JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue

JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)

An International Scholarly Open Access, Peer-reviewed, Refereed Journal

Antimicrobial Activities of Marine Actinobacteria *Streptomyces sp*. isolated from Machilipatnam coast area

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Abstract : Actinobacteria of marine origin are considered to be a good and efficient source of secondary metabolites having been developed for clinical use. *Streptomyces* is the largest genus of actinobacteria belonging to the family Streptomycetaceae. It is gram positive with enhanced GC content. *Streptomyces* is synonymous with the production of numerous naturally originated antibiotics having a multitude of uses. It has been established that the coastal wetland inhabiting actinobacteria possess broad spectrum antimicrobial activities. This forms the basis that they are able to generate newer bioactive substances. In the present investigation the antimicrobial activities of marine actinobacteria- *Streptomyces sp*., namely, *Streptomyces alboniger*, *Streptomyces coelicolor and Streptomyces griseus* that were isolated from the Machilipatnam coast area was studied against the clinical bacteria consisting of Gram positive *Staphylococcus aureus, Streptococcus faecalis* and Gram negative *Proteus vulgaris and Salmonella typhi.* .

IndexTerms **- Actinobacteria, Streptomyces, antimicrobial, bioactive, metaboloites.**

I. INTRODUCTION

The most interesting and appreciable feature of *Streptomyces sp*. is the production of secondary metabolites, specifically antibiotics. Streptomycetaceae family is part of the phylum Actinobacteria , the order Actinomycetales and the genus Streptomyces is the sole member of the family by Anderson and Wellington, (2001). The studies of Chen *et al*., (2021) established the coastal wetland inhabiting actinobacteria exhibiting broad spectrum antimicrobial activities in addition to PKS and NRPS genes. Wang *et al*., (2020) presented the antimicrobial activities of almost 313 novel natural products of marine actinomycetes from 1976 to 2019. Hamed *et al*., (2019) isolated actinomycetes inhabiting the marine regions of the Red sea and the Suez gulf with antagonistic properties. Phongsopitanum *et al*., (2019) established the antagonistic properties of nearly 41 marine actinomycetes of Thailand region. The studies of Subramani and Aalbersberg, (2012) imply that Streptomycetes group amongst actinomycetes establish it to be economically important. This genus is accredited with producing more than fifty percent of the known antibiotics.

The studies of Ramesh and Mathivanan, (2009), Peela et *al*., (2005) established the prevalance of diversified antimicrobial activity and enzyme production in marine originated actinomycetes. This has increased the pronouncement of industrially important marine actinomycetes, the major one being *Streptomyces* from the Bay of Bengal which produced the best enzymes and molecules. The work of Kathiresan *et al*., (2005) caused the isolation of marine actinomycetes from mangroves and estuaries the strains of which proved to act as agrobased fungicides. The relevant antagonistic properties of 107 marine actinomycetes that was screened of the Konkan coast of Maharashtra was established by Gulve and Deshmukh, (2012). Sivakumar *et al*., (2011) were able to isolate 78 types of marine actinomycetes from the Bay of Bengal along the Pudimadaka coast of Andhra Pradesh wherein *Streptomyces* was the most prevailing strain exhibiting anti bacterial and anti fungal activity. Jiang *et al*., (2017) studied chalcomycins from marine derived *Streptomyces* species and their antimicrobial activities. Cho *et al*., (2020) discussed the antibacterial activity of chromomycins from the marine derived *Streptomyces microflavus.*

Dharmaraj, (2010) highlighted that within the marine actinobacterial group *Streptomyces* is represented by the greatest number of species and varieties. Patel *et al*., (2020) observed and studied the antibacterial activity of marine bacterial pigments from the Arabian sea. Elnaby *et al*., (2016) worked on the properties pertaining to antibacterial and anticancer action of silver nanoparticles that are synthesized extracellularly and obtained from marine *Streptomyces rochei* MHM13. Similar studies by Rana *et al*., (2018) were based on the antimicrobial potency of silver nanoparticles generated by *Streptomyces sp.* RHS16 against certain fish pathogens. Dholakiya *et al*., (2017) predicted the antioxidant and antibacterial activities of newer actinobacterial strain *Streptomyces variabilis* RD-5 that was isolated from the gulf of Khambhat Gujarat. Luo *et al*., (2020) worked on certain natural marine antibacterial products as lobophorin L M, spirotetronate antibiotics and ansamycins that were derivatives of the marine *Streptomyces sp*. 4506. The studies of Janardhan *et al.*, (2014), Jiao *et al.*, (2013) established and emphasized marine mangrove based actinomycetes possessing potent ability to produce bioactive compounds. Many researchers have focused on gifted antimicrobial compounds isolated from marine actinomycetes from 1991-2013. The observations of Behie *et al.*(2017), Kamjam

