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SELECTIVE ISOLATION AND CHARACTERIZATION OF *NOCARDIOPSIS FLAVESCENS* VJMS-18 FROM COASTAL REGIONS OF ANDHRA PRADESH, INDIA

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ABSTRACT: Objective: To evaluate the antimicrobial potential of *Nocardiopsis flavescens* isolated from the marine environment. **Methods:** Attempts were made to isolate actinomycete strains from marine coastal regions of Andhra Pradesh, India. The soil samples collected were pre-treated with calcium carbonate, diluted and plated on three different media to isolate actinomycetes. All the isolates were screened for antimicrobial activity by using the Kirby-Bauer disc diffusion method. Among the fifty isolates screened, one potent strain designated as VJMS-18 exhibited high antimicrobial activity. The isolate was identified by using micro-morphological, cultural, biochemical, physiological and molecular approaches. **Results:** The Potent strain was identified as *Nocardiopsis flavescens* VJMS-18. It showed good growth on YMD agar amended with 3% NaCl. Growth pattern of the isolate was studied by culturing it in YMD broth. The culture broth was extracted with ethyl acetate and tested for antimicrobial activity. It exhibited high antimicrobial activity against gram positive and gram negative bacteria as well as fungi. **Conclusion:** The present investigation reveals that *Nocardiopsis flavescens* VJMS-18 isolated from the coastal environment is the potential source of novel bioactive compounds.

INTRODUCTION: Marine domain is the prime repository of biological diversity and the marine microorganisms are recognized to be major sources of novel compounds. Marine life furnishes a repertoire of rare actinomycetes that can be a virtual source of drugs [1]. It is indisputable that new drugs, distinctly antibiotics are desired to halt and contrary to the persistent disperse of antibiotic-resistant pathogens which cause vitality threatening infections [2]. Distinct antibiotics were derived from marine actinomycetes [3] and at present two-thirds of natural antibiotics are obtained from actinomycetes [4].

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Several studies on the diversity of actinomycetes in marine environments have emerged in the isolation of *Nocardiopsis* species [5, 6, 7, 8, 9]. Studies have shown that *Nocardiopsis* is ubiquitously dispersed across a diverse range of environments such as deserts, marine habitats, plant tissues, animal guts and indoor environments [10, 11].

Nocardiopsis species produce different types of pharmacological compounds with anti-oxidant, anti-tumor, anti-inflammatory, anti-bacterial and anti-oxidant properties. On the premise of the phylogenetic position, morphological features and chemotaxonomic properties are shown by *Nocardiopsis*, a new family for the genus referred to as 'Nocardiopsaceae*'* was fabricated [12]. The genus '*Nocardiopsis*' was phylogenetically articulated and characterized as a distinct lineage within the emanation of the order 'Actinomycetales'.

Nocardiopsis is anaerobic, gram-positive, non-acid-fast and catalase-positive actinomycete. Colonies on organic media agar plates are coarsely wrinkled or folded. The substrate and aerial mycelia are well-developed and the latter fragments into spores. Members of the genus *Nocardiopsis* are ecologically adaptable and biotechnologically influential. The plethora of *Nocardiopsis spp.* obtained from different ecological niches (soils, marine forms, marine sediments and mine tailings) produce a variety of antibiotics includes quinoline alkaloids, p-terphenyl derivatives, novobiocin, phenazinediol, cyclic tetrapeptides, nocardio-pyrones, thiopeptides and proteins [13]. As part of our ongoing screening of different habitats of marine ecosystems of Andhra Pradesh, India resulted in the isolation of potent strain VJMS-18 with broad-spectrum activity against different gram positive and gram negative bacteria as well as fungi. An attempt was made in the present study to identify the strain based on the polyphasic taxonomic approach along with its antimicrobial profile.

MATERIALS AND METHODS:

Marine Samples Collection: Soil samples were collected at a depth of 6-10 cm from south-coastal regions of Andhra Pradesh, India [14]. The samples were packed in sterile polyethylene bags and aseptically transported to the laboratory for further analysis.

Pre-treatment of Samples: The samples were subjected to pre-treatment to facilitate isolation of actinomycetes. The collected samples were air dried and pre-treated with calcium carbonate (1%) and incubated at 35 °C for 2 weeks[15].

