ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 16, Issue 11, 2023

Online - 2455-3891
Print - 0974-2441

Research Article

BIOACTIVE METABOLITE PRODUCTION BY *NOCARDIOPSIS SYNNEMATAFORMANS*VLS-10 OF MANGROVE ORIGIN

MARY SWAPNA MOGILI, MUVVA VIJAYALAKSHMI*

Department of Botany and Microbiology, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India.

***Corresponding author: Muvva Vijayalakshmi; Email: muvvavijayalakshmi77@gmail.com**

Received: 21 April 2023, Revised and Accepted: 19 June 2023

ABSTRACT

Objective: Antibiotic resistance of pathogens has become a serious problem all over the world. Therefore, focusing for novel antibiotics is an important endeavor which is very much needed. Around 50 morphologically different *Actinobacteria* isolated from mangrove habitats of Krishna district, Andhra Pradesh, India were screened for antimicrobial activity.

Methods: Among 50 isolates, one strain *Nocardiopsis synnemataformans* VLS-10 was identified based on morphological, physiological, biochemical and molecular approaches. The effect of environmental parameters such as incubation period, pH, temperature and salt concentration and effect of various nutrients such as carbon and nitrogen sources and minerals on the bioactive metabolite production by *N. synnemataformans* VLS-10 was evaluated by employing agar well diffusion assay.

Results: Among the 50 isolates, one strain designated as VLS-10 was efficient to produce potential secondary metabolites. It was identified as *N. synnemataformans* based on polyphasic taxonomy. The present work is mainly aimed to study process optimization parameters to get high yield of bioactive compounds. ISP-2 medium supplemented with sodium chloride @ 9% maintained at pH 7.0 supported maximum yield of secondary metabolites by the strain when incubated at 35°C for 9 days.

Conclusion: Secondary metabolites possessed broad-spectrum activity against human pathogenic bacteria and fungi. Hence, strain *N. synnemataformans*VLS-10 becomes a significant source for antimicrobial compounds.

Keywords: Mangrove *Actinobacteria*, Polyphasic taxonomy, Nutritional parameters, Antagonistic activity, *Nocardiopsis synnemataformans*VLS-10.

© 2023 The Authors. Published by Innovare Academic Sciences Pvt Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/) DOI: http://dx.doi.org/10.22159/ajpcr.2023v16i11.48146. Journal homepage: https://innovareacademics.in/journals/index.php/ajpcr

INTRODUCTION

The rapid emergence of antibiotic resistance among pathogens has led to interest in the discovery of new novel antimicrobial agents. The history of new drug discovery techniques reveals that most novel skeletons come from natural sources, which involves screening of micro-organisms or plant extracts [1,2]. The demand for new drugs in the world is extremely urgent due to the rapid spread of illnesses and antibiotic-resistant pathogens which has led to an emphasis on the extreme environments for isolating novel bioactive strains [3]. Recently, the mangrove ecosystem has gained popularity for the discovery of novel strains and novel medicinal compounds.

The mangroves ecosystems are mostly tropical trees that thrive between the high spring tide mark and near mean sea levels on stable beaches [4]. The microbial diversity of mangrove ecosystems provides information on their ecological role and unique biotechnological possibilities in the field of agriculture, industry, medicine and pharmaceuticals [5]. The mangrove ecosystem is a relatively unknown source of *Actinobacteria* with the potential to produce active secondary metabolites [6]. Despite several researches on bacterial productivity and activity within the mangrove habitats, little is known about their genetic and metabolic diversity.

Actinobacteria are fascinating resource among microorganisms and are proven to be an in exhaustive mine of antimicrobial agents, particularly those potent against pathogenic organisms. Microbial secondary metabolites originated from *Actinobacteria* have had marvelous success in the discovery of new medications [7]. They generate a vast range of secondary metabolites and more than 70% of the naturally derived antibiotics are currently in clinical use. They remain a fundamental source of new chemical diversity and an important part of drug discovery [8].

The mangrove forests are among the world's most productive ecosystems which improves coastal water, produces commercial forest products, supports coastal fisheries and protects coastlines. Due to its properties of high salinity, strong winds, extreme tides, high temperature, anaerobic soils and muddiness, little is known about the bacterial population living in the mangrove especially of *Actinobacteria* with the potential to produce bioactive metabolites.

At present, the discoveries of new natural metabolites are focusing on non-*Streptomyces* or rare *Actinobacteria*. Rare *Actinobacteria* are always referred to the strains that are difficult to isolate and might correspond to the unrivalled source of new natural metabolites [9]. Bioactivity investigations of natural metabolites from the mangrove rare *Actinobacteria* have become popular. Compounds discovered from the mangrove rare *Actinobacteria* are uniquely structured and lead directly to the development of novel antibiotics that are effective against antibiotic-resistant infections [10].

Among rare *Actinobacteria*, *Nocardiopsis* produces a wide variety of chemical classes of compounds with diverse biological activities. The first description of the genus *Nocardiopsis* was in 1976 by Mayer [11]. It belongs to the order *Actinomycetales*, family *Nocardiopsaceae* and morphologically related to members of the genera *Actinomadura* and *Nocardia* [11,12]. This genus is allied with different ecosystems including terrestrial as plant epiphytes and endophytes and in mangrove environments [13]. *Nocardiopsis* species are Gram-positive,

aerobic, halotolerant and catalase positive *Actinobacteria*. They possess nocardioform mycelia with long chains of spores on aerial parts. The genus *Nocardiopsis* remarkably produced a wide variety of chemical classes of compounds with diverse biological activities.

The secondary metabolites produced by *Nocardiopsis* are mainly polyketides [14,15], cyclic peptides [16,17], macrolides [18], diketopiperazines [19,20], pyrones [21,22], alkaloids [23], naphthoquinones [24], phenazines [25] and phenoxazine derivatives [26], which possess a wide spectrum of pharmacological and biological effects, mainly as antibacterial [16], antifungal [27], anticancerous [25], antitumor [28], cytotoxic [24,29], immunomodulatory [18] and protein kinase inhibitory [30]. It is also known for the production of biosurfactants [31].

As part of our ongoing screening, strain VLS-10 isolated from mangrove habitats 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.

METHODS

Mangrove sample collection

Soil samples were collected at a depth of 6–10 cm from mangrove habitats of Krishna district, Andhra Pradesh, India. The samples were packed in sterile polyethylene bags and aseptically transported to the laboratory for further analysis.

Pretreatment of samples

The samples were subjected to pretreatment to facilitate isolation of *Actinobacteria*. The samples were air dried and pretreated with calcium carbonate (1%) and incubated at 35°C for 2 weeks [32].