et al., (2017) have corelated the production of newer bioactive metabolites with actinobacterial adaptations to extreme climatic conditions.

Streptomycetes has the ability to produce cellulose with cellulolytic waste materials used in degradation studies characterized from the marine environments. Actinobacteria isolated from marine sediments and also molluscs form the major source for many extracellular enzymes as studied in Vellar estuary by Patil *et al.*, (2001). This inferred the characterization of 104 isolates of which 77 of them were observed to show inhibitory activity against one of the pathogens. *Actinopolyspora sp.* have shown antagonistic activity against the Gram positive bacteria that were isolated from the west coast of India. On the contrary it exhibited no antagonistic activity against the Gram negative bacteria as reported by Kokare *et al.*, (2004). Marine actinobacteria are featured in the bioprospecting of enzymes secreted with plenty of industrial applications by Sahu *et al.*, (2005). *Streptomyces albus* having good scope for industrial production of extracellular L-glutaminase reported by Umamaheswary *et al.*, (2005). Actinobacterial studies imply and spread information about the antibiotic bioactive producing properties that could be utilized for human welfare as discussed and explained by Sahu *et al.*, (2006). *Streptomyces albidoflavus* has shown anti -tumour properties isolated from the Pichavaram mangroves by Siva Kumar *et al.*, (2005). Marine actinobacterial strains to date remain the most superior ones in research by Lakshmipathy *et al.*, (2010). *Streptomyces* isolated from urban soils have shown to be resistant to multiple antibiotics by Zotchev, (2012). Learn-Han *et al.*, (2012) exemplified the marine derived genus of *Streptomyces* having poduced 289 secondary metabolites basing on the information provided by marinlit database .

Selvakumar *et al*., (2012) characterized the marine microorganisms producing vitamin B12 and the remarkable antagonistic activity of glucose utilizing potential of *Streptomyces sp. Streptomyces avermitillis* is synonymous with avicta a nematicidal product explored by Cabrera *et al.*, (2012). The studies of Antunes *et al.*, (2014) confirmed 7600 compounds being produced under *Streptomyces species*. Rana and Salam, (2014) isolated and characterized 23 marine actinomycetes with atleast 7 bringing in inhibition of both Gram negative and positive strains with a 20 m inhibitory zone. *Streptomyces* species also involved in the control of certain plant pathogens as reported by Al-Askar *et al.*, (2015). The prospective magnitude of marine microorganisms involved in the making of constructive metabolic products being cytotoxic, antibacterial and antifungal was revealed by Sivalingam *et al.*, (2019).Study and observations by various researchers has proved that marine originated *Streptomyces* being abundantly available is a potential source for novel anti microbial activities although microorganisms of mangrove origin gained not enough recognition as pointed by Liua *et al.*, (2008). Nature has risen as most important source for newer antibiotics by Kpehn and Carter, (2005) wherein alternative selected isolation procedures have been suggested by Sathiyaseelan and Saranraj, (2016). Therefore this present research work laying more emphasis on the isolation of the marine actinobacteria, *Streptomyces sp.* with specific antagonistic properties from the sea and mangrove regions of coastal Machilipatnam.

