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ISOLATION, IDENTIFICATION AND ANTIMICROBIAL POTENTIAL OF MARINE *Streptomyces* spp FROM COASTAL REGIONS OF ANDHRA PRADESH, INDIA

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ABSTRACT

Studies on bioactive actinobacteria from saline soil are very scanty and in the present study an initiative has been taken to isolate culturable halophilic actinobacteria and to screen their bioactive potential. The marine sediment samples were collected from the coastal regions of Andhra Pradesh, India and isolations were carried out by using Yeast extract malt extract dextrose (YMD) agar medium. Identification of the strains was carried out by polyphasic taxonomical studies including morphological, cultural, physiological, biochemical characters along with16S rRNA analysis. The isolates were subjected to fermentation and the crude extract was screened for antimicrobial activity by agar diffusion assay. The 16S rRNA sequence of the isolates showed 100% similarity with Streptomyces vinaceusdrappus and Streptomyces rectiverticillatus. The growth was maximal in the designed production medium with the incubation temperature of 37ºC and pH of 7.0 with 3% NaCl. The crude extracts of these isolates also exhibited significant antagonistic activity against fungi (Aspergillus flavus, Candida albicans and Penicillium citrinum) and Gram-negative bacteria (Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris and Xanthomonas campestris) as well as Gram positive bacteria (Staphylococcus aureus, Bacillus megaterium and Bacillus subtilis). The actinobacterial strains isolated from marine sediments were identified as Streptomyces vinaceusdrappus VJMS-4 and Streptomyces rectiverticillatus VJMS-8 which were moderately halophilic and produced extracellular bioactive metabolites, active against fungal and bacterial pathogens. Further studies on purification and characterization of the bioactive compounds from the strains are in progress. The results of this study suggested that the marine actinobacteria from the unexplored Indian coast could provide lead compounds of therapeutic value.

KEY WORDS

Marine ecosystems, Phylogenetic study, Streptomyces vinaceusdrappus VJMS-4, Streptomyces rectiverticillatus VJMS-8, Bioactive compounds

INTRODUCTION

The marine environment is one of the admired and atypical repositories of bioactive natural compounds, which unveil structural / chemical features not found in terrestrial natural products [1]. This marine environment is a potential natural resource of actinobacteria worth investigated in search of new actinobacteria with biopharmaceutical importance. Marine actinobacteria are attracting much attention due to structural diversity, unique biological activity and functionally active secondary metabolites, which are counterparts to terrestrial sources. The recent reports showed that this group of microorganisms still remains dynamic origin of antibiotics. As a result of the increasing ubiquity of antibiotic resistant pathogens and the pharmacological failure of antibiotics, there is need to investigate new bioactive metabolites from

actinobacteria for potential pharmaceutical and industrial applications [2, 3].

Several approaches including cultural, genomic and metagenomic analysis have been engaged for the isolation of novel bioactive compounds from actinobacteria [4]. These members have an unparalleled ability to produce diverse secondary metabolites [5]. They are efficient producers of new secondary metabolites that show a broad range of biological activities including antibacterial, antifungal, anticancer, antitumor, cytotoxic, cytostatic, anti-inflammatory, anti-parasitic, anti-malaria, antiviral, antioxidant, anti-angiogenesis, anti-HIV, neurological, immunosuppressive agents, enzymes, enzyme inhibitors and pigments [6].

In fact, the distribution of actinobacteria in the sea remain largely undescribed, and even today, conclusive evidence that these bacteria play important ecological roles in the marine environment have remained elusive. Early evidence supporting the existence of marine actinobacteria came from the description of *Rhodococcus marinonascene*, the first marine actinobacterium characterized [7]. Several other novel bioactive compounds were discovered from aquatic actinobacteria including rifamycin from *Micromonospora* sp. [8]; salinosporamide-A, an anticancer metabolite from *Salinispora* sp. [9]; marinomycins from *Marinophilus* sp. [10]; abyssomicin-C from *Verrucosispora* sp. and marinopyrroles from *Streptomyces* sp [11]. It is evident from these reports that marine actinobacteria not only have several new species, but also have plenty of novel structures with potent bioactivities [12]. These natural products investigation and functional gene screening of actinobacteria associated with marine organisms revealed that they can synthesize numerous natural products including polyketides, isoprenoids, phenazines, peptides, indolocarbazoles, sterols and others [13]