Isolation of the mangrove *Actinobacteria*

Mangrove *Actinobacteria* were isolated by serial dilution method [33,34]. Stock solution was prepared by diluting 1 g of soil sample in 100 mL of sterile 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):

1. Yeast extract Malt extract Dextrose Agar-yeast extract: 4.0; malt extract: 10.0; dextrose: 4.0; and agar: 20.0 [35]
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; and agar: 18.0 [36]
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; and agar: 18 [37].

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 *Actinobacteria* (rough, chalky, powdery appearance with radiating growth and leathery texture) were observed [38,39]. The pure cultures were maintained on YMD agar slants at 4°C. The actinobacterial strains thus isolated were screened for the production of antimicrobial compounds. Among the isolates tested for bioactive compounds, the isolate designated as VLS-10 was found potent compared to other strains.

Taxonomy of potential actinobacterial strain

Taxonomic studies were performed based on morphological, cultural, biochemical, physiological and molecular analyses.

Morphological and cultural characters of strain VLS-10

Morphology studies were performed using the methods described by Shirling and Gottlieb [35]. The spore bearing hyphae and arrangement of spores were observed by cover slip method. The morphology of the mycelium and spore surface was observed using scanning electron microscope (SEM: JOELJSM 5600, Japan) [40].

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. In each individual medium, the color and growth of aerial and substrate mycelium as well as formation of soluble pigments were noted after incubation at 35°C for 7 days.

Physiological and Biochemical characters of strain VLS-10

The ability of selected isolate to utilize ten different carbon sources was determined on YMD agar plates amended with carbon sources @ 1%. The plates were incubated at 35°C for 7 days [35]. 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%) [35]. Biochemical tests such as H_2S production [41], gelatin liquefaction, starch hydrolysis, catalase production, indole, methyl red, Voges–Proskauer, citrate utilization, nitrate reduction [42], casein hydrolysis and triple sugar iron tests were also performed. The sensitivity of the isolate to different antibiotics was also determined by paper disk method [43].

Screening of the strain VLS-10 for extracellular enzyme production

The strain VLS-10 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, 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 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 s, annealing temperature of 55°C for 60 s and extension at 72°C for 60 s and an addition of 5 min at 72°C as final extension). The amplification reactions were carried out with a total volume of 50 µL 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 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 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).

Pairwise sequence alignment

The gene sequence of isolate VLS-10 was aligned using BLAST against the gene library available for *Nocardiopsis* species in the NCBI and the GenBank. Pairwise evolutionary distances were computed by molecular evolutionary genetics analysis (MEGA-6) software.

Multiple sequence alignment

The phylogenetic analysis of isolate was conducted using maximum parsimony method with BLAST and CLUSTAL W. The closely related

homologous isolates were identified, retrieved, and compared to the sequence of isolated strain using CLUSTAL W available with the MEGA 6 Version [44].

Nucleotide sequence accession numbers

The 16S rRNA gene sequence of the isolate VLS-10 was registered in the GenBank database.

Nutritional parameters affecting the bioactive metabolite production by the strain

Bioactive metabolite production by the strain was optimized using different 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 the isolate and its antimicrobial activity against Gram-positive bacteria (*Bacillus megaterium, Streptococcus mutans* and *Staphylococcus aureus*), Gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae, Xanthomonas campestries* and *Pseudomonas aeruginosa*), and fungi (*Aspergillus flavus*, *Candida alibicans* and *Penicillium citrinum*) were recorded by culturing it in ISP-2 broth for 10 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, biomass of strain and production of antimicrobial metabolites were determined. Biomass was measured as dry weight of cell mass (mg/100 mL culture medium). The supernatant was extracted with ethyl acetate, vacuum dried in a rotavapor and used for testing the antimicrobial activity against bacteria and fungi by agar well diffusion method [45].

Assay of antimicrobial activity

The antimicrobial activity of solvent extract was tested by agar diffusion assay. Ethyl acetate itself served as negative control. The wells were made 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 observed by the presence of a zone of inhibition surrounding the well. Each test was repeated 3 times and the antimicrobial activity was expressed as mean of diameter of inhibition zones (mm) when compared to control. In 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.

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 the pH of production medium from 5.0 to 9.0. The optimal pH achieved at this step was used for further study [46]. Similarly, the optimum temperature for bioactive metabolite yield was measured by incubating the production medium at temperatures ranging from 20 to 40°C [47].

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 fermentation medium amended with different concentrations of sodium chloride (0–12%) at optimum pH and temperature for 8 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 arabinose, galactose, dulcitol, maltose, mannitol, starch, sucrose, lactose, fructose, cellulose and sorbitol @1% were added to the medium 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, the impact of different nitrogen sources on yield of antimicrobials of the strain was studied by supplementing nitrogen source in the medium with different nitrogen sources such as peptone, glycine, urea, glutamine, asparagine, cysteine, L-arginine, ammonium sulfate, tryptone, beef extract and sodium pyruvate in the medium replacing nitrogen source. Further, the impact of different levels of optimized nitrogen source (0.5–2.0%) was studied to enhance antimicrobial metabolite production [48].

Test organisms

The antimicrobial metabolites produced by the strain under optimized conditions were tested against bacteria (*S. aureus* (MTCC 3160), *B. megaterium* (NCIM 2187), *S. mutans* (MTCC 497), *X. campestris* (MTCC 2286), *K. pneumoniae* (MTCC 109), *P. aeruginosa* (ATCC 9027) and *E. coli* (ATCC 9027)) and fungi *A. flavus* (ATCC 189), *C. albicans* (MTCC 183) and *P. 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.

RESULTS AND DISCUSSION

The present study was designed to investigate the mangrove sediment regions of Andhra Pradesh for novel *Actinobacteria* and their antimicrobial properties. Fifty actinobacterial strains isolated from the mangrove habitats were screened for antimicrobial activity. The strain designated as VLS-10 was found to exhibit high antagonistic activity against the micro-organisms tested. The strain VLS-10 exhibited typical morphological characteristics of the genus *Nocardiopsis.* Morphological and micromorphological observation of the strain revealed that aerial hyphae were abundant with well-developed irregular branches penetrating agar thus formed compact colonies on the agar surface (Fig. 1). Synnemata were formed from spiral aerial hyphae that wrapped together to form long ropes. The hyphae fragmented into small rod-shaped elements at later stages. Single zig-zag hyphae were also observed, which fragmented in a similar way to form rod-shaped elements. Soluble pigment production by the strain was not found on tyrosine agar medium.

Cultural characteristics

The cultural characteristics of the strain are represented in Table 1. The strain VLS-10 exhibited good growth on 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 and Czapek-Dox agar. White aerial mycelium and pale yellow substrate mycelium were found on ISP-2, nutrient agar and humic acid vitamin-B agar. The strain could not grow on ISP-1, ISP-4, ISP-5, ISP-7, starch casein salts agar and glucose tryptone agar media.