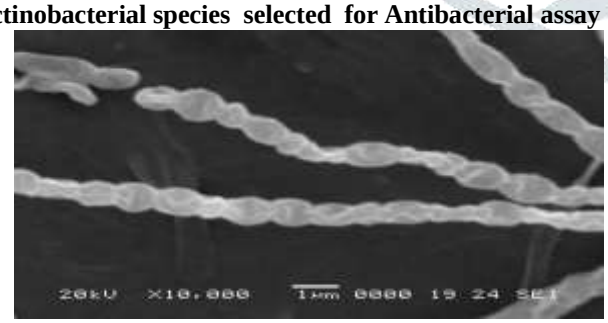
Materials and methods: Antibacterial activity

The antagonistic activities of the thus isolated actinobacteria during the investigative period were evaluated against the clinical bacteria being Gram positive *Staphylococcus aureus* and *Streptococcus faecalis* and Gram negative *Proteus vulgaris* and *Salmonella typhi.*

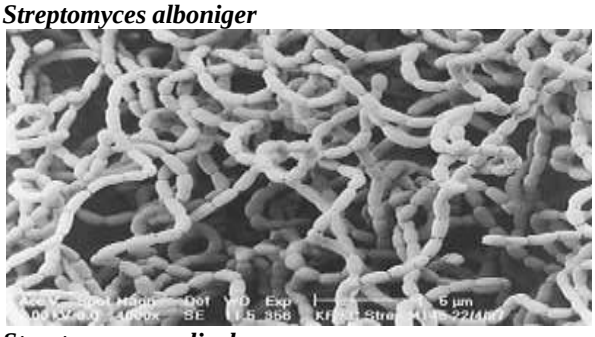
Assay for antagonistic activity

The often occuring marine actinobacteria, *Streptomyces alboniger, Streptomyces coelicolor* and *Streptomyces griseus* that were isolated during the period of investigation were selected to assess their antagonistic activity against the clinical bacteria considered. The test conducted by the cross streak method on modified nutrient agar medium as given by Dholakiya *et al*. (2017). The surface of the modified agar was streaked singly and later left to incubate at 28 ± 2°C for a period of one week according to Balagurunathan and Subramaniam (2001).The actinobacterial growth was observed and identified as being ribbon like wherein the clinical bacterial streaking at right angles to the actinobacterial streak. They were further subjected to incubation at 28°C. The zone of inhibition measured based on the time frame ranging from 24 to 48 hrs wherein the control plate was devoid of the actinobacterial inoculum in comparision to the usual bacterial growth.The inhibitory zone of > 15 mm is measured as an important activity apprehensive in the combat against a wide range of pathogens.

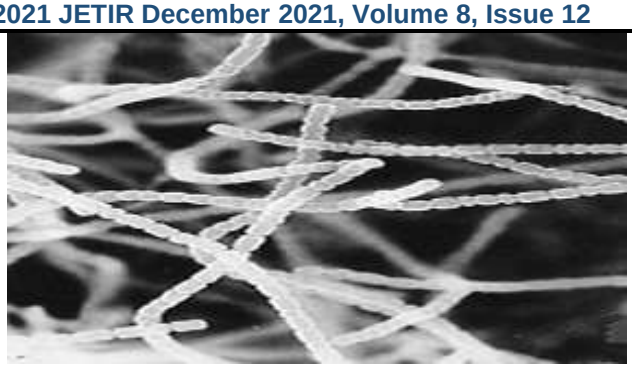
Actinobacterial species selected for Antibacterial assay



Streptomyces alboniger



Streptomyces coelicolor



Streptomyces griseus

Extraction of Antibacterial Principle

The particular opposed actinobacterial isolates infused into a broth of casein agar in a shake flask method with speed being 200-250 rpm . The incubatory duration was 1 week at 28ºC. Broth cultures subjected to filtration twice done initially by a supreme quality whatman filter paper and later by the millipore filter procured from millipore millex HV hydrophilic PVDF comprising of size of 0.45µm. The filtrates transferred under sterile conditions into the flask stored at 4°C to proceed with assessment and investigation. This was followed by the slow and individual addition of the solvents alcohol, chloroform, ethyl acetate and methanol to a similar volume of the filtrate. These were mixed well and subjected to a 5000 rpm centrifugation wherein the time period was 10 min by Remya and Vijayakumar, (2008).

Antibiotic disc preparation

The method adopted to study the evaluation of the antibacterial extracts with diverse cultures of bacteria was of Kirby-Bauer disc diffusion. This is also considered and categorized as the antibiotic susceptibility test. Sterilized filter paper discs were used on to which 200 µl of the extract was impregnated and then completely dried. This was done under aseptic conditions.