Isolation of the Marine Actinomycetes: Marine actinomycetes were isolated by serial dilution method [16, 17]. The stock solution was prepared by diluting 1 g of the soil sample in 100 ml of sterile saline water and shaken well-using vortex mixer. From the stock solution, 10^{-3} and 10^{-4} dilutions were made by serial dilution method. The diluted sample (0.1 ml) was spread on the surface of three different media (g/L):

- Yeast extract Malt extract Dextrose Agar - yeast extract: 4.0; malt extract: 10.0; dextrose: 4.0; agar: 20.0. [18]
- Starch Casein Agar - starch: 10.0; casein: 0.3; KNO_3: 2.0; NaCl: 2.0; K_2HPO_4: 2.0; $MgSO_4.7H_2O$: 0.05; $CaCO_3$: 0.02; $FeSO_4.7H_2O$: 0.01; agar: 18.0. [19]
- Humic-Acid Vitamin-B Agar - Humic acid: 1.0; Na_2HPO_4: 0.5; KCL: 1.7; $MgSO_4.7H_2O$: 0.05; $FeSO_4.7H_2O$: 0.01; $CaCO_3$: 0.01; Agar: 18. [20]

Nystatin (50 mg/mL) and streptomycin (25 mg/mL) were added to each medium to inhibit fungal and bacterial contamination respectively. Plates were incubated at 35 °C for 7-20 days. The colonies showing the characteristics of actinomycetes (rough, chalky, powdery appearance with radiating growth and leathery texture) were observed [21, 22]. The pure cultures were maintained on YMD agar slants at 4 °C. The isolated actinomycete strains were screened for their ability to produce antimicrobial compounds. Among the 50 isolates tested for bioactive compounds, the isolate designated as VJMS-18 was found potent compared to other strains.

Taxonomy of Potential Actinomycete Strain: Taxonomic studies were performed based on micro-morphological, cultural, biochemical, physiological and molecular analyses.

Morphological and Cultural Characters of Strain VJMS-18: Morphology studies were performed by using the methods described by Shirling and Gottlieb [18]. The spore-bearing hyphae and arrangement of spores were observed by the coverslip method. The morphology of the mycelium and spore surface was observed using a scanning electron microscope (SEM: JOELJSM 5600, Japan) [23].

The cultural characteristics were examined by growing isolate on different International Streptomyces Project (ISP) media: Tryptone yeast extract agar (ISP-1), yeast extract-malt extract dextrose agar (ISP-2), oatmeal agar (ISP-3), inorganic salts starch agar (ISP-4), glycerol asparagine agar (ISP-5), tyrosine agar (ISP-7) and Non-ISP media including czapek-Dox agar, nutrient agar, starch casein salts agar, glucose tryptone agar and humic-acid vitamin-B agar. In each medium color and growth of aerial and substrate mycelium as well as the formation of soluble pigments were noted after incubation at 35°C for 7 days.

Physiological and Biochemical Characters of Strain VJMS-18: The ability of the selected isolate to utilize 10 different carbon sources was determined on YMD agar plates amended with carbon sources @ 1%. The plates were incubated at 35 °C for 7 days [18]. The production of melanoid pigments was tested on ISP-7. The actinomycete isolate was also tested for its ability to grow at different concentrations of NaCl (0-12%), at different temperatures (30-45 °C) and different levels of pH (5-9 %) [18]. Biochemical tests such as H_2S production [24], gelatin liquefaction, starch hydrolysis, catalase production, indole, methyl red, Voges - Proskauer, citrate utilization, nitrate reduction [25], casein hydrolysis and triple sugar iron tests were also carried out. The sensitivity of the isolate to different antibiotics was also determined by paper disc method [26].

Screening of the Strain VJMS-18 for Extra-cellular Enzyme Production: The strain VJMS-18 was inoculated on the agar medium incorporated with substrates such as carboxyl methyl cellulose, starch casein agar, skim milk, asparagine, glutamine and tween 20 for the production of enzymes including cellulase, amylase, protease, asparaginase, glutaminase and lipase respectively. Plates were incubated at 35 °C for 7 days. Appropriate indicator solutions were flooded to determine the production of enzymes.