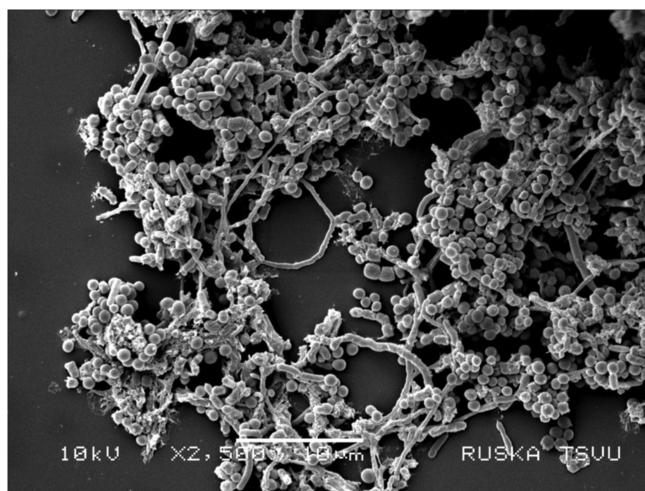

Fig. 1: Scanning electron microscopic photograph of isolate VLS-10

Table 1: Cultural characteristics of the strain VLS-10

Name of the medium	Growth	AM*	SM**	Pigmentation
Tryptone yeast extract agar (ISP-1)	-	-	-	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	Moderate	White	Pale yellow	No
Nutrient agar	Good	White	Pale yellow	No
Starch casein salts agar	-	-	-	No
Glucose tryptone agar	-	-	-	No
Humic-acid Vitamin-B agar	Good	White	Pale yellow	No

AM: Aerial mycelium, SM: Substrate mycelium.: No growth, ISP: International *Streptomyces* project

Biochemical characteristics of VLS-10

The strain VLS-10 exhibited positive response to indole, methyl red, Voges–Proskauer, nitrate reduction, urease production and starch hydrolysis but negative for citrate utilization test, hydrogen sulfide and catalase production, triple sugar iron and gelatine liquefaction. The details of morphological, physiological and biochemical characteristics of the isolate are given (Table 2). Utilization of carbon sources by the strains could be used as an aid for species determination [41]. The strain VLS-10 efficiently utilized the carbon sources such as D-glucose, maltose, sucrose, galactose, dulcitol, mannitol and starch but could not utilize fructose, lactose, sorbitol, and cellulose (Table 3). It was also reported that antibiotic sensitivity of some nocardioform bacteria could be used as one of the valuable criteria for taxonomic differentiation [49]. Antibiotic susceptibility testing showed that the isolate was susceptible to imipenem, chloramphenicol, clindamycin, tetracycline, and cefixime but resistant to gentamicin, vancomycin, cefepime, amikacin and penicillin (Table 4).

Physiological characteristics

The physiological tests are indispensable tools for classification and identification of *Actinobacteria* [50-53]. The strain VLS-10 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 optimum growth at 9% NaCl; hence, the strain could be placed in intermediate salt tolerance group [54] (Table 5). The strain VLS-10 could also produce enzymes such as L-asparaginase, glutaminase, amylase and urease.

MOLECULAR CHARACTERIZATION OF THE STRAIN VLS-10

The 16S rRNA sequence data supported the assignment of this isolate VLS-10 to the genus *Nocardiopsis* and species *Synnemataformans*. The partial 16S rRNA sequence of the strain VLS-10 was obtained and submitted to the GenBank database under an accession number MW450583. The partial sequence was aligned and compared with all the 16S rRNA gene sequence available in the GenBank database using the multisequence advanced BLAST comparison tool that is available in the website of National Center for Biotechnology Information. The highest 16S rRNA sequence similarity value of 100% was obtained for the *Nocardiopsis synnemataformans*. The phylogenetic analysis of the 16S rRNA gene sequence was aligned using the CLUSTAL W program from the MEGA 6 Version. Phylogenetic tree was constructed using MEGA software Version 6 using maximum parsimony method [55-57]. The topologies of the constructed tree were evaluated by bootstrap analysis with 1000 resamplings by maximum parsimony tool. Sequence comparison of the strain VLS-10 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 *N. synnemataformans* (Fig. 2).

Growth pattern and antimicrobial profile of the strain

The growth pattern and antimicrobial profile of *N. synnemataformans* VLS-10 were studied at regular intervals up to 10 days in batch culture. The

Table 2: Morphological and biochemical characteristics of the isolate VLS-10

Character	Response
Morphological characters	VLS-10
Sporophore morphology	Recti flexible
Color of aerial mycelium	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 VLS-10

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 negative/no growth

Table 4: Antibiotic sensitivity of the strain VLS-10

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

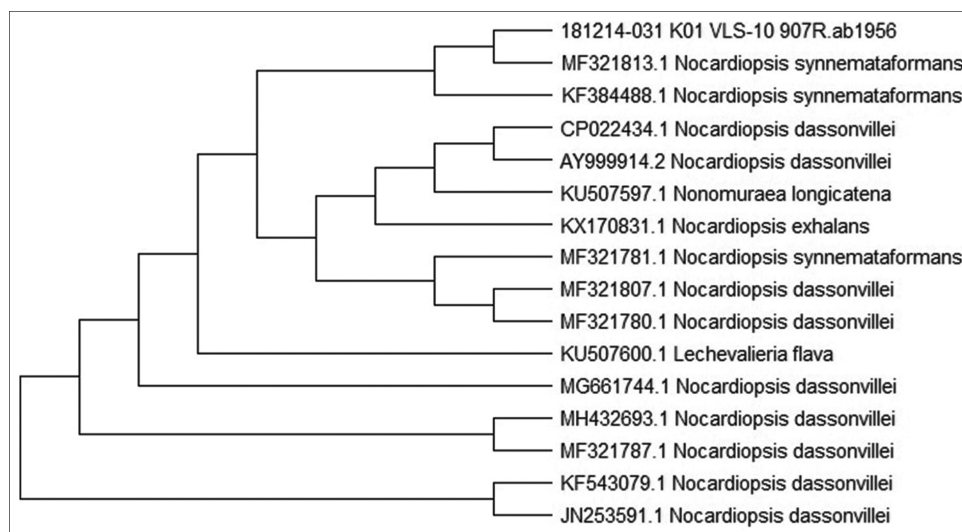

Fig. 2: Maximum parsimony tree based on partial 16s rRNA gene sequence showing relationship between isolate VLS-10 and related members of the genus *Nocardiopsis*

