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ANTIMICROBIAL METABOLITE PRODUCTION BY *KOCURIA ASSAMENSIS* VLS-2 ISOLATED FROM SOUTH-COASTAL REGIONS OF ANDHRA PRADESH, INDIA

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ABSTRACT: An attempt was made to identify actinomycete strains of the South coastal region of Andhra Pradesh, India along with the study of their antimicrobial potential. Among the 50 actinomycete strains isolated, one strain (VLS-2) showed strong antimicrobial activity against Gram-positive and Gram-negative bacteria as well as fungi. It was identified as *Kocuria assamensis* based on morphological, biochemical, physiological as well as molecular characteristics. ISP-1 medium supplemented with sodium chloride @ 7% maintained at pH 7.0 supported the maximum yield of secondary metabolites by the strain when incubated at 35^{o}C for six days. Production of antimicrobials by the strain varied significantly with different carbon and nitrogen sources. The present study reveals the potentiality of actinomycete strains isolated from South-coast of Andhra Pradesh, India which shows further scope to investigate their novel bioactive compounds by employing both natural product chemistry and modern biotechnological aspects.

INTRODUCTION: Oceans occupy 71% of the Earth's surface holding 97% of the planet's water and nearly 87% of life with fundamentally pristine fauna and flora [1] and are an immense source for undiscovered organisms including microorganisms and novel natural products. The marine natural products are the major source for new pharmaceuticals due to their huge chemical diversity. Extensive research on marine natural products over the past three decades has divulged that marine rare actinomycetes are best fertile sources of unique and diverse metabolites [2]. The unfamiliar and underexplored environments including marine ecosystems are propitious sources of rare actinobacteria that are reputed to be fruitful sources of novel compounds [3].

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'Rare actinobacteria' are defined as the actinomycete strains less frequently isolated than that of the routinely isolated *Streptomyces* spp., even yet they may not literally be scarce in the environment. It is indubitable that new drugs, distinctly antibiotics, are urgently needed to arrest the antibiotic resistant pathogens [4]. The rise of emerging diseases and antibiotic-resistant human pathogenic bacteria such as multidrug resistant (MDR) strains of *Mycobacterium tuberculosis*, vancomycin resistant enterococci (VRE), methicillin resistant *Staphylococus aureus* (MRSA), *Pseudomonas aeruginosa* and *Candida albicans* [5] lead to focus extreme environments for isolating novel bioactive strains [6].

Marine-derived rare actinomycetes are reputed to be a virtually rich source of diverse chemicals, structurally unique secondary metabolites and novel therapeutic compounds [7, 2]. Only 11 rare actinomycete genera had been reported by 1970, while 220 genera were reported by 2013 [3]. High-throughput metagenome sequencing methods

extended our knowledge and revealed the influence of many novel actinobacteria that were not earlier detected in culture studies [8, 9, 10]. The recovery of rare actinomycetes in conventional culture methods is generally less as compared to S*treptomyces* strains [8]. However, the current knowledge of marine actinomycete physiological, chemical and structural features permitted the design of selective isolation media [8]. A total of 13,700 bioactive metabolites were reported from actinomycete, of which 10,400 were derived from streptomycetes and 3300 from rare actinomycetal strains [1].

Actinomycetes are Gram-positive bacteria of the order *Actinomycetales*; characterized by filamentous morphology, DNA with high G+C content, presence of LL-Diaminopimelic acid (LL-DAP) and the presence or absence of characteristic sugars in the cell wall. They are ubiquitous and form a perpetual and persistent population in diverse ecosystems [11, 12]. Innovating new actinomycete taxa from varied habitats with unique metabolic activity often led to the discovery of novel antimicrobial agents. Distinct antimicrobial agents have been isolated and characterized from actinomycetes including aminoglycosides, anthracyclines, glycopeptides, macrolides, beta-lactams, polyenes, phenazine and tetracyclines. As part of our ongoing screening, strain VLS-2 isolated from South coastal regions of Andhra Pradesh, India exhibited broad spectrum activity against 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 and to optimize the cultural conditions for enhancing the productivity of strain.

MATERIALS AND METHODS:

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

P**retreatment of Samples:** The samples were subjected to pretreatment to facilitate the isolation of actinomycetes. The samples collected were air-dried and pretreated with calcium carbonate (1%) and incubated at 35°C for two weeks [13].

Isolation of Marine Actinomycetes: Marine actinomycetes were isolated by serial dilution method [14, 15]. Stock solution was prepared by diluting 1 g of soil sample in 100 mL of sterile saline water and shaking 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):

1. Yeast extract-Malt extract-Dextrose-Agar: yeast extract: 4.0; malt extract: 10.0;dextrose: 4.0; agar: 20.0 [16]
2. 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 [17]
3. 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 .0 [18].

