotic Staurosporine derived from *S. champavatii* (KSKU5).

1. Introduction

The rapid emergence and global spread of multidrug-resistant (MDR) pathogens have created an urgent need for the discovery and development of novel antibiotics with unique mechanisms of action. Several clinically important human pathogens, including Staphylococcus aureus, Salmonella paratyphi, Streptococcus pneumoniae, Pseudomonas aeruginosa, Enterococcus faecalis, Clostridium difficile, and Mycobacterium tuberculosis have developed resistance to currently available antimicrobial agents. The mechanisms underlying antibiotic resistance include enzymatic inactivation of drugs, acquisition of resistance genes such as those encoding β-lactamases, and enzymatic modification of aminoglycosides (Ramirez and Tolmasky, 2010; Blair et al., 2015).The increasing prevalence of antibiotic resistance, combined with the diminishing efficacy of existing drugs, highlights the urgent need to explore new ecological niches for novel antimicrobial agents (Ventola, 2015; Hutchings et al., 2019). Actinomycetes, particularly members of the genus Streptomyces, are well known for their ability to produce a wide range of bioactive secondary metabolites, contributing to more than two-thirds of naturally derived antibiotics (Bérdy, 2012; van der Meij et al., 2017; Kumar et al., 2020; Porika et al., 2014).

In recent years, extreme and underexplored environments have gained attention as promising sources of novel actinomycetes with unique metabolic capabilities (Tiwari and Gupta, 2012; Jose and Jebakumar, 2013). Coal mine soils, characterized by harsh physicochemical conditions, represent such a niche that may harbor diverse and stress-adapted actinomycetes. In the present study, actinomycetes were isolated from various coal mine soil habitats, with a notably higher prevalence observed in the Bhupalpally coal mining region compared to other sampling sites. Among the isolates, *Streptomyces champavatii* KSKU5 emerged as a potent strain exhibiting significant antimicrobial activity. This finding underscores the ecological significance of coal mine environments as reservoirs of actinomycetes capable of producing biologically active secondary metabolites with antibacterial, anti-Candidal, and potential anticancer properties (Demain and Sanchez, 2009; Subramani and Aalbersberg, 2012). Furthermore, the utilization of cost-effective substrates such as sugarcane molasses, corn steep liquor, and soybean meal as alternative carbon and nitrogen sources offers a feasible and economical strategy for large-scale production of these bioactive compounds (Pandey et al., 2000). This study highlights the importance of coal mine ecosystems as promising sources of diverse actinomycetes with notable antimicrobial potential. Although the bioactive compounds identified may be previously reported, their occurrence in a unique ecological niche and their demonstrated

biological activities reinforce their significance for future biotechnological and pharmaceutical applications.

2. Materials and Methods

2.1. Collection of soil samples from underground Coalmines

Soil samples from coal mines were collected from seven significant sites in Telangana, including Bellampally (BPL), Bhupalpally (BHPL), Godavarikhani (GDK), Kothagudem (KGDM), Sathupally (SPL), Madhamari-Kalyankhani (MM-KK), and Ravindhrakhani (RK). The samples were extracted from a depth ranging from 10 to 15 cm, placed into sterile polythene bags with zip locks, and transported to the laboratory for the purpose of isolating actinomycetes.

2.2. Isolation of actinomycetes

Strains of actinomycetes sourced from different coal mine locations were isolated and subjected to serial dilutions ranging from 10-3 to 10-8. A volume of one milliliter from each dilution was inoculated onto suitable media, including yeast extract-malt extract agar, glycerol asparagine agar, oatmeal agar, and starch casein agar. These media were autoclaved for 15 minutes at 121°C, with the addition of 30µg/l of both nystatin and streptomycin to prevent the growth of fungi and bacteria, followed by the application of the pour plate technique.

2.3. Purification and Maintenance of Isolates

Selected colonies were purified by repeated streaking on fresh Starch Casein Agar plates. Pure cultures were maintained on agar slants at 4 °C.