Table 5: Physiological and enzymatic characters of the strain VLS-10

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 9%
Enzymatic activity	
Amylase	P
Protease	N
Cellulase	N
Asparaginase	P
Glutaminase	P
Lipase	N
Urease	P

*P: Positive, N: Negative

stationary phase of the strain extended from 216 h to 240 h of incubation (Fig. 3). The secondary metabolites obtained from 9-day-old culture showed high antimicrobial activity against the test microbes. The antimicrobial metabolites produced from 11-day-old *Nocardiopsis litoralis*VSM-8 [58], 8-day old *Nocardiopsis flavescens*VJMS-18 [59], 5-day old *Nocardiopsis halotolerans*VJPR-2 [60] and 4-day-old *Nocardia metallicus*VJSY-14 [61] exhibited high antagonistic activity. The secondary metabolites obtained from 6-day-old *Streptomyces vinaceusdrappus*VJMS-4, *Streptomyces rectiverticillatus*VJMS-8 [62] and 5-day old *Streptomyces albogriseolous*VJMS-7 [63] showed high antimicrobial activity against the test microbes.

Influence of culture media on bioactive metabolite production by the strain

The influence of different media on the production bioactive metabolites is shown in Fig. 4. Among the media tested, yeast extract malt extract dextrose broth supported the production of bioactive metabolites followed by glycerol asparagines broth. Oskay showed that activity of actinobacterial isolates could be increased or decreased remarkably under different cultural conditions. The actinobacterial strains such as *Nocardiopsis arvandica* [64] and *Nocardiopsis sinuspersici* [65] also showed optimum level of antibiotic production in yeast extract malt extract dextrose broth.

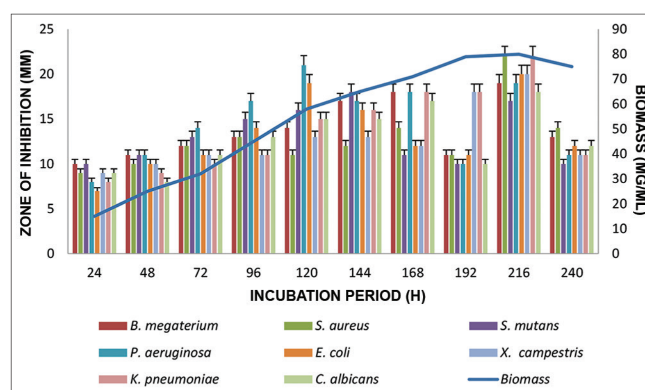

Fig. 3: Influence of incubation period on bioactive metabolite production by *Nocardiopsis synnemataformans*VLS-10. Data are statistically analyzed and found significant at 5%

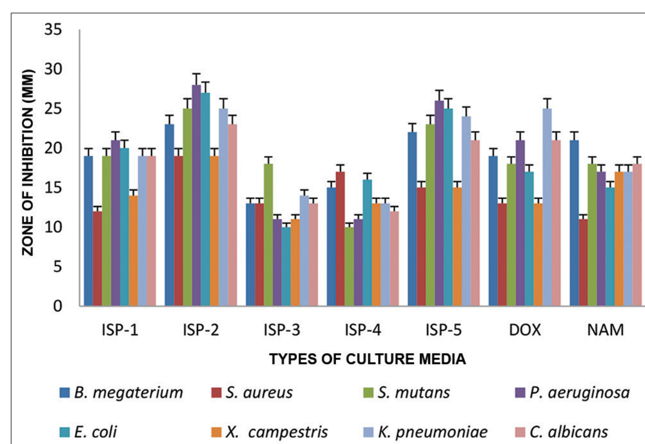

Fig. 4: Influence of cultural media on bioactive metabolite production by *Nocardiopsis synnemataformans*VLS-10. Data are statistically analyzed and found significant at 5%

Influence of initial pH and incubation temperature on bioactive metabolite production by the strain

The various environmental requirements influence growth and bioactive metabolite production by *Actinobacteria*. Maximum

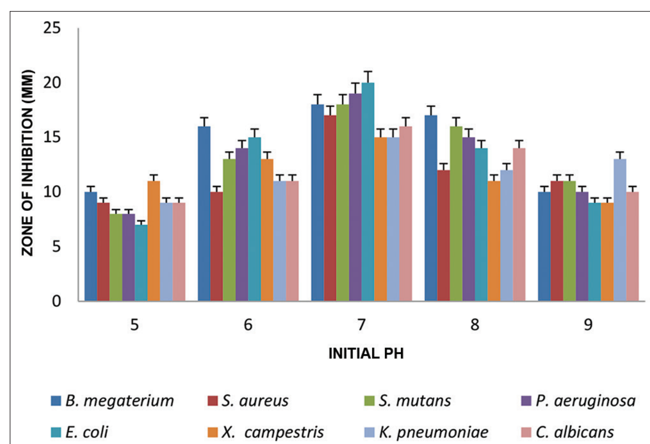

Fig. 5: Influence of initial pH on bioactive metabolite production by *Nocardiopsis synnemataformans*VLS-10. Data are statistically analyzed and found significant at 5%

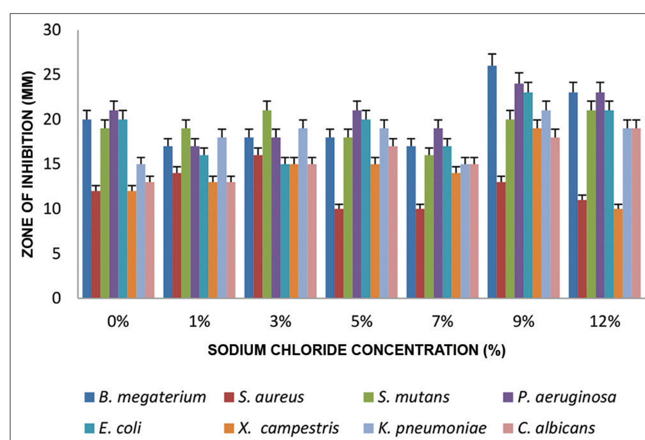

Fig. 7: Influence of Sodium chloride concentration on bioactive metabolite production by *Nocardiopsis synnemataformans*VLS-10. Data are statistically analyzed and found significant at 5%