Molecular Identification: The genomic DNA used for the polymerase chain reaction (PCR) was prepared from the colonies grown on YMD agar for 3 days. The total genomic DNA extracted from the isolate was isolated by employing the DNA purification Kit (Pure Fast® Bacterial Genomic DNA purification kit, Helini Biomolecules, India) according to the manufacturer protocol. Conditions of the PCR were standardized with initial denaturation at 94 °C for 3 min followed by 30 cycles of amplification (Denaturation at 94 °C for 60 sec, annealing temperature of 55 °C for 60 sec and extension at 72 °C for 60 sec and an addition of 5 min at 72°C as final extension). The amplification reactions were carried out with a total volume of 50µl in a gradient PCR (Eppendorf, Germany). Each reaction mixture contained 1 µl of DNA, 1 µl of 10 *P* mol forward 16S actino specific primer (5'AAATGGAGGAAGGTGGGGAT-'3), 1 µl of 10 *P* mol reverse 16S action specific primer (5'- AGGAGGTGATCCAACCGCA-'3), 25 µl of master mix and 22 µl of molecular grade nuclease-free water. The separation was carried out at 90 Volts for 40 min in TAE buffer with 5 µl of ethidium bromide. PCR product was analyzed using agarose gel (1%) and the fragment was purified (Helini Pure Fast PCR clean up kit, Helini Biomolecules, India) as per the manufacturer's instructions. The bands were analyzed under UV light and documented using Gel Doc. The direct sequencing of PCR products was performed by the dideoxy chain termination method using 3100-Avant genetic analyzer (Applied Biosystems, USA).

PairWise Sequence Alignment: The gene sequence of the isolate VJMS-18 was aligned using BLAST against the gene library available for *Nocardiopsis* species in the NCBI and the GenBank. Pairwise evolutionary distances were computed by MEGA-6 software.

Multiple Sequence Alignment: The phylogenetic analysis was conducted using the maximum parsimony method of the isolate using BLAST and CLUSTAL W. The closely related homologous isolates were identified, retrieved and compared to the sequence of the isolated strains using CLUSTAL W available with the MEGA 6 Version [27].

Nucleotide Sequence Accession Numbers: The 16S rRNA gene sequence of the isolate VJMS-18 was registered in the GenBank database.

Growth Pattern of VJMS-18 Strain: The selected antagonistic isolate VJMS-18 was inoculated into YMD broth and incubated at 35 ºC for ten days on a rotary shaker at 180 rpm. The flasks were harvested at 24 h interval and the growth of the isolate was determined by taking the dry weight of biomass. After incubation, the broth was filtered through Whatman no.1 filter paper. To the culture filtrate, an equal volume of ethyl acetate was added and shaken vigorously for about 20 min. The solvent extract was then concentrated in the rotary vacuum and tested for antimicrobial activity against selected microorganisms listed below:

Bacteria: *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (ATCC 6633), *Bacillus megaterium* (NCIM 2187), *Xanthomonas*

campestris (MTCC 2286), *Proteus vulgaris* (ATCC 6380), *Pseudomonas aeruginosa* (ATCC 9027) and *Escherichia coli* (ATCC 9027).

Fungi: *Aspergillus flavus* (ATCC 189), *Candida albicans* (MTCC 183) and *Penicillium citrinum* (MTCC 6849).

The bacterial cultures were plated on nutrient agar medium and incubated at 37 °C for 24 h and fungal cultures were plated on Sabouraud dextrose agar medium at 35 °C for 48 h.

Assay of Antimicrobial Activity: The antimicrobial activity of solvent extract obtained from isolate VJMS-18 was tested by agar diffusion assay. Ethyl acetate itself was used as negative control. The wells were made by using sterile cork borer (6 mm diameter). The activity was evaluated by adding 80 µl of the extract to solidified agar medium seeded with test organisms. The plates were incubated at 37 °C for 24 h during which activity was evidenced by the presence of a zone of inhibition surrounding the well.

Each test was repeated three times and the antimicrobial activity was expressed as the mean of the diameter of inhibition zones (mm) produced by the secondary metabolites when compared to control. In the case of fungi, the petri plates were incubated at 30 °C for 2 days. At the end of 48 h, inhibition zones formed in the medium were measured.

RESULTS AND DISCUSSION: The present study was designed to investigate the marine coastal regions of Andhra Pradesh for novel actinomycetes and their antimicrobial properties. Fifty actinomycete strains (VJMS1-50) isolated from the marine habitats were screened for antimicrobial activity. One strain designated as VJMS-18 was found to exhibit high antagonistic activity against the microorganisms tested. The strain VJMS-18 exhibited typical morphological characteristics of the genus *Nocardiopsis.*

Morphological and micromorphological observation of the strain revealed that aerial hypha was abundant with well-developed long fragmented hyphae with rod-shaped smooth surfaced spores. Soluble pigment production by the strain was not found on tyrosine agar medium.