2.4. Screening for Antibiotic Production

Primary screening of actinomycete isolates for antimicrobial activity was performed using the cross-streak method against test microorganisms viz. S. *aureus, E.coli, S. pneumonia*, *S. paratyphi, B. subtilis, E. aerogenes, P. aeruginosa, M. luteus*, *B. cereus, K. pneumonia C.albicans.* Isolates showing inhibitory activity were subjected to secondary screening using the agar well diffusion method.

2.5. Identification and characterization of selected actinomycetes isolates

2.5.1. Colony morphology

Cultural characteristics were examined by cultivating the organism on oatmeal agar, glycerol asparagine agar, yeast extract-malt extract agar, inorganic salt starch agar, and starch casein agar media for 14 days at a temperature of 30°C. The morphological characteristics, including aerial hyphae, spore chains, spore masses, spore surfaces, the coloration of both aerial and substrate mycelia, as well as the production of diffusible pigments, were evaluated by growing the organism on ISP-4 medium for 7 days and analyzed using a light microscope.

2.5.2. Biochemical and Molecular identification of streptomycetes

The isolated actinomycetes strains from the coalmine soil samples were identified by conventional biochemical tests in support of Bergey's Manual of systematic Bacteriology 1st and 2nd edition (Krieg, 1984, Taiwo and Oso, 2004). The promising strain was further identified by molecular methods.

2.5.3. Isolation of DNA and 16S rDNA sequencing

The promising treptomycetes were cultured in starch casein broth at 28°C, and the cells were harvested for DNA extraction. Genomic DNA was isolated using a lysis buffer containing RNase and lysozyme in Tris-EDTA (pH 9.0), followed by incubation, NaCl addition, SDS treatment, and heat lysis. After cooling, potassium acetate was added, and the mixture was centrifuged. DNA was precipitated with isopropanol and assessed on a 1% agarose gel, showing high-quality genomic DNA. The 16S rDNA gene was amplified using primers 27F and 1492R, producing a ~1500 bp PCR product. The amplicon was purified and sequenced using the BDT v3.1 Cycle Sequencing Kit on an ABI 370x1 Genetic Analyzer. Forward and reverse sequences were aligned to obtain a consensus sequence, which was subjected to BLAST analysis against the NCBI database. The top ten similar sequences were aligned using ClustalW, and a phylogenetic tree was constructed using MEGA 7.

2.6. Fermentation and Production of Secondary Metabolites

A loopful of the selected potent isolate was inoculated into seed medium and incubated at 28-30 °C for 48 h under shaking conditions (120rpm). The seed culture was then transferred into production medium and incubated under similar conditions to allow for secondary metabolite production.

2.7. Extraction of Bioactive Compounds

After fermentation, the culture broth was extracted with an equal volume of ethyl acetate using a separating funnel. The organic phase was collected and concentrated under reduced pressure using a rotary evaporator to obtain crude extracts.

2.8. Production and extraction of antimicrobial compound

The production of bioactive compound was carried out in production medium i.e., C Medium broth and Spore-inoculated Streptomyces suspension and incubating at 200 rpm for 7 days at 280 ±20C in a rotating shaker. After the growth, the antimicrobial compound was extracted with ethyl acetate and was separated in a rotavapor, the left-over residue was collected as antimicrobial compound. The crude extract was then subjected to purification using column chromatography. Elution was carried out using solvent systems of varying polarity. Fractions were collected and analyzed for antimicrobial activity, and active fractions were pooled for further analysis.

2.9. Structural Characterization

2.9.1. FT-IR Spectral studies

The compound KSKU5 of Streptomyces sp is required to undergo spectral IR testing. The IR range was recorded using a PerkinElmer Spectrum 100 FT-IR spectrophotometer, employing the KBr pellet method, with samples scanned across a range of 400-4000 cm-1.

2.9.2. NMR spectral analysis

NMR spectra were obtained using a Bruker Ultra Shield 500 Plus TM spectrometer equipped with a 5mm PABBO probe, employing deuterated methanol or DMSO as the solvent, while tetramethyl silane served as the internal standard. The chemical shifts were measured in parts per million (ppm, δ), and the coupling constants were recorded at 500 mHertz (MHz). The specifics of the experimental conditions were as follows: Operating temperature was set at 280°C; Proton spectra were

captured at 64K (F2), with an acquisition time (AQ) of 3.17 seconds, a spectral width (SW) of 20.66 ppm, a delay time (D1) of 1 second, and a number of scans (NS) equal to 128. (Maskey et al., 2006).