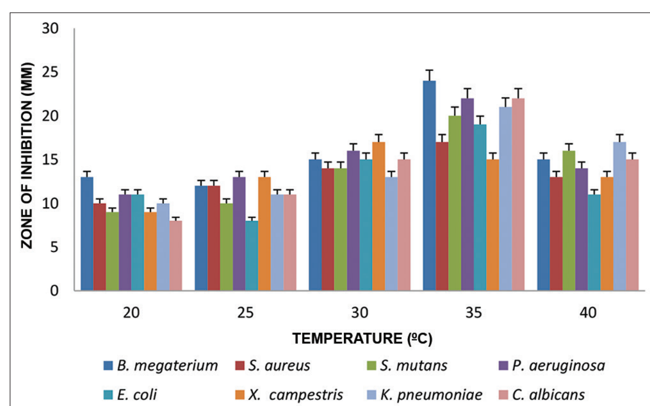

Fig. 6: Influence of incubation temperature on bioactive metabolite production by *Nocardiopsis synnemataformans*VLS-10. Data are statistically analyzed and found significant at 5%

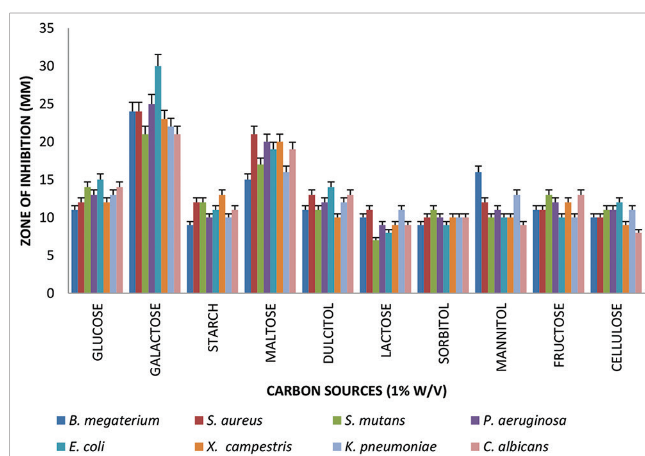

Fig. 8: Influence of carbon sources on bioactive metabolite production by *Nocardiopsis synnemataformans*VLS-10. Data are statistically analyzed and found significant at 5%

growth and antimicrobial metabolite production was obtained at pH 7.0 (Fig. 5). The actinobacterial strains such as *N. metallicus* DSM 44598 [66], *Nocardiopsis dassonvillei* [67] and *Nocardiopsis luteus* [68] showed optimum level of antibiotic production at 7.0 pH. 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 *Actinobacteria* belonging to the genus *Nocardiopsis* including *Nocardiopsis algeriensis* [69] and *N. halotolerans* [70] showed optimum levels of antibiotic production at 35°C.

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 to 12%. Sodium chloride @9% was found to be optimum for antimicrobial compound production by *N. synnemataformans* VLS-10 (Fig. 7). Further, increase in salt concentration resulted in reduced antimicrobial activity. The requirement of sodium chloride for production of bioactive metabolites seems to be different among actinobacterial strains. Optimum sodium chloride concentration for antimicrobial metabolite production was reported to be 9% for *Nocardiopsis nikkonensis* [71] and *Nocardiopsis xinjiangensis* [72].

Influence of carbon sources on bioactive metabolite production by the strain

Effect of different carbon sources was evaluated for their impact on antimicrobial metabolite production (Fig. 8). Among the various carbon sources tested, galactose was found to be best 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 galactose was the most preferred carbon source for biomass and bioactive metabolite production by this strain, different levels of galactose (0.5–2.0%) were tested to determine optimal concentration for bioactive metabolite production (Fig. 9). Galactose @1.0% supplemented to the medium promoted bioactive metabolite production.

Influence of nitrogen sources on bioactive metabolite production by the strain

Different nitrogen sources were found to have significant effect on secondary metabolite production by *N. synnemataformans*VLS-10. High antimicrobial activity was obtained in culture medium supplemented with methionine, followed by sodium pyruvate and peptone (Fig. 10). Methionine @1% supported high metabolite production (Fig. 11). Antibiotic production was found to be governed by nitrogen sources [73] and utilization of nitrogen sources for the production of bioactive metabolites seems to be different among actinobacterial strains.

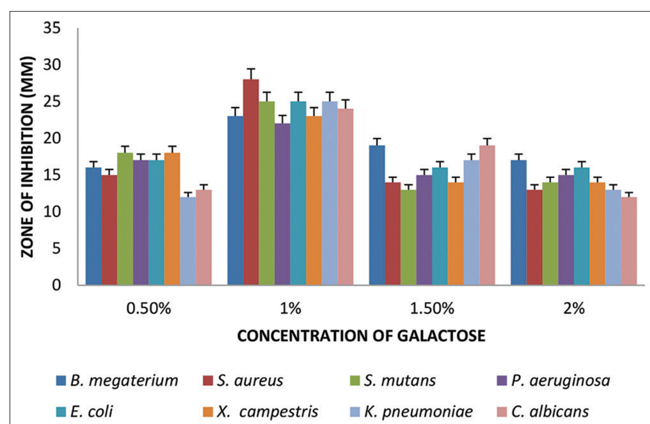

Fig. 9: Influence of Galactose on bioactive metabolite production by *Nocardiopsis synnemataformans*VLS-10. Data are statistically analyzed and found significant at 5%

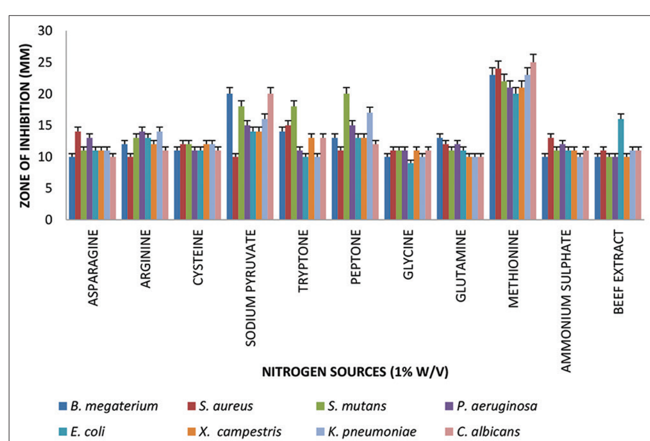

Fig. 10: Influence of nitrogen sources on bioactive metabolite production by *Nocardiopsis synnemataformans*VLS-10. Data are statistically analyzed and found significant at 5%