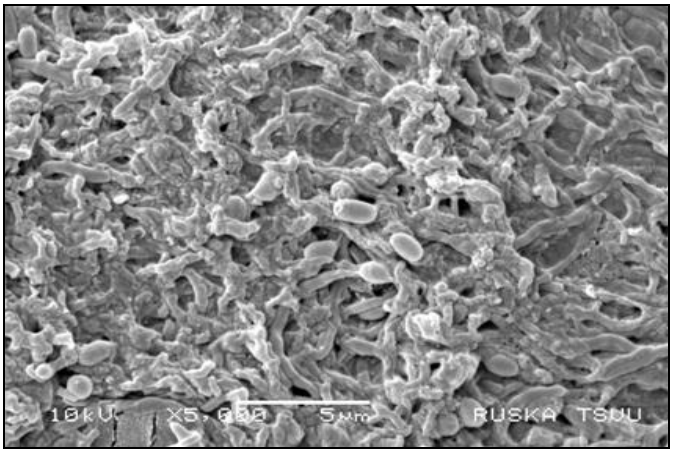

FIG. 1: SCANNING ELECTRON MICROSCOPIC PHOTOGRAPH OF *NOCARDIOPSIS FLAVESCENS* VJMS-18

Cultural Characteristics: The cultural characteristics of the strain are represented in **Table 1**. The strain VJMS-18 exhibited good growth on ISP-1, ISP-2, nutrient agar and humic-acid vitamin-B agar. The growth was moderate on ISP-4 agar while it was poor on ISP-3, ISP-5, Czapek-Dox agar, ISP-7 and glucose-tryptone agar.

TABLE 1: CULTURAL CHARACTERISTICS OF THE STRAIN VJMS-18

Name of the medium	Growth	AM*	SM**	Pigmentation
Tryptone yeast extract agar (ISP-1)	Good	Creamy white	Pale yellow	No
Yeast extract-malt extract dextrose agar (ISP-2)	Good	Creamy white	Pale yellow	No
Oat-meal agar (ISP-3)	-	-	-	No
Inorganic salts starch agar (ISP-4)	Moderate	white	Pale yellow	No
Glycerol asparagine agar (ISP-5)	-	-	-	No
Tyrosine agar (ISP-7)	-	-	-	No
Czapek-Dox agar	-	-	-	No
Nutrient agar	Good	Creamy white	Pale yellow	No
Starch casein salts agar	Good	Creamy white	Pale yellow	No
Glucose tryptone agar	-	-	-	No
Humic-acid vitamin-B agar	Good	Creamy white	Pale yellow	No

AM- Aerial mycelium, SM- Substrate mycelium.-: No growth, ISP: International Streptomyces Project

Creamy white aerial mycelium and pale yellow substrate mycelium were found on ISP-1, ISP-2, nutrient agar and humic acid vitamin-B agar. The strain could not grow on ISP-3, ISP-5, ISP-7, Czapek-Dox agar, and glucose tryptone agar media.

Biochemical Characteristics of VJMS-18: The strain VJMS-18 exhibited a positive response to indole, methyl red, vogues-proskauer, citrate utilization tests, nitrate reduction and urease production but negative for hydrogen sulphide, catalase production, triple sugar iron and gelatine liquefaction. The details of morphological, physiological and biochemical characteristics of the isolate are given **Table 2**.

TABLE 2: MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF THE ISOLATE VJMS-18

Character	Response
Morphological characters	VJMS-18
Sporophore morphology	Recti flexible
Color of aerial mycelium	Creamy white
Color of substrate mycelium	Pale yellow
Biochemical characters	
Catalase production	-
Urease production	+
Hydrogen sulfide production test	-
Nitrate reduction	+
Starch hydrolysis	-
Gelatin liquefaction	-
Methyl red test	+
Voges proskauer test	+
Indole production	+
Citrate utilization	-

TABLE 3: UTILIZATION OF CARBON SOURCES BY THE STRAIN VJMS-18

Character	Response
Utilization of carbon sources (w/v)*	
Lactose	++
Maltose	+++
Arabinose	-
Sucrose	+ +
Sorbitol	+++
D-Glucose	++
Galactose	++
Fructose	-
Starch	++
Mannitol	++
Cellulose	-
Mannose	-

* Growth of the strain measured as the dry weight of the mycelium '+++' -good growth; '++' -moderate growth; '+'-weak growth; '-'indicates negative/no growth.