2.9.3. Mass spectrophotometric analysis

Extensive data was collected utilizing the SYNAPT G2 High-Definition Mass Spectrometry (HDMS) system, which was linked to a capillary (Waters ZsprayTMLackSpray) employing electrospray ionization. The capillary voltage was set at 3.0 kV, with cone voltages at 69V, a temperature of 800°C, a desolvation temperature of 3000°C, and a desolvation gas flow rate of 475 L/h. The samples were analyzed in positive ion mode across a mass range of 50 to 2000. (Maskey et al., 2006).

2.10. Antimicrobial Activity of Extracted Compound

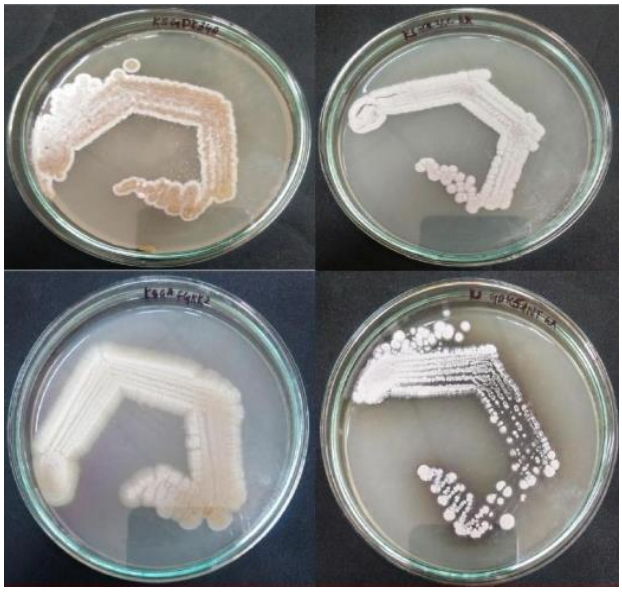
The antimicrobial activity of the purified compound was evaluated using the agar well diffusion method against test pathogens Different concentrations of the compound were tested. Standard antibiotics were used as positive controls, and solvent served as negative control. Zones of inhibition were measured after incubation. Minimum inhibitory concentration (MIC) was determined.

3. Results and Discussion

3.1. Isolation of Actinomycetes

In the present investigation, in order to isolate an actinomycetes with an enhanced yield of antimicrobial compound, soil samples from various coalmine sites in Telangana, were collected and different actinomycetes (fig.1) were isolated and biochemical tests were performed (Table.1) and the promising strain was further identified using molecular methods.

Fig 1. Colony morphology of Actinomycetes isolates from coalmine soil samples



3.2. Biochemical and Molecular identification of potent actinomycetes isolate

Biochemical tests of the isolates viz. KSKU3, KSKU5, KSKU19 and KSKU23 were performed and the results are presented in tabale.1. Among the strains, the *Streptomyces* sp (Fig. 2) showing wide range of antimicrobial activity was further characterized using molecular techniques. PCR amplification of the 16S rDNA chromosome and its structure were administered for molecular identification. The strain sequence was equated to existing EMBL data base species and phylogenetic (Fig.3) relatedness was determined using Maximu likelihood method and received accession number MH553077. Further, SEM image of the strain was taken at various magnifications (Fig.4).

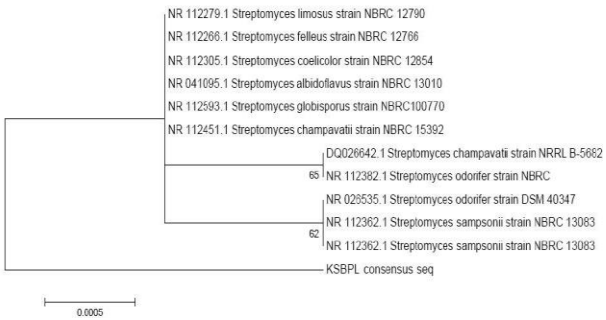
Table 1. Biochemical characteristics of Actinomycetes isolates.