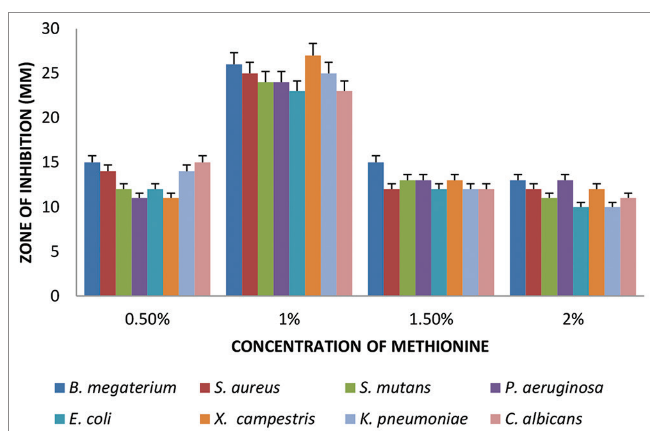

Fig. 11: Influence of methionine on bioactive metabolite production by *Nocardiopsis synnemataformans*VLS-10. Data are statistically analyzed and found significant at 5%

CONCLUSION

In the present study, *N. synnemataformans*VLS-10 isolated from mangrove habitats of Krishna district, Andhra Pradesh, India exhibited high antimicrobial activity when cultured in modified ISP-2 broth with malt extract (1%), galactose (1%), methionine (1%) and sodium chloride (9%) with pH 7.0 and incubated at 35°C for 240 h. Among the bacteria tested, *B. megaterium*, *E. coli*, *P. aeruginosa* and *K. pneumoniae* are highly sensitive to the metabolites followed by *S. aureus*, *X. campestris* and *S. mutans* while *C. albicans* exhibited high sensitivity followed by *A. flavus* and *P. citrinum* with respect to fungi.

ACKNOWLEDGMENT

Authors are thankful to UGC, New Delhi for providing BSR fellowship to carry out this work and to the Department of Botany and Microbiology for providing laboratory facilities.

AUTHORS CONTRIBUTION

Concept and design of the study, data collection, data analysis and manuscript writing were done by first and corresponding author.

CONFLICTS OF INTERESTS

We declare that we have no conflicts of interest.

AUTHORS FUNDING

UGC, New Delhi.

REFERENCES

1. Ganesan A. The impact of natural products upon drug discovery. Curr Opin Chem Biol 2008;12:306-317
2. Baltz RH. Antimicrobials from actinomycetes. Back to the future. Microbe 2007;2:125-31.
3. Jiang Y, Xu P, Lou K, Xu LH, Liu ZH. Problems and countermeasure on development of pharmaceuticals from *Actinobacteria* resource. Microbiology 2008;35:272-4.
4. Holguin G, Vazquez P, Bashan Y. The role of sediment microorganisms in the productivity, conservation, and rehabilitation of mangrove ecosystems: An overview. Biol Fertil Soils 2001;33:265-78. doi: 10.1007/s003740000319
5. Lageiro MM, Moura MJ, Reis A, Costa-Ferreira MJ. Microbial proteases application in leather industry. J Biotechnol 2007;131:S239-40. doi: 10.1016/j.jbiotec.2007.07.717
6. Hong K, Gao AH, Xie QY, Gao H, Zhuang L, Lin HP. *Actinobacteria* for marine drug discovery isolated from mangrove soils and plants in China. Mar Drugs 2009;7:24-44.
7. Naine J, Srinivasan MV, Devi SC. Novel anticancer compounds from marine actinomycetes: A review. J Pharm Res 2011;4:1285-7.
8. Raja A, Prabakarana P. Actinomycetes and drug-an overview. Sci Alert 2011;1:72-84.
9. Baltz RH. Combinatorial biosynthesis of novel antibiotics and other secondary metabolites in actinomycetes. SIM News 2006;56:148-60.
10. Lam KS. Discovery of novel metabolites from marine actinomycetes. Curr Opin Microbiol 2006;9:245-51. doi: 10.1016/j.mib.2006.03.004, PMID 16675289
11. Meyer J. Nocardiopsis, a new genus of the order *Actinomycetales.* Int J Syst Evol Microbiol 1976;26:487-93. doi: 10.1099/00207713-26-4-487
12. Rainey FA, Ward-Rainey N, Kroppenstedt RM, Stackebrandt E. The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: Proposal of Nocardiopsaceae fam. nov. Int J Syst Evol Microbiol 1996;46:1088-92. doi: 10.1099/00207713-46-4-1088
13. Bennur T, Kumar AR, Zinjarde S, Javdekar V. *Nocardiopsis*. *Nocardiopsis* species: Incidence, ecological roles and adaptations. Microbiol Res 2015;174:33-47. doi: 10.1016/j.micres.2015.03.010, PMID 25946327
14. Raju R, Piggott AM, Quezada M, Capon RJ. Nocardiopsins C and D and nocardiopyrone A: Newpolyketides from an Australian marine-derived *Nocardiopsis* sp. Tetrahedron 2013;69:692-8.
15. Dashti Y, Grkovic T, Abdelmohsen UR, Hentschel U, Quinn RJ. Actinomycete metabolome induction/suppression with N-Acetylglucosamine. J Nat Prod 2017;80:828-36. doi: 10.1021/acs.jnatprod.6b00673, PMID 28355070
16. Engelhardt K, Degnes KF, Kemmler M, Bredholt H, Fjaervik E, Klinkenberg G, *et al*. Production of a new thiopeptide antibiotic, TP-1161, by a marine *Nocardiopsis* species. Appl Environ Microbiol 2010;76:4969-76. doi: 10.1128/AEM.00741-10, PMID 20562278
17. Shin J, Seo Y, Lee HS, Rho JR, Mo SJ. A new cyclic peptide from