Marine actinobacteria are one of the best sources of secondary metabolites and the vast majority of these compounds are derived from the single genus *Streptomyces*, whose species are distributed widely in the marine and terrestrial habitats and are of commercial interest due to their unique capacity to produce novel metabolites [14].The genus, *Streptomyces* is responsible for the formation of more than 60% of known industrially important compounds such as β-lactams, tetracyclines, aminoglycosides, glycopeptides, macrolides, aminocyclitols, cyclic peptides, lincosamides, glycolipopeptides, streptogramines, ansamycines, chloramphenicol and phenylpropanoids [15, 16].

As part of our ongoing screening of different habitats of marine ecosystems of Andhra Pradesh, India resulted in the isolation of potent strains VJMS-4 and VJMS-8 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 strains based on the polyphasic taxonomic approach along with their antimicrobial profile.

MATERIALS AND METHODS

Sampling and isolation of actinobacteria: The marine sediment samples were collected at the depth of 6 to 10 cm from the coastal regions of Andhra Pradesh, India and brought to the laboratory in sterilized zip lock bags and air-dried at room temperature. The air-dried soil samples were pretreated with calcium carbonate (10:1 w/w) and incubated at 37°C for 4 days [17].

Selective isolation of actinobacteria:

The pretreated soil samples were serially diluted in sterile distilled water and plated on selective media such as Yeast extract malt extract dextrose (YMD) agar media supplemented with 50% sea water and 3% NaCl (18). The medium was adjusted to pH 7.0 and 0.1 mL of diluted sample was spread over YMD agar supplemented with nystatin (25 µg/mL) and streptomycin (25 µg/mL) to reduce fungal and bacterial contamination respectively and incubated at 30±2°C for 3 weeks. Actinobacterial colonies were picked out [19], subcultured and preserved on YMD agar slants at 4°C [20]. The isolated actinobacterial strains were screened for their ability to generate bioactive compounds. Among the 40 isolates tested for bioactive compounds, two isolates designated as VJMS-4 and VJMS-8 were found potent compared to other strains.

Identification of the potent strains VJMS-4 and VJMS-8 by polyphasic taxonomy:

Morphological, Cultural, Physiological and biochemical characteristics of the strains

The potent actinobacterial strains were characterized by cultural, morphological, physiological, biochemical and molecular methods. The colors, nature of mycelium and spore arrangement of the strains were observed by slide culture technique [17]. The morphological

characteristics were assessed using scanning electron microscopy (SEM: Model- JOELJSM 5600, Japan) of 4-day old culture grown on YMD agar medium. The strains were grown on seven International Streptomyces Project (ISP) media and four non-ISP media to observe the cultural characteristics such as color of aerial mycelium, substrate mycelium, pigment production, and spore formation [18]. Melanin pigment production was observed by culturing the strains on tyrosine agar (ISP-7) medium [19]. Hydrolysis of starch, nitrate reduction [20] and H_2S production were also evaluated [21]. Physiological characteristics such as influence of pH (5-9), temperature (20-45°C) and effect of NaCl on growth of strains were analyzed. The susceptibility of the strains to different antibiotics was also determined by paper disc method [22]. The ability of the strains to produce industrially important enzymes such as amylase, cellulase, asparaginase, protease and glutaminase was also tested.

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 was extracted from the strains employing the DNA purification Kit (Pure Fast® Bacterial Genomic DNA purification kit, Helini Bio molecules, India) according to the manufacturer protocol. Conditions of the PCR were standardized with initial denaturation at 94°C for 3 minutes followed by 30 cycles of amplification (Denaturation at 94°C for 60 seconds, annealing temperature of 55°C for 60 seconds, and extension at 72°C for 60 seconds and an addition of 5 minutes 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 actino 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 minutes in TAE buffer with5 µL of ethidium bromide. PCR product `was analyzed using 1% agarose gel, and the fragment was purified (Helini Pure Fast PCR clean up kit, Helini Bio molecules, 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 dideoxy chain termination method using 3100-Avant genetic analyzer (Applied Biosystems, USA).