S.No	Name of the test	Ksku3	Ksku5	Ksku19	Ksku23
1	Catalase	+	+	+	+
2	Urease	+	+	+	+
3	Oxidase	-	-	-	+
4	Nitrate reduction	-	-	-	-
5	Gelatin hydrolysis	-	-	-	-
6	Casein hydrolysis	+	+	+	+
7	Starch hydrolysis	+	+	+	+

Fig 2. Streptomyces champavatii(KSKU5)

It

Fig 3. Phylogenetic analysis by Maximum likelihood Method



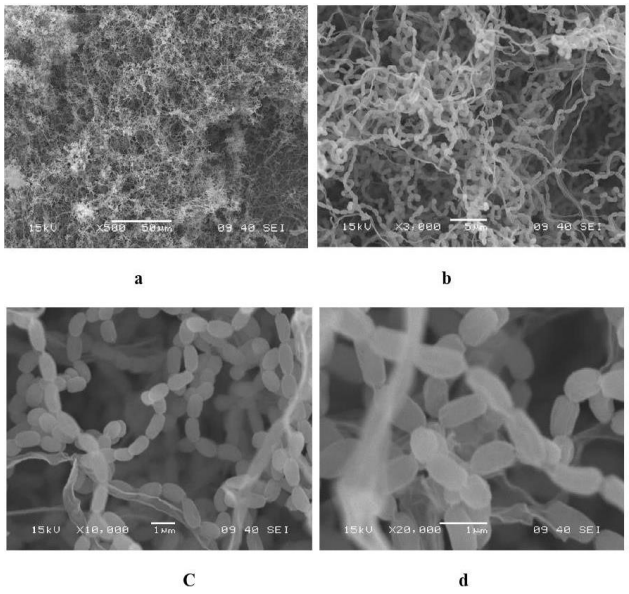
3.3. Extraction and purification of antimicrobial compound

The antimicrobial compound from culture broth of Streptomyces champavati (KSKU5) was extracted using ethyl acetate and subjected to column chromatography and the purified compound was further subjected to thin layer chromatography.

3.4. Structural elucidation of purified antimicrobial compound

The purified antimicrobial compound obtained from the culture extract was subjected to comprehensive spectral analysis, including Fourier Transform Infrared Spectroscopy (FTIR), Nuclear Magnetic Resonance (NMR), and Mass Spectrometry (MS), to elucidate its chemical structure.

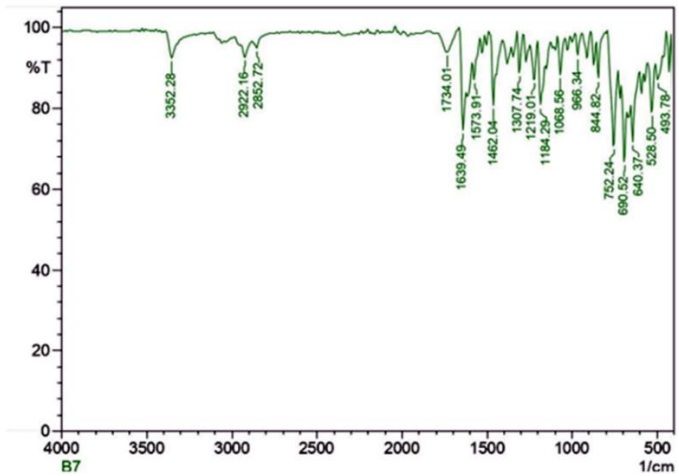
Fig 4. SEM image of Streptomyces champavatii. (KSKU5



3.4.1. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectrum of the purified antimicrobial compound exhibited a broad peak at 3352.28 cm^{-1} corresponding to O–H stretching. Peaks at 2922.16 cm^{-1} and 2852.72 cm^{-1} indicate aliphatic C–H stretching. A strong band at 1734.01 cm^{-1} confirms the presence of a carbonyl (C=O) group, while the peak at 1639.49 cm^{-1} suggests C=C or amide functionality. Absorption bands at 1573.91 cm^{-1} and 1462.04 cm^{-1} are attributed to aromatic ring vibrations. Peaks in the region 1307–1184 cm^{-1} correspond to C–N and C–O stretching, and those between 1089–966 cm^{-1} indicate C–O–C and =C–H bending. The bands at 844–690 cm^{-1} further confirm aromatic substitution. The spectrum indicates the presence of hydroxyl, carbonyl, aromatic, and amine/ether groups, characteristic of bioactive secondary metabolites (Fig.5).