Utilization of carbon sources by the strains could be used as an aid for species determination [28]. The strain VJMS-18 efficiently utilized the carbon sources such as D-glucose, lactose, maltose, sucrose, galactose, sorbitol, mannitol and starch but could not utilize fructose, arabinose, cellulose and mannose **Table 3**. It was also reported that the antibiotic sensitivity of some nocardioform bacteria as one of the valuable criteria for their taxonomic differentiation [29]. Antibiotic susceptibility testing showed that the isolate was susceptible to imipenem, chloramphenicol, clindamycin, tetracycline and cefixime but resistant to gentamicin, vancomycin, cefepime, amaickin and penicillin **Table 4**.

TABLE 4: ANTIBIOTIC SENSITIVITY OF THE STRAIN VJMS-18

Antibiotic sensitivity	Response
Gentamicin (10 µg)	R
Vancomycin (30 µg)	R
Penicillin (10 µg)	R
Clindamycin (25 µg)	S
Chloramphenicol (50 µg)	S
Cefepime (30 µg)	R
Imipenem (10 µg)	S
Cefixime (30 µg)	S
Tetracycline (30 µg)	S
Amikacin (10 µg)	R

*S-Sensitive; R-Resistant

Physiological Characteristics: The physiological tests are indispensable tools for classification and identification of actinomycetes and influencing their growth [30, 31, 32, 33].

TABLE 5: PHYSIOLOGICAL AND ENZYMATIC CHARACTERS OF THE STRAIN VJMS-18

Physiological characters	Response
Gram reaction	+
Production of melanin pigment	-
Range of temperature for growth	30-45 °C
Optimum temperature for growth	35 °C
Range of pH for growth	5.0-9.0
Optimum pH for growth	7.0
NaCl tolerance	Up to 12%
Enzymatic activity	
Amylase	P
Protease	P
Cellulase	N
Asparaginase	P
Glutaminase	P
Lipase	N

*P–Positive; N–Negative.

The strain VJMS-18 can grow in the pH range of 6-9 with the optimum being 7.0. The temperature range for growth was 30-45 °C with the optimum at 35°C. The strain exhibited salt tolerance up to 12% with optimum growth at 3% NaCl; hence the strain

could be placed in intermediate salt tolerance group [34] **Table 5**. The strain VJMS-18 could also produce enzymes like L-asparaginase, glutaminase, amylase and protease.

Molecular characterization of the strain VJMS-18: The 16S rRNA sequence data supported the assignment of this isolate VJMS-18 to the genus *Nocardiopsis* and species *flavescens*. The partial 16S rRNA sequence of the strain VJMS-18 was obtained and submitted to the GenBank database under an accession number MH423862. The partial sequence was aligned and compared with all the 16S rRNA gene sequence available in the GenBank database by using the multisequence advanced BLAST comparison tool that is available in the website of National Centre for Biotechnology Information. The highest 16S rRNA sequence similarity value of 100% was obtained for the *Nocardiopsis flavescens*. The phylogenetic analysis of the 16S rRNA gene sequence was aligned using the CLUSTAL W programme from the MEGA 6 Version.

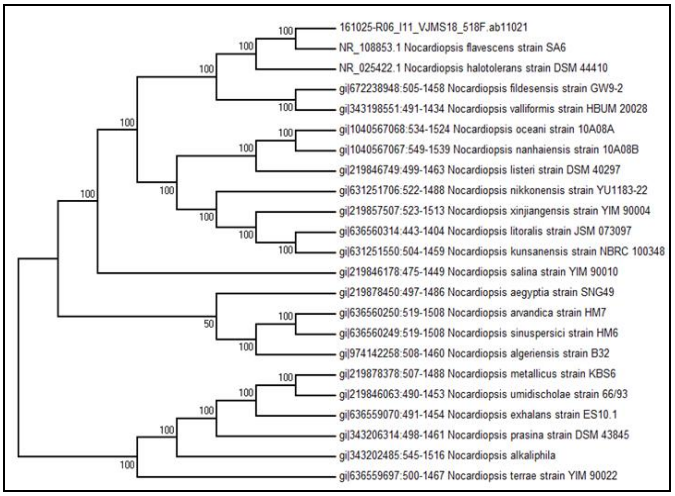

FIG. 2: MAXIMUM PARSIMONY TREE-BASED ON PARTIAL 16S rRNA GENE SEQUENCE SHOWING RELATIONSHIP BETWEEN ISOLATE VJMS-18 AND RELATED MEMBERS OF THE GENUS *NOCARDIOPSIS*

Phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software Version 6 using maximum parsimony method [35, 36, 37]. The topologies of the constructed tree were evaluated by bootstrap analysis with 1000 resamplings by maximum parsimony tool. Sequence comparison of the strain VJMS-18 with the corresponding sequences of the close representative strains of *Nocardiopsis* from the GenBank database showed that this strain formed a close distinct phyletic line with clade encompassed by *Nocardiopsis flavescens, Nocardiopsis halotolerens* and *Nocardiopsis fidesensis* **Fig. 2**.

Growth Pattern and Antimicrobial Potential of *Nocardiopsis flavescens* VJMS-18: The growth curve and antimicrobial profile of *Nocardiopsis flavescens* VJMS-18 were studied at regular intervals up to 10 days in batch culture. The stationary phase of the isolate extended from 192 h to 216 h of incubation. The secondary metabolites obtained from 8-day-old culture showed high antimicrobial activity against the test microbes **Fig. 3**. Usha kiranmayi *et al.*, (2016) noted the production of antimicrobial metabolites from an 11-day-old culture of *Nocardiopsis litoralis* VSM-8 [38]. Saba *et al.*, (2016) stated that secondary metabolites obtained from the 4-day-old culture of *Nocardia metallicus* VJSY-14 showed high antimicrobial activity [39]. Prasad *et al.*, (2016) reported that 5-day old-culture of *Nocardiopsis halotolerans* VJPR-2 exhibited high antagonistic activity [40]. Kavitha *et al.* (2009) noted the production of antimicrobial metabolites from a 4-day-old culture of *Nocardia levis* MK-VL_113 [41].

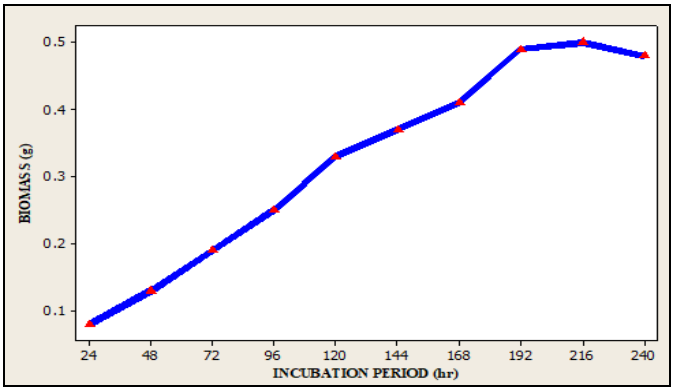

FIG. 3: GROWTH PATTERN OF THE STRAIN *NOCARDIOPSIS FLAVESCENS* VJMS-18

The secondary metabolites obtained from five-day-old culture of *Arthrobacter kerguelensis* VL-RK-09 [42] and *Streptomyces cellulosae* VJDS-7 [43] showed high antimicrobial activity against the test microbes. The antimicrobial spectrum of the isolate cultured on YMD broth is presented in **Table 6**. The metabolites extracted from 8-day-old culture broth showed maximum activity against *B. megaterium*, *B. subtilis*, *S aureus*, *E. coli* and *P. vulgaris*. In case of fungi, *A. flavus* showed high sensitivity when compared to *C. albicans* and *P. citrinum*.

TABLE 6: ANTI-BACTERIAL AND ANTI-FUNGAL ACTIVITY OF STRAIN *NOCARDIOPSIS FLAVESCENS* VJMS-18

	Test organism	Zone of Inhibition (mm)
	Bacteria	
1	*Staphylococcus aureus*	21
2	*Escherichia coli*	19
3	*Xanthomonas campestris*	17
4	*Pseudomonas aeruginosa*	17
5	*Bacillus megaterium*	20
6	*Bacillus subtilis*	22
7	*Proteus vulgaris*	18
	Fungi	
8	*Candida albicans*	16
9	*Aspergillus flavus*	17
10	*Penicillium citrinum*	15

CONCLUSION: The present investigation highlights the anti-microbial potential of *Nocardiopsis flavescens* VJMS-18. Further study on optimization, purification and chemical characterization of bioactive compounds of the isolate is in progress.

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CONFLICT OF INTEREST: Nil

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