Pair wise sequence alignment

The gene sequences of the strains were aligned using BLAST against the gene library available for *Streptomyces* 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 strains using BLAST and CLUSTAL W. The closely related homologous strains were identified, retrieved and compared to the sequence of the isolated strains using CLUSTAL W available with the MEGA 6 Version [23].

Nucleotide sequence accession numbers

The 16S rRNA gene sequence of the strains VJMS-4 and VJMS-8 were registered in the GenBank database.

Growth pattern of the strains VJMS-4 and VJMS -8

For determination of growth pattern, the strains were inoculated into 250 ml flasks containing 100 ml YMD broth and incubated at 30±2°C on a rotary shaker at 180 rpm. The flasks were harvested at 24 hr interval and growth of the strains was determined by taking dry weight of biomass. The culture filtrates obtained after separating the biomass were extracted with ethyl acetate and antimicrobial activity of the crude extracts was determined by agar well diffusion method.

Extraction of metabolites and antimicrobial assay

The antimicrobial activities of the strains were determined by agar well diffusion assay. The homogenous culture suspension prepared by suspending 3-day-old culture in sterile saline was used to inoculate YMD broth (seed medium) and the culture was incubated at 30°C for 48hr on a rotator shaker at 180 rpm. Seed culture at the rate of 10% was transferred to YMD broth (fermentation medium). The fermentation was carried out at 30±2°C for 120 hr under agitation at 180 rpm. The antimicrobial compound was recovered from the filtrate by solvent extraction method. Ethyl acetate was added to the filtrate (1:1) and shaken vigorously. The ethyl acetate extract was evaporated to dryness in water bath and the residue thus obtained was used to determine antimicrobial activity. Ethyl acetate itself was used as negative control. About 80 µl of the crude extract and 80 µl of negative control were poured into separate wells. The standard antibiotic disc was placed on the agar surface

as a positive control. Plates were incubated at 37°C for 48 hr and inhibition zones (mm) were measured. The experiment was carried out in triplicates for each test organism and the mean values were tabulated.

Test organisms

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).

RESULTS AND DISCUSSION

Among the 40 actinobacterial strains isolated from marine ecosystem of Guntur district, Andhra Pradesh, predominantly two strains VJMS-4 and VJMS-8 were found to be potent and exhibited strong antimicrobial activity against Gram-positive, Gram-negative bacteria and fungi. These strains exhibited typical morphological characteristics of genus *Streptomyces.* Morphological and micro morphological observation of the strains studied by SEM revealed that VJMS-4 showed sporulation with fragmented mycelium and smooth spore surface, while strain VJMS-8 exhibited heavy sporulation with rough surface (Figs.1 and 2).

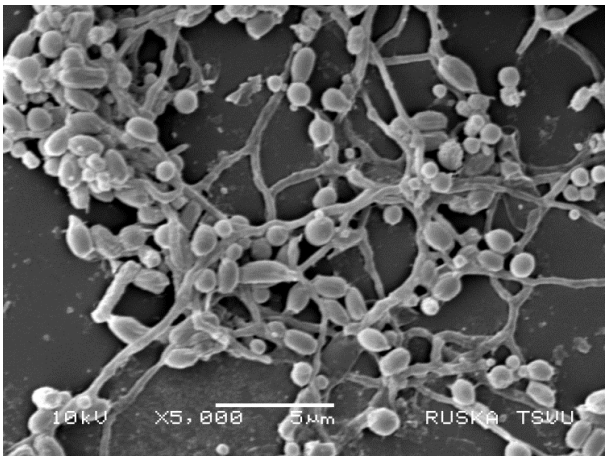

Fig1 Scanning Electron Microscopic photograph of *Streptomyces vinaceusdrappus* VJMS-4