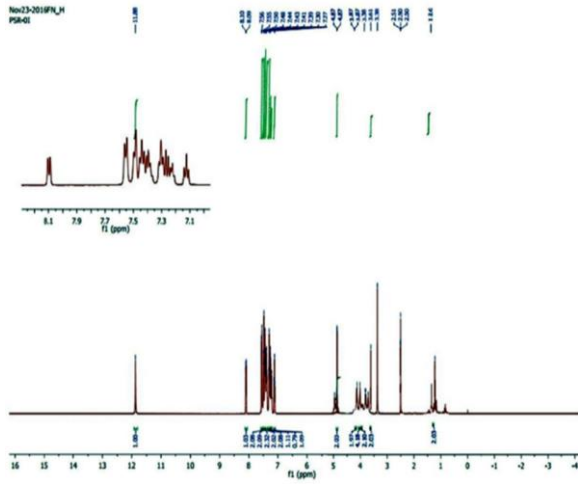
Fig 5. FTIR Spectra of antimicrobial compound isolated from *S. champavatii*



3.4.2 NMR Spectra of the purified antimicrobial compound

The ^{1}H NMR spectrum showed signals in the aromatic region (δ 7.0–8.0 ppm), indicating a substituted aromatic ring system. A downfield signal at δ ~11.9 ppm suggests the presence of an exchangeable proton (–OH/–NH). Peaks in the δ 3.0–5.0 ppm region correspond to protons attached to electronegative atoms, while signals in the δ 1.0–2.5 ppm range indicate aliphatic protons. Overall, the spectrum supports a substituted aromatic compound with aliphatic and heteroatom-linked groups (Fig.6).

Fig 6. NMR Spectra of antimicrobial compound isolated from *S. champavatii*



3.4.3. ^{13}C Spectrum of staurosporine product produced by Streptomyces champavatii (KSKU5)

The ^{13}C NMR spectrum showed signals in the δ 120–150 ppm region indicating aromatic carbons, and a downfield signal around δ 160–180 ppm corresponding to a carbonyl group. Peaks in the δ 50–80 ppm range suggest C–O/C–N carbons, while signals at δ 10–40 ppm indicate aliphatic carbons. Overall, the data confirm the presence of aromatic, carbonyl, and aliphatic functionalities.

Fig 7. T C13 NMR Spectra of antimicrobial compound isolated from *S. champavatii*

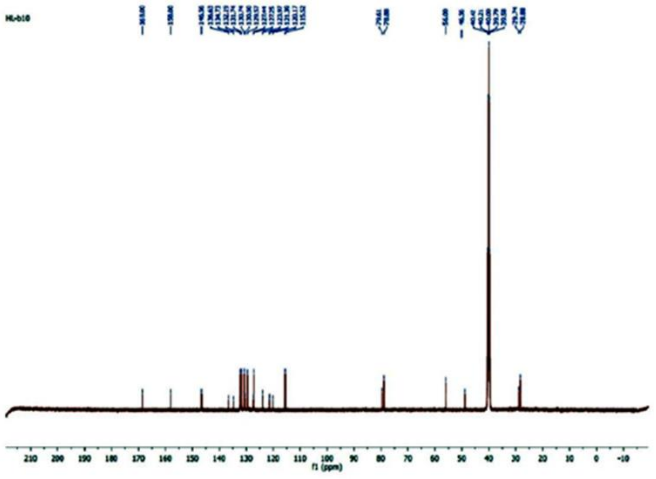


Table 2. Characterization of antimicrobial compound *S. champavatii* (KSKU5)