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 $35^{O}C$ for 7–20 days. The colonies showing the characteristics of actinomycetes (rough, chalky, powdery appearance with radiating growth and leathery texture) were observed [19, 20]. The pure cultures were maintained on YMD agar slants at 4°C. The actinomycete strains thus isolated were screened for their ability to produce antimicrobial compounds. Among the 50 isolates tested for bioactive compounds, the isolate designated as VLS-2 was found to be potent compared to other strains.

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

Morphological and Cultural Characters of Strain VLS-2: Morphology studies were performed using the methods described by Shirling and Gottlieb [16]. The spore-bearing hyphae and arrangement of spores were observed by the cover slip method. The morphology of mycelium and spore surface was observed using a scanning electron microscope (SEM: JOELJSM 5600, Japan) [21].

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), oat-meal 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. Color and growth of aerial and substrate mycelium and the formation of soluble pigments were noted on all media after incubation at 35°C for 7 days.

Physiological and Biochemical Characters of Strain VLS-2: 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 [16]. The production of melanoid pigments was tested on ISP-7. The isolate was also tested for its ability to grow at different concentrations of NaCl (0-12%), at different temperatures (30-45°C) and at different levels of pH (5-9 %) [16]. Biochemical tests such as H_2S production [22], gelatin liquefaction, starch hydrolysis, catalase production, indole, methyl red, Voges-Proskauer, citrate utilization, nitrate reduction [23], 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 [24].

Screening of the Strain VLS-2 for Extracellular Enzyme Production: The strain VLS-2 was inoculated on 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, urease 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 colonies grown on YMD agar for 3 days. The total genomic DNA extracted from the isolate was isolated using 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 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 a 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 with 5 µL of ethidium bromide. The PCR product was analyzed using agarose gel (1%) and the fragment was purified (Helini Pure Fast PCR clean up kit, Helini Biomolecules, India) 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 a 3100-Avant genetic analyzer (Applied Biosystems, USA).

Pair Wise Sequence Alignment: The gene sequence of VLS-2 was aligned using BLAST against the gene library available for *Kocuria* species in the NCBI and the GenBank. Pairwise evolutionary distances were computed by MEGA-6 software.

Multiple Sequence Alignment: The phylogenetic analysis was conducted employing the maximum parsimony method using BLAST and CLUSTAL W. The closely related homologous isolates were identified, retrieved and compared to the sequence of strains isolated using CLUSTAL W available with the MEGA 6 Version [25].

Nucleotide Sequence Accession Numbers: The 16S rRNA gene sequence of isolate VLS-2 was registered in the GenBank database.

Nutritional Parameters Affecting the Bioactive Metabolite Production by the Strain: Bioactive metabolite production by the strain was optimized by altering parameters such as incubation period,

pH, temperature, sodium chloride, carbon, nitrogen sources and minerals.

Growth Pattern and Effect of Incubation time on Bioactive Metabolite Production by the Strain: Growth pattern of *Kocuria assamensis* VLS-2 and its antimicrobial activity against Gram positive bacteria (*Bacillus megaterium, Streptococcus mutans* and *Staphylococcus aureus*), Gram negative bacteria (*Escherichia coli, Klebisella pneumoniae, Xanthomonas campestris* and *Pseudomonas aeruginosa*) and fungi (*Aspergillus flavus, Candida albicans* and *Penicillium citrinum*) were recorded by culturing the strain in ISP- 2 broth for 8 days. The strain was inoculated into 250 ml flasks containing 100 ml ISP-2 broth and incubated at 35°C on a rotary shaker at 120 rpm. At every 24 h interval, the biomass of strain and production of antimicrobial metabolites were determined the cell mass's dry weight (mg/100ml culture medium) was measured. The supernatant was extracted with ethyl acetate, vacuum dried in a rotavapor and used for testing antimicrobial activity against bacteria and fungi through agar well diffusion method [26].

Assay of Antimicrobial Activity: The antimicrobial activity of solvent extract obtained from *Kocuria assamensis* VLS-2 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 extract to the 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 antimicrobial activity was expressed as mean of diameter of inhibition zones (mm) when compared to the control. In case of fungi, the Petri plates were incubated at 30°C for 2 days. At the end of 72 h, inhibition zones formed in the medium were measured.

Influence of Initial pH and Incubation Temperature on Bioactive Metabolite Production by the Strain: Influence of initial pH on bioactive metabolite production by the strain was determined by adjusting pH of production medium from 5.0-9.0. The optimal pH achieved at this step was used for further study [27]. Similarly the optimum temperature for bioactive metabolite yield was measured by incubating the production medium at temperatures ranging from 20-40ºC [28].

Influence of Sodium Chloride on Bioactive Metabolite Production by the Strain: The influence of salinity on bioactive metabolite production by the strain was recorded by culturing the strain in the fermentation medium amended with different concentrations of sodium chloride (0-12%) at optimum pH and temperature for eight days. The salt concentration in which the strain exhibits optimum level of bioactive metabolites was fixed for further studies.