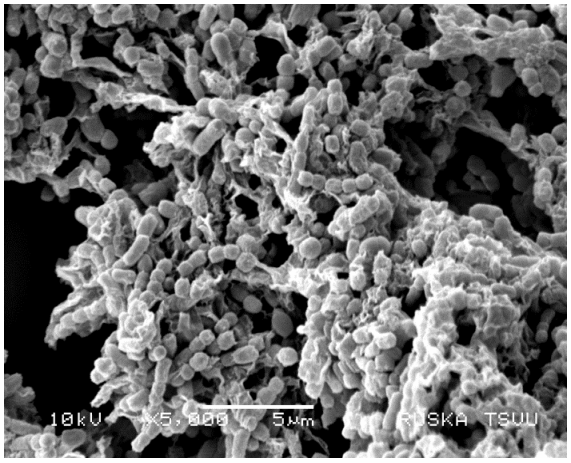

Fig2: Scanning Electron Microscopic Photograph of *Streptomyces rectiverticillatus* VJMS-8

Identification of the strains VJMS-4 and VJMS-8

The cultural characteristics of the strains are represented in table 1 and 2. The strain VJMS-4 exhibited good growth on tryptone yeast extract agar (ISP-1), YMD agar (ISP-2), glycerol asparagine agar (ISP-5) and starch casein salts agar (non-ISP) while the growth was moderate on oat meal agar. The color of aerial mycelium was white and substrate mycelium was pale yellow on the different media tested. No pigment production was observed on the media tested. The strain VJMS-8 exhibited good growth on tryptone yeast extract-agar, YMD agar, glycerol asparagine agar, starch casein salts agar, nutrient agar and humic acid vitamin-B agar media while the growth was moderate on oat meal agar media. The growth was poor on czapek-dox agar media. The color of aerial mycelium was Gray and substrate mycelium was pale yellow on different media. Soluble black colored pigment production was observed on the media tested.

Biochemical characteristics of the strains:

The physiological and biochemical characteristics were significant tools for identification of actinobacteria, which influence the growth rate [21-23]. Several tests were conducted for identifying strains VJMS-4 and VJMS-8 (table 3). The strain VJMS-4 exhibited a positive response to catalase production, indole production, urease activity and gelatin liquefaction but negative to methyl red, Voges-Proskauer, citrate utilization and H_2S production. The other strain VJMS-8 exhibited a positive response to indole production, urease activity, gelatin liquefaction and H_2S production but negative to methyl red, Voges-Proskauer, citrate utilization and catalase activity. Both the strains could also produce enzymes like L-asparaginase, amylase and cellulase.

Physiological characteristics of the strains:

Both the strains VJMS-4 and VJMS-8 showed good growth at pH range between 5 and 9 with the optimum being 7, and the range of temperature for growth was 30-40°C with the optimum being 35°C. Sodium chloride tolerance of the strains was also studied as the salt concentration has a profound effect on the production of antibiotics by microorganisms. Both the strains could grow well in the medium supplemented with 1% and 3% sodium chloride and showed tolerance up to 9%. VJMS-8 strain showed good growth even in the absence of NaCl. VJMS-4 strain utilized a wide range of carbon sources such as maltose, sucrose, D-glucose, sorbitol, lactose and mannitol supported good growth of the strain. The strain was resistant to the majority of antibiotics tested and showed sensitivity to penicillin, amikacin, vancomycin, gentamicin and chloramphenicol. Though the strain VJMS-8 utilized a wide range of carbon sources such as maltose, sucrose, D-glucose, sorbitol, dulcitol, mannitol and galactose supported good growth of the strain. The strain was resistant to the majority of antibiotics tested and showed sensitivity to gentamicin, imipenem, vancomycin, clindamycin, chloramphenicol and tetracycline.

Table 1: Cultural characteristics of the strain VJMS-4

Name of the medium	Growth	AM	SM	Pigmentation
Tryptone yeast extract agar (ISP-1)	Good	White	Pale yellow	No
Yeast extract malt extract dextrose agar (ISP-2)	Good	White	Pale yellow	No
Oat-meal agar (ISP-3)	Moderate	White	Pale yellow	No
Inorganic salts Starch Agar (ISP-4)	-	-	-	No
Glycerol Asparagine agar (ISP-5)	Good	White	Pale yellow	No
Tyrosine agar (ISP-7)	-	-	-	No
Czapek-dox agar	-	-	-	No
Nutrient agar	-	-	-	No
Starch casein salts agar	Good	White	Pale yellow	No
Glucose tryptone agar	-	-	-	No