S.No.	Characters	Staurosporine (*S. champavatii* (KSKU5)
1	Colour	White
2	Nature	Viscous
3	Solubility	Ethyl acetate, DMSO
4	Melting point	140
5	pH stability	4--7
6	Temperature stability	30-40
7	Rf value (TLC) butanol-ethyl acetate water (9:9:1)	0.52
8	IR value	3325, 2922, 1639, 1462 and 1059 cm-1
10	1H NMR	1-4, 2-5, 3.8, 4.05, 7.55, 8.09
11	13C NMR	56-90, 46-90 and 90-160

The antimicrobial compound from *Streptomyces champavatii* (KSKU5) was obtained as a white, viscous substance, soluble in ethyl acetate and DMSO. It showed a melting point of ~140°C, with stability at pH 4–7 and 30–40°C. The Rf value (0.52) in butanol:ethyl acetate:water (9:9:1) indicates moderate polarity. FTIR peaks at 3325, 2922, 1639, 1462, and 1059 cm^{-1} suggest hydroxyl, aliphatic, carbonyl, and aromatic groups. The ^{1}H NMR signals (δ 1–8 ppm) confirm aliphatic and aromatic protons, while ^{13}C NMR (δ 46–160 ppm) indicates aliphatic, oxygenated, and aromatic carbons. These data confirm the compound as Staurosporine (table.2).

Table 3. Anti-bacterial activity and Anti-candidal activity of purified compound

S.No.	Characters	Staurosporine (*S. champavatii* (KSKU5)
1	Colour	White
2	Nature	Viscous
3	Solubility	Ethyl acetate, DMSO
4	Melting point	140
5	pH stability	4--7
6	Temperature stability	30-40
7	Rf value (TLC) butanol-ethyl acetate water (9:9:1)	0.52
8	IR value	3325, 2922, 1639, 1462 and 1059 cm-1
10	1H NMR	1-4, 2-5, 3.8, 4.05, 7.55, 8.09
11	13C NMR	56-90, 46-90 and 90-160

Fig 8. Expected structure of staurosporine produced by *Streptomyces champavatii* (KSKU5)

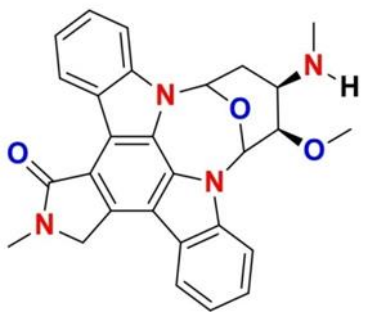


Table 3. Anti bacterial activity and Anti candidial activity of purified compound

S.No.	Name of the test pathogens	Conc. In µg/ml	Zone of inhibition (mm) Staurosporine
1	*B. subtilis*	600	9
		900	12
2	*B. cereus*	600	8
		900	14
3	*S. aureus*	600	10
		900	19
4	*S. pneumonia*	600	8
		900	12
5	*M. luteus*	600	7
		900	13
6	*E. coli*	600	10
		900	18
7	*E. aerogenes*	600	6
		900	10
8	*K. pneumonia*	600	11
		900	19
9	*S. paratyphi*	600	10
		900	17
10	*P. aeruginosa*	600	10
		900	18
11	*C. albicans*	600	9
		900	15

Actinomycetes represent a major component of soil microbiota and are often considered transitional organisms exhibiting characteristics of both bacteria and fungi. They are well known for producing a wide range of bioactive metabolites, including antibiotics, anticancer agents, immunosuppressants, and industrial enzymes (Naine et al., 2011; Newman and Cragg, 2007; Kekuda et al., 2010). Approximately 80% of known antibiotics have been derived from actinomycetes, particularly from the genus Streptomyces (Thakur et al., 2007). These organisms are a prolific source of diverse secondary metabolites with significant activity against pathogenic microorganisms (Durand et al., 2019; Grasso et al., 2016; Xiao et al., 2017).

In the present study, different soil samples were collected from various coal mine regions of Telangana, including Bellampalli, Bhupalpally, Godavarikhani, Kothagudem, Mandamarri Kalyankhani, Sathupally, and Ravindhrakhani. Among the isolates, strain BHPL-KSKU5 from Bhupalpally exhibited the highest antibacterial and antifungal activity. Bhupalpally soil yielded a greater diversity of actinomycetes compared to other sampling sites.