Influence of Carbon and Nitrogen Sources on Bioactive Metabolite Production by the Strain: Various carbon sources such as galactose, dulcitol, maltose, mannitol, starch, sucrose, lactose, fructose, cellulose and sorbitol @1% were added to the optimized medium by replacing the carbon source. The influence of varying concentrations of the best carbon source (0.5-2.0%) on bioactive metabolite production was also examined. Likewise, impact of different nitrogen sources on the yield of antimicrobials of the strain was studied by supplementing different nitrogen sources like peptone, glycine, urea, glutamine, asparagine, cystiene, L-arginine, ammonium sulphate, tryptone, beef extract and sodium pyruvate to optimized medium by replacing nitrogen source. Further, the impact of different levels of optimized nitrogen source (0.5- 2.0%) was studied to enhance antimicrobial metabolite production [29].

Test Organisms: The antimicrobial metabolites produced by strain under optimized conditions were tested against bacteria (*Staphylococcus aureus* (MTCC 3160), *Bacillus megaterium* (NCIM 2187), *Streptococcus mutans* (MTCC 497), *Xanthomonas campestris* (MTCC 2286), *Klebisella pneumoniae* (MTCC 109), *Pseudomonas aeruginosa* (ATCC 9027) and *Escherichia coli* (ATCC 9027)) and fungi *Aspergillus flavus* (ATCC 189), *Candida albicans* (MTCC 183) and *Penicillium citrinum* (MTCC 6849) using agar plate diffusion assay.

Statistical Analysis: Statistical analysis was carried out for antimicrobial metabolite production

by the strain using One-way Analysis of Variance (ANOVA).

RESULTS AND DISCUSSION: The present study was designed to investigate the marine coastal regions of Andhra Pradesh for novel actinomycetes and their antimicrobial properties. 50 actinomycete strains isolated from marine habitats were screened for antimicrobial activity. One strain designated as VLS-2 was found to exhibit high antagonistic activity against the microorganisms tested.

The strain VLS-2 exhibited typical morphological characteristics of the genus *Kocuria.* Morphological and micromorphological observation of the strain revealed that aerial hyphae are smooth, circular and short rods, uniform edged, translucent, extremely mucoid and orange in colour.

The vegetative hyphae were in pale yellow, opaque, smooth with irregular edges. The spherical cells (diameter 1.0-1.5 µm) occur in pairs, tetrads and packets. Soluble pigment production by the strain was not found on tyrosine agar medium.

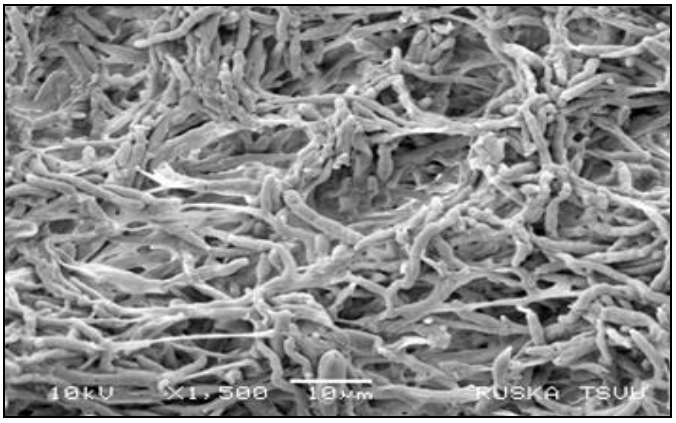

FIG. 1: SCANNING ELECTRON MICROSCOPIC PHOTOGRAPH OF *KOCURIA ASSAMENSIS* VLS-2

Cultural Characteristics: The cultural characteristics of the strain are represented in **Table 1**. The strain VLS-2 exhibited good growth on ISP-1, ISP-2 and glucose tryptone agar media. The growth was moderate on nutrient agar and Czapek-Dox agar but poor on ISP-5. Yellow-brown aerial mycelium and pale-yellow substrate mycelium were found on ISP-1, ISP-2 and glucose tryptone agar media. The strain could not grow on ISP-3, ISP-4, ISP-7, Starch casein salts agar and humic acid vitamin-B agar.