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

Table 2: Cultural characteristics of the strain VJMS-8

Name of the medium	Growth	AM	SM	Pigmentation
Tryptone yeast extract agar (ISP-1)	Good	Gray	Pale yellow	Black
Yeast extract malt extract dextrose agar (ISP-2)	Good	Gray	Pale yellow	Black
Oat-meal agar (ISP-3)	Moderate	Gray	Pale yellow	Black
Inorganic salts Starch Agar (ISP-4)	-	-	-	No
Glycerol Asparagine agar (ISP-5)	Good	Gray	Pale yellow	Black
Tyrosine agar (ISP-7)	-	-	-	No
Czapek-dox agar	Poor	Gray	Pale yellow	Black
Nutrient agar	Good	Gray	Pale yellow	Black
Starch casein salts agar	Good	Gray	Pale yellow	Black
Glucose tryptone agar	-	-	-	No

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

Table 3: Morphological, biochemical and physiological characteristics of the strains

Character	Response	
Morphological characters	**VJMS-4**	**VJMS-8**
Sporophore morphology	Recti flexible	Recti flexible
Color of aerial mycelium	White	Gray
Color of substrate mycelium	Pale yellow	Pale yellow
Biochemical characters		
Catalase production	+	-
Urease production	+	+

Character		Response
Hydrogen sulfide production test	-	+
Nitrate reduction	-	-
Starch hydrolysis	+	+
Gelatin liquefaction	+	+
Methyl red test	-	-
Voges proskauer test	-	-
Indole production	+	+
Citrate utilization	-	-
Physiological characters		
Gram reaction	+	+
Production of melanin pigment		+
Range of temperature for growth	30-45 °C	30-45 °C
Optimum temperature for growth	35°C	35 °C
Range of pH for growth	5-9	5-9
Optimum pH for growth	7	7
NaCl tolerance	Up to 9%	Up to 9%
Utilization of carbon sources (w/v)*		
Lactose	++	+
Maltose	+	+++
Dulcitol	+	+
Sucrose	+ +	+
Sorbitol	+++	+++
D-Glucose	++	++
Galactose	+	+++
Fructose	-	-
Starch	+	+
Mannitol	+++	+
Cellulose	-	-
Antibiotic sensitivity		
Gentamicin (10µg)	S	S
Vancomycin (30µg)	S	S
Penicillin (10µg)	R	R
Clindamycin (25µg)	R	R
Chloramphenicol (50µg)	S	S
Cefepime (30 µg)	R	R
Imipenem(10µg)	R	S
Cefixime (30µg)	R	R
Tetracycline (30µg)	R	S
Amikacin (10µg)	S	R
Enzymatic activity		
Amylase	P	P
Protease	N	N
Cellulase	P	P

Character	Response	
Asparaginase	P	P
Glutaminase	N	N

*** Growth of the strain measured as dry weight of the mycelium '+++'-good growth; '++'-moderate growth; '+'-weak growth; '-'indicates negative/no growth; S–Sensitive; R–Resistant; P–Positive; N–Negative.**

Molecular characterization

Based on the 16S rRNA sequence data, the strain VJMS-4 belong to the genus *Streptomyces vinaceusdrappus* and the strain VJMS-8 belong to *Streptomyces rectiverticillatus.* The partial 16S rRNA sequences of the strains were submitted to the GenBank database with accession numbers MG309762 and MG309763. The partial sequence was aligned and compared with all the 16S rRNA gene sequence available in the GenBank database using the multi-sequence advanced BLAST comparison tool. The phylogenetic analysis of the 16S rRNA gene sequence was aligned using the CLUSTAL W program from the MEGA 6 Version. Phylogenetic trees (Figs. 3 and 4) were constructed using MEGA software Version 6 using maximum parsimony method [24].