a marine-derived bacterium of the genus *Nocardiopsis*. J Nat Prod 2003;66:883-4. doi: 10.1021/np030075r, PMID 12828484
18. Raju R, Piggott AM, Conte M, Tnimov Z, Alexandrov K, Capon RJ. Nocardiopsins: New FKBP12-binding macrolide polyketides from an Australian marine-derived actinomycete, *Nocardiopsis* sp. Chemistry 2010;16:3194-200. doi: 10.1002/chem.200902933, PMID 20112311
19. Raju R, Piggott AM, Huang XC, Capon RJ. Nocardioazines: A novel bridged diketopiperazine scaffold from a marine-derived bacterium inhibits P-glycoprotein. Org Lett 2011;13:2770-3. doi: 10.1021/ol200904v, PMID 21513295
20. Fu P, Liu P, Qu H, Wang Y, Chen D, Wang H, *et al*. A-pyrones and diketopiperazine derivatives from the marine-derived actinomycete *Nocardiopsis* dassonvillei HR10-5. J Nat Prod 2011;74:2219-23. doi: 10.1021/np200597m, PMID 21958359
21. Kim Y, Ogura H, Akasaka K, Oikawa T, Matsuura N, Imada C, *et al*. Nocapyrones: And pyrones from a marine-derived *Nocardiopsis* sp. Mar Drugs 2014;12:4110-25. doi: 10.3390/md12074110, PMID 25007160
22. Kim MC, Kwon OW, Park JS, Kim SY, Kwon HC. Nocapyrones H-J, 3, 6-disubstituted pyrones from the marine actinomycete *Nocardiopsis* sp. KMF-001. Chem Pharm Bull (Tokyo) 2013;61:511-5. doi: 10.1248/cpb.c12-00956, PMID 23386029
23. Tian S, Yang Y, Liu K, Xiong Z, Xu L, Zhao L. Antimicrobial metabolites from a novel halophilic actinomycete *Nocardiopsis* terrae YIM 90022. Nat Prod Res 2014;28:344-6. doi: 10.1080/14786419.2013.858341, PMID 24236566
24. Ding ZG, Zhao JY, Li MG, Huang R, Li QM, Cui XL, *et al*. spironaphthoquinones from a tin mine tailings-derived alkalophilic *Nocardiopsis* species. J Nat Prod 2012;75:1994-8.
25. Gao X, Lu Y, Xing Y, Ma Y, Lu J, Bao W, *et al*. A novel anticancer and antifungus phenazine derivative from a marine actinomycete BM-17. Microbiol Res 2012;167:616-22. doi: 10.1016/j.micres.2012.02.008, PMID 22494896
26. Lu CH, Li YY, Wang HX, Wang BM, Shen YM. A new phenoxazine derivative isolated from marine sediment actinomycetes, *Nocardiopsis* sp. 236. Drug Discov Ther 2013;7:101-4. PMID 23917857
27. Tian SZ, Pu X, Luo G, Zhao LX, Xu LH, Li WJ, *et al*. Isolation and characterization of new p-Terphenyls with antifungal, antibacterial and antioxidant activities from halophilic actinomycete *Nocardiopsis* gilva YIM 90087. J Agric Food Chem 2013;61:3006-12. doi: 10.1021/jf400718w, PMID 23441911
28. He J, Roemer E, Lange C, Huang X, Maier A, Kelter G, *et al*. Structure, derivatization, and antitumor activity of new griseusins from *Nocardiopsis* sp. J Med Chem 2007;50:5168-75. doi: 10.1021/jm070170n, PMID 17877337
29. Cho JY, Williams PG, Kwon HC, Jensen PR, Fenical W. Lucentamycins AD. cytotoxic peptides from the marine-derived actinomycete *Nocardiopsis* lucentensis. J Nat Prod 2007;70:1321-8. doi: 10.1021/np070101b, PMID 17630797
30. Kase H, Iwahashi K, Matsuda YK. K-252a, A potent inhibitor of protein kinase C from microbial origin. J Antibiot (Tokyo) 1986;39:1059-65. doi: 10.7164/antibiotics.39.1059, PMID 3759657
31. Khopade A, Biao R, Liu X, Mahadik K, Zhang L, Kokare C. Production and stability studies of the biosurfactant isolated from marine *Nocardiopsis* sp. B4. Desalination 2012;285:198-204. doi: 10.1016/j.desal.2011.10.002
32. El-Nakeeb MA, Lechevalier HA. Selective isolation of aerobic actinomycetes. Appl Microbiol 1963;11:75-7. doi: 10.1128/am.11.2.75-77.1963, PMID 13937509
33. Valli S, Suvathi SS, Aysha OS, Nirmala P, Vinoth KP, Reena A. Antimicrobial potential of Actinomycetes species isolated from marine environment. Asian Pac J Trop Biomed 2012;2:469-73. doi: 10.1016/S2221-1691(12)60078-1, PMID 23569952
34. Chaudhary HS, Yadav J, Shrivastava AR, Singh S, Singh AK, Gopalan N. Antibacterial activity of actinomycetes isolated from different soil samples of Sheopur (A city of central India). J Adv Pharm Technol Res 2013;4:118-23. doi: 10.4103/2231-4040.111528, PMID 23833752
35. Shirling EB, Gottlieb D. Methods for characterization of *Streptomyces* species. Int J Syst Evol Microbiol 1966;16:313-40. doi: 10.1099/00207713-16-3-313
36. Jose PA, Jebakumar SR. Phylogenetic diversity of actinomycetes cultured from coastal multipond solar saltern in Tuticorin, India. Aquat Biosyst 2012;8:23. doi: 10.1186/2046-9063-8-23, PMID 22950748
37. Boughachiche F, Reghioua S, Oulmi L, Zerizer H, Kitouni M, Boudemagh A. Isolation of actinomycetales producing antimicrobial substances from the Sebkha Ain Mlila. Sci Technol 2005;23:5-10.
38. Bizuye A, Moges F, Andualem B. Isolation and screening of antibiotic producing actinomycetes from soils in Gondar town, North West Ethiopia. Asian Pac J Trop Dis 2013;3:375-81. doi: 10.1016/S2222-1808(13)60087-0
39. Nonomura H, Ohara Y. Distribution of actinomycetes in soil. XI. Some new species of the genus *Actinomadura*, *Lechevalier*. J Ferment Technol 1971;49:904-12.
40. Das S, Lyla PS, Khan SA. Characterization and identification of marine actinomycetes existing systems, complexities and future directions. Natl Acad Sci Lett 2008;31:149-60.
41. Pridham TG, Gottlieb D. The utilization of carbon compounds by some actinomycetales as an aid for species determination. J Bacteriol 1948;56:107-14. doi: 10.1128/jb.56.1.107-114.1948, PMID 16561537
42. Kavitha A, Vijayalakshmi M, Sudhakar P, Narasimha G. Screening of actinomycete strains for the production of antifungal metabolites. Afr J Microbiol Res 2010;4:27-32.
43. Gordon RE. Some criteria for the recognition of *Nocardia madurae* (Vincent) Blanchard. J Gen Microbiol 1966;45:355-64. doi: 10.1099/00221287-45-2-355, PMID 5969755
44. Cowan ST. Cowan, Steel's, Manual for the Identification of Medical Bacteria. 2nd ed. Cambridge: University Press; 1974.
45. Cappuccino JG, Sherman N. Microbiology: A Laboratory Manual. Harlow: Benjamin; 2002.
46. Oskay M. Effects of some environmental conditions on biomass and antimicrobial metabolite production by *Streptomyces* sp. KGG32. Int J Agric Biol 2011;13:7-24.
47. Ripa FA, Nikkon F, Zaman S, Khondkar P. Optimal conditions for antimicrobial metabolites production from a new *Streptomyces* sp. RUPA-08PR isolated from Bangladeshi soil. Mycobiology 2009;37:211-4. doi: 10.4489/MYCO.2009.37.3.211, PMID 23983535
48. Kathiresan K, Balagurunathan R, Selvam MM. Fungicidal activity of marine actinomycetes against phytopathogenic fungi. Indian J Biotechnol 2005;4:271-6.
49. Goodfellow M, Orchard VA. Antibiotic sensitivity of some nocardioform bacteria and its value as a criterion for taxonomy. J Gen Microbiol 1974;83:375-87. doi: 10.1099/00221287-83-2-375, PMID 4610085
50. Kampfer P, Kroppenstedt RM, Dott W. A numerical classification of the genera *Streptomyces* and *Streptoverticillium* using miniaturized physiological tests. J Gen Microbiol 1991;137:1831-91. doi: 10.1099/00221287-137-8-1831
51. Hasegawa T, Yamano T, Yoneda M. *Streptomyces inusitatus* sp. nov. Int J Syst Bacteriol 1978;28:407-10. doi: 10.1099/00207713-28-3-407
52. Kim BS, Sahin N, Minnikin DE, Screwinska JZ, Mordarski M, Goodfellow M. Classification of thermophilic *streptomycetes* including the description of *Streptomyces thermoalcalitolerance* sp. nov. Int J Syst Evol Microbiol 1999;49:7-17.
53. Shimizu M, Nakagawa Y, Sato Y, Furumai T, Igarashi Y, Onaka H, *et al*. Studies on endophytic actinomycetes (1) *Streptomycetes* sp. Isolated from rhododendron and its antimicrobial activity. J Gen Plant Pathol 2000;66:360-6. doi: 10.1007/PL00012978
54. Tresner HD, Hayes JA, Backus EJ. Differential tolerance of Streptomycetes to sodium chloride as a taxonomic aid. Appl Microbiol 1968;16:1134-6. doi: 10.1128/am.16.8.1134-1136.1968, PMID 5675504
55. Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;24:189-204.
56. Takahashi K, Nei M. Efficiencies of fast algorithms of phylogenetic inference under the criteria of maximum parsimony, minimum evolution, and maximum likelihood when a large number of sequences are used. Mol Biol Evol 2000;17:1251-8. doi: 10.1093/oxfordjournals.molbev.a026408, PMID 10908645
57. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28:2731-9. doi: 10.1093/molbev/msr121, PMID 21546353
58. Ushakiranmayi M, Vijayalakshmi M. Optimization of culture conditions by response surface methodology and unstructured kinetic modeling for bioactive metabolite production by *Nocardiopsis* litoralis VSM-8. 3 Biotech 2016;6:210.
59. Swapna MM, Vijayalakshmi M. Selective isolation and characterization of *Nocardiopsis flavescens*VJMS-18 from coastal regions of Andhra Pradesh, India. Int J Pharm Sci Res 2019;10:3800-7.
60. Prasadarao N, Vijayalakshmi M. Exploration of potent actinobacterium *Nocardiopsis halotolerans*VJPR-2 isolated from mangrove habitats. Asian J Pharm Clin Res 2016;9:4.
61. Saba Y, Vijayalakshmi M, Kumar R M. Taxonomic characterization of