TABLE 1: CULTURAL CHARACTERISTICS OF THE STRAIN VLS-2

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

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

Biochemical Characteristics of VLS-2: The strain VLS-2 exhibited a positive response to methyl red, citrate utilization test, nitrate reduction, gelatine liquefaction, catalase production, hydrogen sulphide and starch hydrolysis but negative for indole, Vogues-Proskauer, urease production and triple sugar iron tests. The details of morphological, physiological and biochemical characteristics of the isolate are given in **Table 2**. The utilization of carbon sources by the strain could be used as an aid for species determination [22]. The strain efficiently utilized carbon sources such as D-glucose, maltose, sucrose, galactose, dulcitol, lactose and sorbitol but could not utilize fructose, starch and cellulose **Table 3**. It was also reported that some 'nocardioform bacteria' antibiotic sensitivity was one of the valuable criteria for their taxonomic differentiation [30]. Antibiotic susceptibility testing showed that the isolate was susceptible to vancomycin, chloramphenicol, clindamycin, tetracycline and cefixime but resistant to gentamicin, imipenem, cefepime, amikacin and penicillin **Table 4.**

TABLE 2: MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF THE ISOLATE VLS-2

Character	Response
Morphological characters	**VLS-2**
Sporophore morphology	Recti flexible
Color of aerial mycelium	Yellow-brown
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	+
Vogesproskauer test	-
Indole production	-
Citrate utilization	+

TABLE 3: UTILIZATION OF CARBON SOURCES BY THE STRAIN VLS-2

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

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

TABLE 4: ANTIBIOTIC SENSITIVITY OF THE STRAIN VLS-2

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

*S–Sensitive; R–Resistant.

Physiological Characteristics: The physiological tests are indispensable tools for classification and identification of actinomycetes [31-34]. The strain VLS-2 can grow in the pH range of 6-9 with optimum being 7.0. The temperature range for growth was 30-45°C with optimum at 35°C. The strain exhibited salt tolerance up to 12 % with the optimum level at 7% NaCl; hence, the strain could be placed in the intermediate salt tolerance group [35] **Table 5**. The strain VLS-2 could also produce enzymes like L-asparaginase, glutaminase, amylase, caseinase and pectinase.

TABLE 5: PHYSIOLOGICAL AND ENZYMATIC CHARACTERS OF THE STRAIN VLS-2

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 7%
Enzymatic activity	
Amylase	P
Protease	N
Cellulase	N
Asparaginase	P
Glutaminase	P
Lipase	N
Urease	P
Pectinase	P
Caseinase	P

*P–Positive; N–Negative.

Molecular Characterization of the Strain VLS-2: The 16S rRNA sequence data supported the assignment of strain VLS-2 to genus *Kocuria* and species *assamensis*. The partial 16S rRNA sequence of strain VLS-2 was obtained and submitted to the GenBank database under an accession number MW450581. The partial sequence was aligned and compared with all 16S rRNA gene sequences available in the GenBank database using the multisequence advanced BLAST comparison tool available in the National Centre for Biotechnology Information website. The highest 16S rRNA sequence similarity value of 100% was obtained for *Kocuria assamensis*. The phylogenetic analysis of 16S rRNA gene sequence was aligned using the CLUSTAL W programme from MEGA 6 Version. Phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software Version 6 using Maximum parsimony method [36-38]. The topologies of constructed tree were evaluated by bootstrap analysis with 1000 resamplings by the Maximum parsimony tool. Sequence comparison of the strain VLS-2 with corresponding sequences of the close

representative strains of *Kocuria* from the GenBank database showed that this strain formed a close distinct phyletic line with clade encompassed by *Kocuria assamensis, Kocuria polaris* and *Kocuria kristinae* **Fig. 2.**