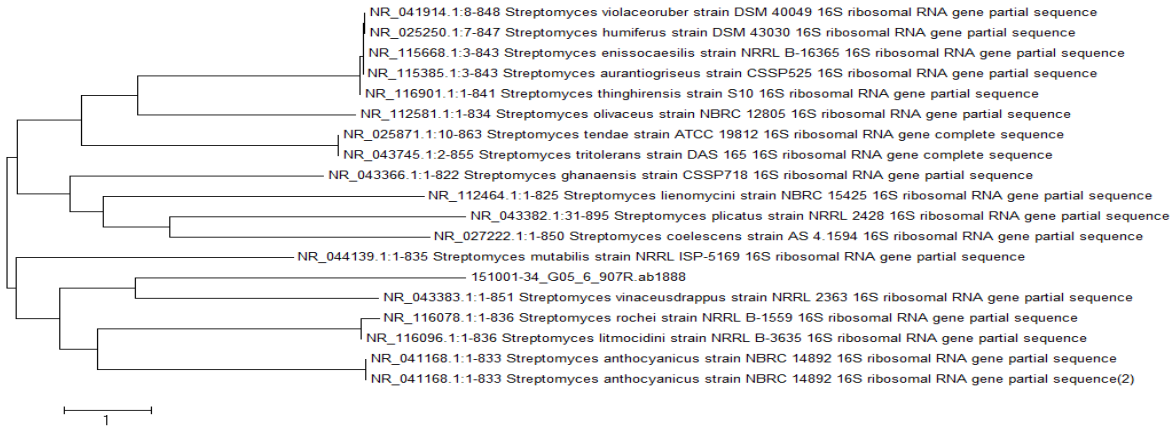

Fig:3 Maximum parsimony tree based on partial 16S rRNA gene sequence showing relationship between strain VJMS-4 and related members of the genus *Streptomyces*

Fig:4 Maximum parsimony tree based on partial 16S rRNA gene sequence showing relationship between strain VJMS-8 and related members of the genus *Streptomyces*

Growth pattern and antimicrobial profile of *Streptomyces vinaceusdrappus* VJMS-4 and *Streptomyces rectiverticillatus* VJMS-8

The growth curve and antimicrobial profile of *S. vinaceusdrappus* VJMS-4 and *S. rectiverticillatus* VJMS-8 were studied at regular intervals up to 8 days in batch

culture. The stationary phase of the strains extended from 144 hr to 168 hr of incubation (Figs. 5 and 6). The secondary metabolites obtained from 6-day-old culture showed high antimicrobial activity against the test microbes. Munaganti *et al*. (2015) noted the production of antimicrobial metabolites from 5-day-old culture of *Rhodococcus erythropolis* VL-RK-05 [25]. Naragani *et al*. (2014) reported that 5-day old-culture extracts of *Streptomyces violaceoruber* VLK-4 evidenced the production of antimicrobial compounds [26]. The secondary metabolites obtained from 4-day-old culture of *Nocardia levis* MK-VL-113 isolated from laterite soils of Guntur showed high antimicrobial activity against the test microbes [27]. The antimicrobial spectrum of the strains cultured on YMD broth for 8 days was tabulated (Table 4). The metabolites extracted from the 6-day-old culture broth showed maximum activity against *B. megaterium*, *B. subtilis*, *X.campestris, S aureus, E. coli* and *P.vulgaris*. In case of fungi, *C. albicans* showed high sensitivity when compared to the other fungi tested.

Table 4: Antibacterial and antifungal activity of *Streptomyces vinaceusdrappus* VJMS-4 and *Streptomyces rectiverticillatus* VJMS-8

Test organism name	Zone of Inhibition (mm)	
Bacteria	**VJMS-4**	**VJMS-8**
Staphylococcus aureus	32	21
Escherichia coli	21	25
Xanthomonas campestris	17	16
Pseudomonas aeruginosa	15	15
Bacillus megaterium	31	24
Bacillus subtilis	16	22
Proteus vulgaris	16	32
Fungi		
Candida albicans	18	21
Aspergillus flavus	17	18
Penicillium citrinum	14	18