rare *Actinobacteria* isolated from mangrove ecosystem. Int J Curr Res 2016;8:31060-6.
62. Swapna MM, Vijayalakshmi M. Antimicrobial potential of *Streptomyces albogriseolous* VJMS-7 isolated from coastal regions of Guntur district, Andhra Pradesh, India. Asian J Microbiol Biotechnol Environ Sci 2019;21:688-94.
63. Swapna MM, Vijayalakshmi M. Isolation, identification and antimicrobial potential of marine *Streptomyces* spp from coastal regions of Andhra Pradesh, India. Int J Pharm Biol Sci 2018;8:1136-45.
64. Hamedi J, Mohammadipanah F, Pötter G, Spröer C, Schumann P, Göker M, *et al*. Nocardiopsis *arvandica* sp. nov., isolated from sandy soil. Int J Syst Evol Microbiol 2011;61:1189-94. doi: 10.1099/ijs.0.022756-0, PMID 20562242
65. Hamedi J, Mohammadipanah F, von Jan M, Pötter G, Schumann P, Spröer C, *et al*. *Nocardiopsis sinuspersici* sp. nov., isolated from sandy rhizospheric soil. Int J Syst Evol Microbiol 2010;60:2346-52. doi: 10.1099/ijs.0.018366-0, PMID 19933587
66. Schippers A, Bosecker K, Willscher S, Spröer C, Schumann P, Kroppenstedt RM. Nocardiopsis *metallicus* sp. nov., a metal-leaching actinomycete isolated from an alkaline slag dump. Int J Syst Evol Microbiol 2002;52:2291-5. doi: 10.1099/00207713-52-6-2291, PMID 12508899
67. Tsujibo H, Miyamoto K, Hasegawa T, Inamori Y. Amino acid compositions and partial sequences of two types of alkaline serine proteases from *Nocardiopsis dassonvillei* subsp. Prasina OPC-210. Agric Biol Chem 1990;54:2177-9, PMID 1368615
68. Hussein EI, Jacob JH, Shakhatreh MA, Al-Razaq MA, Juhmani AF, Cornelison CT. Detection of antibiotic-producing *Actinobacteria* in the sediment and water of Main thermal springs (Jordan). GERMS 2018;8:191-8. doi: 10.18683/germs.2018.1146, PMID 30775338
69. Bouras N, Meklat A, Zitouni A, Mathieu F, Schumann P, Sproer C. *Nocardiopsis algeriensis* sp. nov., an alkalitolerant actinomycete isolated from Saharan soil. Antonie Leeuwenhoek 2015;8:313-20.
70. Al-Zarban SS, Abbas I, Al-Musallam AA, Steiner U, Stackebrandt E, Kroppenstedt RM. *Nocardiopsis halotolerans* sp. nov., isolated from salt marsh soil in Kuwait. Int J Syst Evol Microbiol 2002;52:525-9. doi: 10.1099/00207713-52-2-525, PMID 11931165
71. Yamamura H, Ohkubo SY, Ishida Y, Otoguro M, Tamura T, Hayakawa M. *Nocardiopsis nikkonensis* sp. nov., isolated from a compost sample. Int J Syst Evol Microbiol 2010;60:2967-71. doi: 10.1099/ijs.0.016956-0, PMID 20118291
72. Li MG, Li WJ, Xu P, Cui XL, Xu LH, Jiang CL. *Nocardiopsis xinjiangensis* sp. nov., a halophilic actinomycete isolated from a saline soil sample in China. Int J Syst Evol Microbiol 2003;53:317-21. doi: 10.1099/ijs.0.02055-0, PMID 12656190
73. Francois V, Stephane. Nitrogen source governs the patterns of growth and pristinamycin production in *Streptomyces pristinaespiralis*. Microbiology (Reading) 2001;147:2447-59.