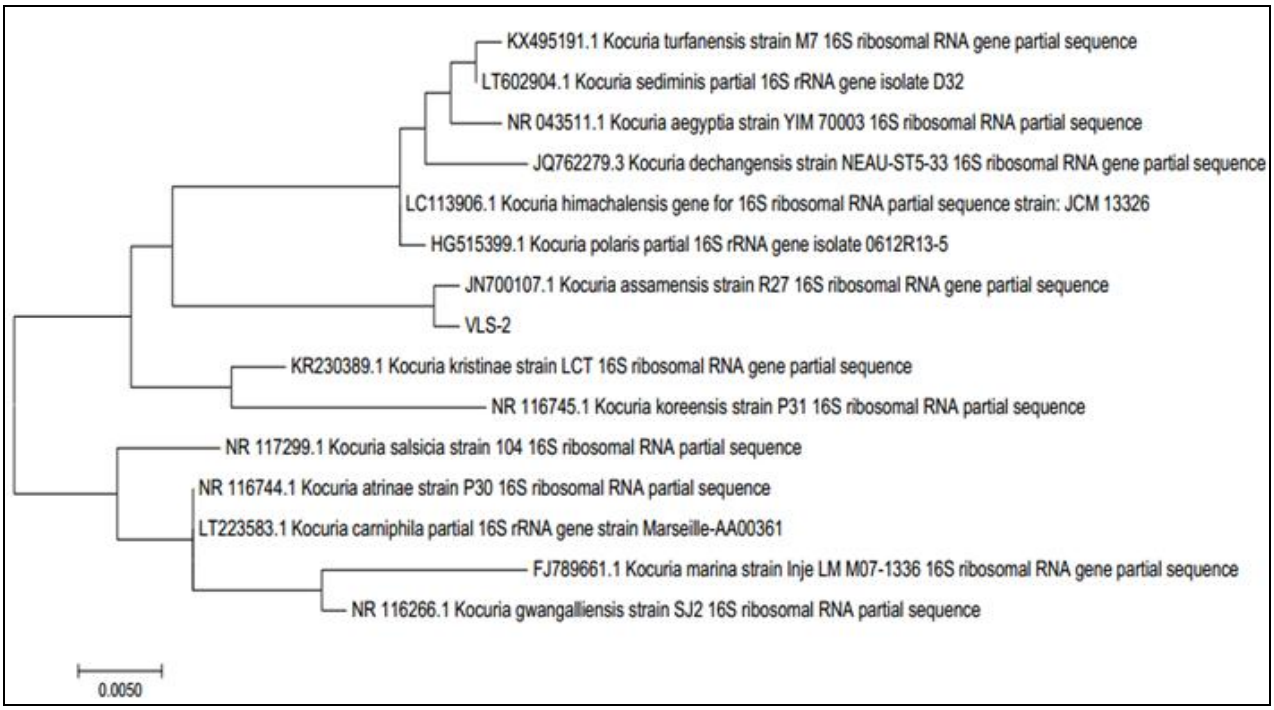

FIG. 2: MAXIMUM PARSIMONY TREE BASED ON PARTIAL 16S rRNA GENE SEQUENCE SHOWING THE RELATIONSHIP BETWEEN ISOLATE VLS-2 AND RELATED MEMBERS OF THE GENUS *KOCURIA*

Growth Pattern and Antimicrobial Profile of the Strain: The growth pattern and antimicrobial profile of *Kocuria assamensis*VLS-2 were studied at regular intervals up to 8 days in batch culture. The stationary phase of the strain extended from 144 h to 168 h of incubation **Fig. 3**. The secondary metabolites obtained from 6-day-old culture showed high antimicrobial activity against the test microbes. The antimicrobial metabolites produced from 11-day-old *Nocardiopsis litoralis*VSM-8 [39], 8-day old *Nocardiopsis flavescens*VJMS-18 [40], 5-day old *Nocardiopsis halotolerans* VJPR-2 [41] and 4-day old *Nocardia metallicus* VJSY-14 [42] exhibited high antagonistic activity. The secondary metabolites obtained from 6-day old culture of *Streptomyces vinaceusdrappus* VJMS-4, *Streptomyces rectiverticillatus* VJMS-8 [43] and 5-day old culture of *Streptomyces albogriseolous* VJMS-7 [44] showed high antimicrobial activity against the test microbes.

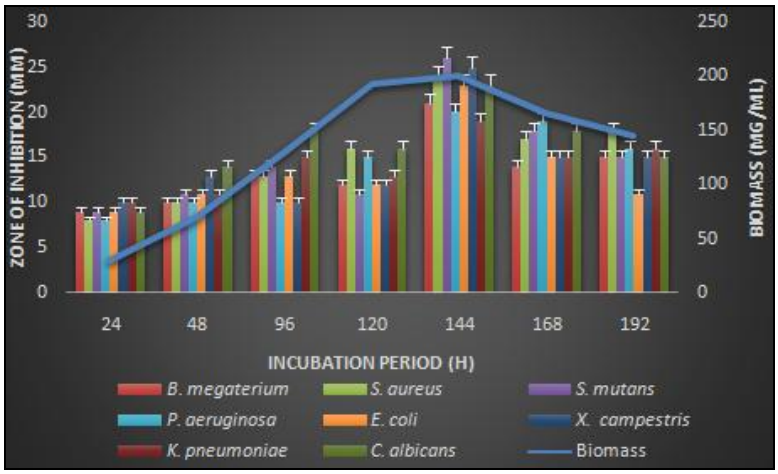

FIG. 3: INFLUENCE OF INCUBATION PERIOD ON BIOACTIVE METABOLITE PRODUCTION BY *KOCURIA ASSAMENSIS*VLS-2. Data are statistically analyzed and found significant at 5%.

Influence of Culture Media on Bioactive Metabolite Production by the Strain: The influence of different media on the production of bioactive metabolites was recorded in **Fig. 4.** Among the media tested, tryptone yeast extract broth supported the production of bioactive metabolites followed by yeast extract malt extract dextrose broth. Oskay *et al.* (2011) reported that the activity of actinomycete isolates could be increased or decreased remarkably under different cultural conditions.