Morphological characterization on starch casein agar revealed spore colors predominantly white (40%), followed by grey (20%), brown (20%), and pale yellow (15%). The major genera identified included Streptomyces, Amycolatopsis, Saccharopolyspora, Microbacterium, and Micromonospora, consistent with earlier reports (Pathalam et al., 2017).

Physiological characterization indicated that the selected strain, identified as Streptomyces champavatii (KSKU5), showed optimal growth at pH 6–8, with no growth at pH 9–10. The strain tolerated 1–3% NaCl, exhibited reduced growth at 4%, and no growth at 5% NaCl, in agreement with previous findings (Palanichamy et al., 2011). Molecular identification using 16S rRNA gene sequencing revealed 99% similarity with *S. champavatii* (NRRLB strain), confirming its taxonomic position (Meena et al., 2013; Dhanasekaran et al., 2005; Vijayakumar et al., 2012).Spectral analysis (FTIR, ^{1}H NMR, and ^{13}C NMR) indicated the presence of functional groups such as amide (–NH), carbonyl (C=O), aromatic (C=C), amine (C–N), and ether (C–O). GC–MS analysis revealed compounds such as isobutyl acetate, butyl acetate, and ethylbenzene, which are commonly associated with Streptomyces metabolites (Rajesh et

al., 2013; Lunavath et al., 2013). The purified compound appeared white and viscous, with a melting point of 140°C, and showed stability at pH 4–7 and 30–40°C. Based on combined spectral data, the compound was identified as Staurosporine. The purified compound exhibited notable antimicrobial activity, showing considerable inhibition against both bacterial and fungal pathogens. The highest activity was recorded against Staphylococcus aureus (20mm), *Pseudomonas aeruginosa* (20mm), and *Escherichia coli* (20 mm), followed by *Salmonella paratyphi* (17 mm), Bacillus cereus (14 mm), Micrococcus luteus (13 mm), Klebsiella pneumonia (10mm), *Bacillus subtilis* (12 mm), Streptococcus pneumoniae (11 mm), and *Enterobacter aerogenes* (10 mm). In terms of fungal pathogens, significant activity was noted against Candida albicans (15 mm) and *Cryptococcus neoformans* (13 mm). Previous studies have also reported similar antimicrobial properties of compounds derived from Streptomyces (Vijayakumar et al., 2012). Candida species are integral components of the normal human microbiota found in the gastrointestinal tract, oral cavity, and vagina; however, in immunocompromised individuals, they can lead to infections that range from superficial mucocutaneous infections to systemic candidiasis (Apsari et al., 2019; Truong et al., 2019). Their ability to adapt to various host environments enhances their potential for pathogenicity. The maximum antimicrobial activity of the compound produced from *S. champavatii* (KSKU5) showed the following effect on *S. aureus* (20mm) followed by *E.coli* (20mm), *S. pneumonia* (14mm), *S. paratyphi* (14mm), *B. subtilis* (13mm), E. aerogenes (9mm), *P. aeruginosa* (20mm), M. luteus (11mm), *B. cereus* (10mm), *K. pneumonia* (10mm) followed by its antifungal inhibitory effects on C.albicans(15mm) and *C. neoformans* (13mm).The zone of inhibition of different microorganisms is shown in table.3

4. Conclusion

The current research effectively illustrated the isolation and characterization of an antibiotic-producing actinomycete, specifically identified as Streptomyces champavatii, sourced from coal mine soil. The bioactive compound that was isolated was recognized as Staurosporine through spectral analysis. This compound demonstrated considerable antimicrobial activity against various pathogenic microorganisms, suggesting its broad-spectrum effectiveness. These results underscore the potential of uncharted and extreme environments, such as coal mine ecosystems, as abundant sources of novel bioactive metabolites. Furthermore, the study emphasizes the significance of actinomycetes as prolific producers of compounds that are important in pharmaceuticals.

In summary, the identified strain and its metabolite present promising opportunities for the development of new antimicrobial agents aimed at addressing the increasing challenge posed by multidrug-resistant pathogens.

Competing interests:

The authors declare that they have no competing interests

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