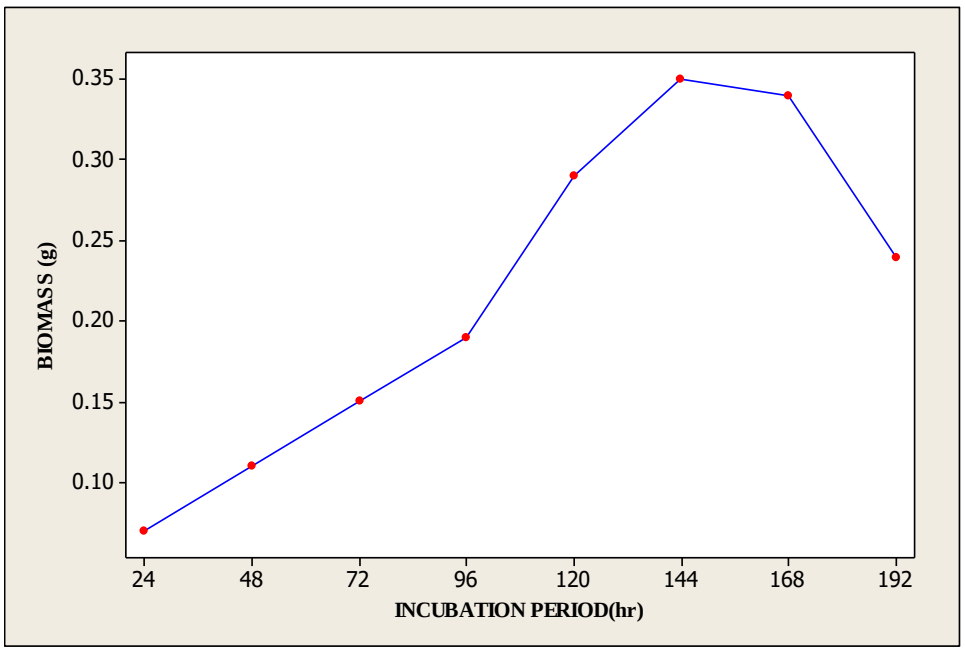

Fig. 5: Growth pattern of the strain *Streptomyces vinaceusdrappus* VJMS-4

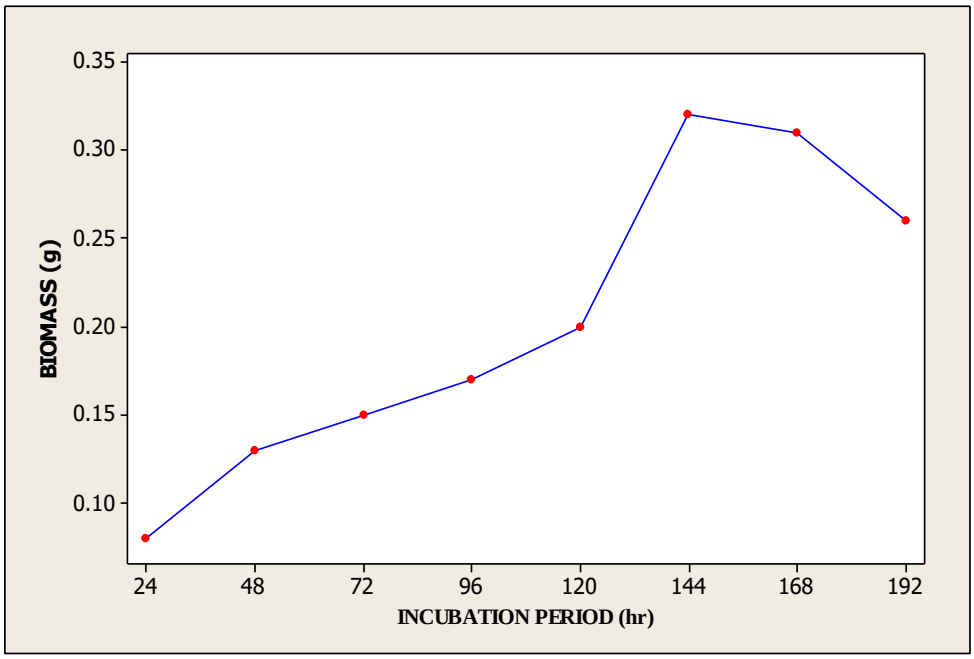

Fig. 6: Growth pattern of the strain *Streptomyces rectiverticillatus* VJMS-8

CONCLUSION

The present investigation highlights the antimicrobial potential of *S.vinaceusdrappus* VJMS-4 and *S. rectiverticillatus* VJMS-8. Further study on optimization, purification and chemical characterization of bioactive compounds of the strains are in progress.

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