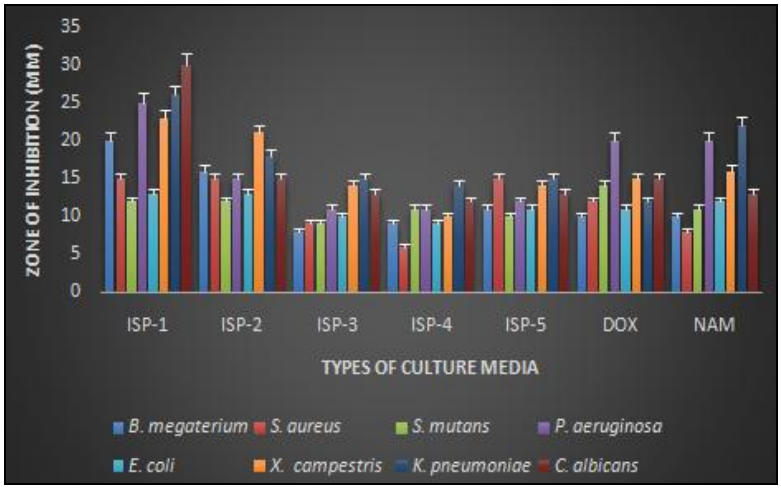

FIG. 4: INFLUENCE OF CULTURE MEDIA ON BIOACTIVE METABOLITE PRODUCTION BY KOCURIA ASSAMENSISVLS-2. Data are statistically analyzed and found significant at 5%.

Influence of Initial pH and Incubation Temperature on Bioactive Metabolite Production by the Strain: Various environmental requirements influence actinomycetes' growth and bioactive metabolite production.

Maximum growth and antimicrobial metabolite production were obtained at pH 7.0 **Fig. 5.** The actinomycete strains like *Kocuria pelopila* [45], *Kocuria himachalensis* [46] and *Kocuria polaris* [47] showed optimum level of antibiotic production at pH 7.0. The influence of temperature on bioactive metabolite production by the strain is presented in **Fig. 6**.

Good growth, as well as antimicrobial metabolite production was obtained at 35°C. The organism appeared to be mesophilic in terms of its optimum temperature for growth.

Several strains of actinomycete belonging to the genus *Kocuria* including *Kocuria marina* [48] and *Kocuria carniphila* [49] showed optimum levels of antibiotic production at 35ºC.

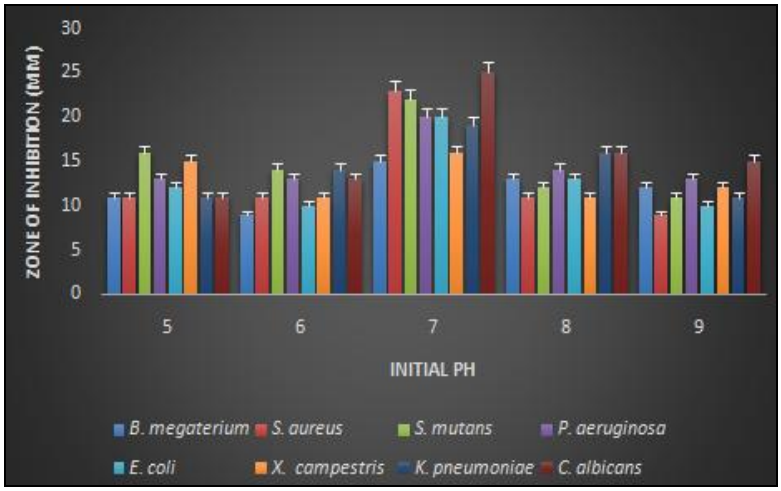

FIG. 5: INFLUENCE OF INITIAL pH ON BIOACTIVE METABOLITE PRODUCTION BY *KOCURIAASSAMENSIS*VLS-2. Data are statistically analyzed and found significant at 5%.

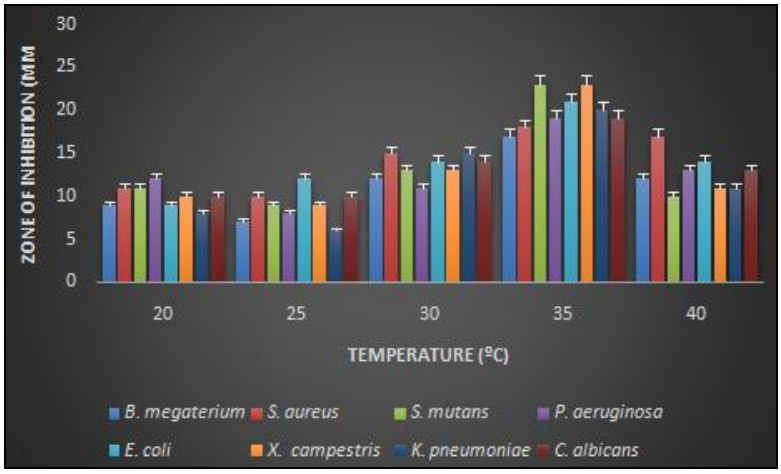

FIG. 6: INFLUENCE OF INCUBATION TEMPERATURE ON BIOACTIVE METABOLITE PRODUCTION BY *KOCURIA ASSAMENSIS*VLS-2. Data are statistically analyzed and found significant at 5%.

Influence of Sodium Chloride on Bioactive Metabolite Production by the Strain: Optimum salt requirement for bioactive metabolite production was examined by supplementing the production medium with different salt concentrations ranging from 0-12%. Sodium chloride at a concentration of 7% was found to be optimum for antimicrobial metabolite production by *Kocuria assamensis* VLS-2 **Fig. 7**. Further increase in salt concentration led to reduced antimicrobial activity. The requirement of sodium chloride for bioactive metabolite production seems to differ among actinomycete strains. Optimum sodium chloride concentration for antimicrobial metabolite production was reported to be 7% for *Kocuria palustris* TAGA 27^{T} [50], *Kocuria rosea* and *Kocuria varians* [51].

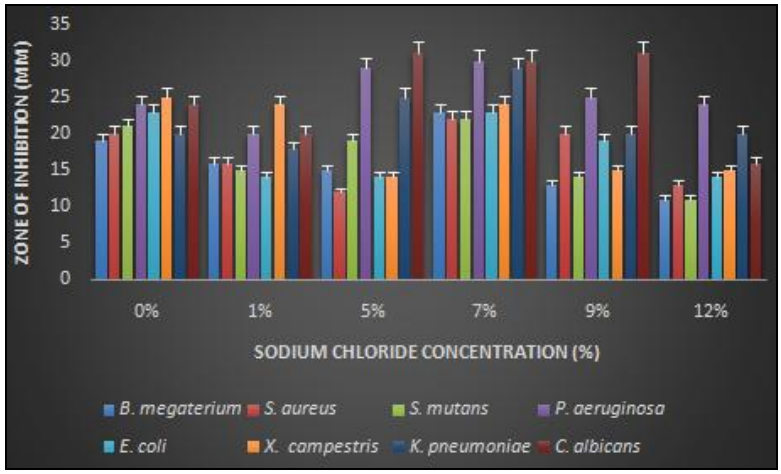

FIG. 7: INFLUENCE OF SODIUM CHLORIDE CONCENTRATION ON BIOACTIVE METABOLITE PRODUCTION BY *KOCURIA ASSAMENSIS*VLS-2. Data are statistically analyzed and found significant at 5%.

Influence of Carbon Sources on Bioactive Metabolite Production by the Strain: The effect of different carbon sources was evaluated for their impact on antimicrobial metabolite production **Fig. 8.**

Among the various carbon sources tested, dulcitol was the best one for bioactive metabolite production. Kavitha *et al.* (2009) reported that *Nocardia levis* MK-VL_113 isolated from laterite soils utilized sucrose as the sole carbon source for antibiotic production. As dulcitol was the most preferred carbon source for biomass and bioactive metabolite production by the strain, different levels of dulcitol (0.5-2.0%) were tested to determine optimal concentration for bioactive metabolite production **Fig. 9**.

Dulcitol@ 1.0% supplemented in the medium promoted bioactive metabolite production.

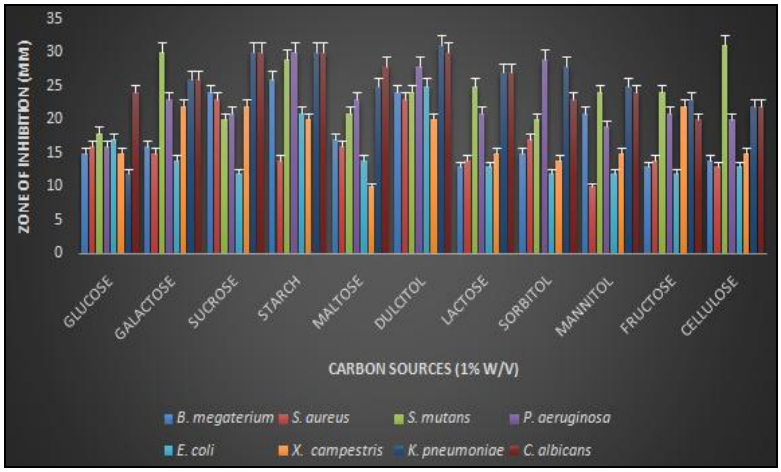

FIG. 8: INFLUENCE OF CARBON SOURCES ON BIOACTIVE METABOLITE PRODUCTION BY KOCURIA ASSAMENSISVLS-2. Data are statistically analyzed and found significant at 5%.

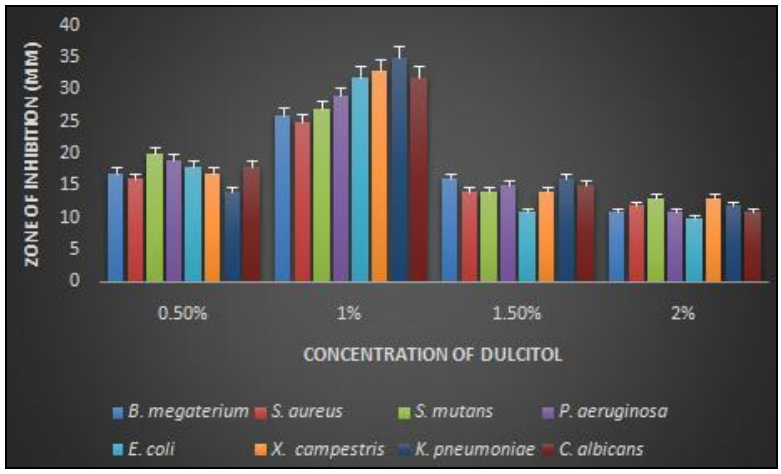

FIG. 9: INFLUENCE OF DULCITOL CONCENTRATION ON BIOACTIVE METABOLITE PRODUCTION BY KOCURIA ASSAMENSISVLS-2. Data are statistically analyzed and found significant at 5%.

Influence of Nitrogen Sources on Bioactive Metabolite Production by the Strain: Different nitrogen sources were found to have a significant effect on secondary metabolite production by *Kocuria assamensis* VLS-2. High antimicrobial activity was obtained in culture filtrates supplemented with tryptone followed by cysteine and asparagine **Fig. 10**. Tryptone@1% supported high metabolite production **Fig. 11.** Antibiotic production was found to be governed by nitrogen sources [52] and utilization of nitrogen sources for the production of bioactive metabolites seems to be different among actinomycete strains.

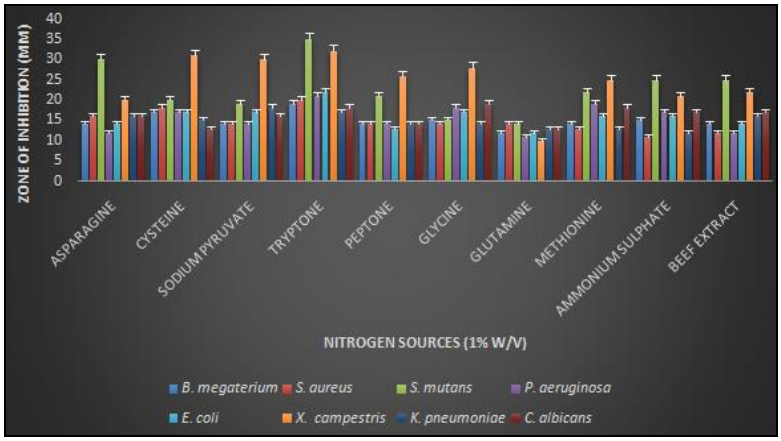

FIG. 10: INFLUENCE OF NITROGEN SOURCES ON BIOACTIVE METABOLITE PRODUCTION BY *KOCURIA ASSAMENSIS*VLS-2. Data are statistically analyzed and found significant at 5%.

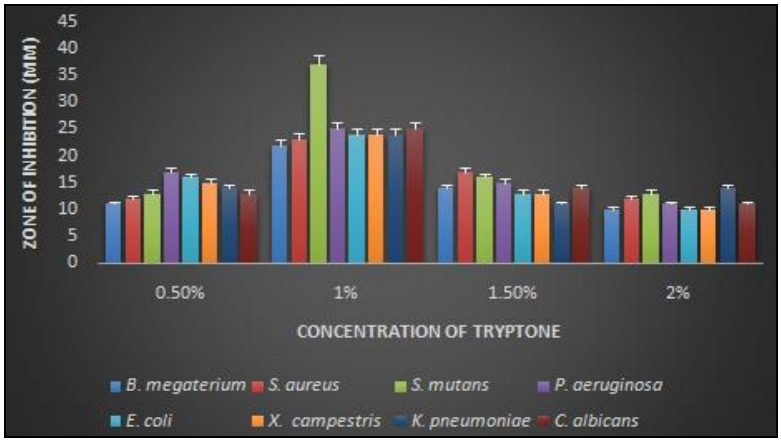

FIG. 11: INFLUENCE OF TRYPTONE CONCENTRATION ON BIOACTIVE METABOLITE PRODUCTION BY *KOCURIA ASSAMENSIS*VLS-2. Data are statistically analyzed and found significant at 5%.

CONCLUSION: In the present study *Kocuria assamensis* VLS-2 isolated from south-coastal regions of Andhra Pradesh, India exhibited high antimicrobial activity when cultured in modified ISP-1 broth with dulcitol(1%), tryptone (1%) and sodium chloride (7%) with pH 7.0 and incubated at 35ºC for 168 h. Among the bacteria tested, *Xanthomonas campestris, Pseudomonas aeruginosa, Streptococcus mutans, Escherichia coli* and *Klebisella pneumoniae* were highly sensitive to the metabolites followed by *Staphylococcus aureus, Bacillus megaterium* and while *Candida albicans* exhibited high sensitivity followed by *Aspergillus flavus* and *Penicillium citrinum* with respect to fungi.

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