Antibacterial assay

The assay involved the nutrient agar plates being readied under aseptic conditions. This was followed by impregnating the selected bacteria comprising of 18-24 hours previous culture. The swab method used for speading the bacterial culture with cotton swabs was adopted. The antibiotic discs thus extracted and impregnated were slowly placed with the pathogenic bacterial swab culture. The incubation time for the plates considered was1 to 2 days thereby checked the zone of inhibition making a note of the zone of clearance in mm. Among the tested ethyl acetate extracts shown good growth inhibition against the test organisms. Therefore for the assessment and analytical studies TLC and HPLC techniques were done on the ethyl acetate extracts.

Purification of Antibacterial Compounds: Thin Layer Chromatography

Thin layer chromatography with silica gel plates collected from Merck Art. 5735 Kiesselgel 60 F 254 used for partial purification of antibacterial principles to detect the compounds. The dried crude extracts of the selected three actinobacteria dissolved in methanol individually spotted on the silica plate developed in the chromatographic solvent system ratio being 3:1:1 v / v consisting n-butanol-acetic acid -water (BAW). The developed thin layer chromatographic plates air dried overnight to remove any traces of the solvents. Thus separated compounds were visualized under UV at 254 nm absorbance also at 365 nm fluorescence. The active spots were detected by bioautography by Betina (1973), Choma and Grzelak (2011). The retention factors (RF) of the active spots were further calculated.

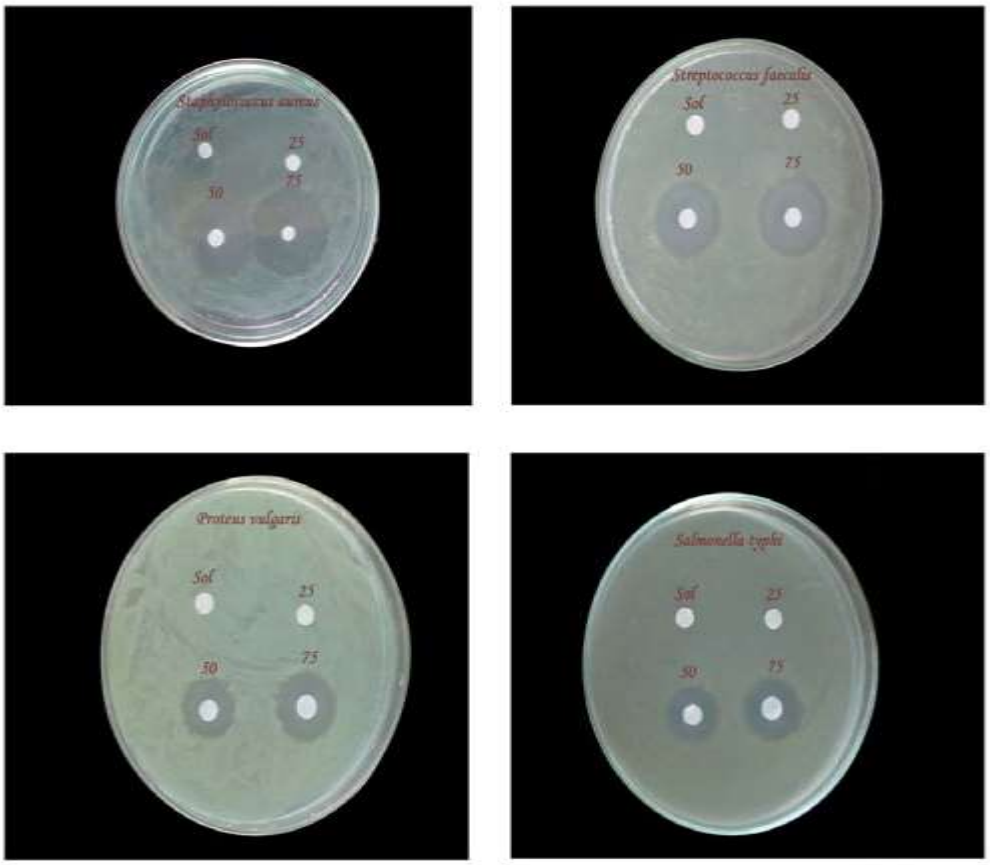
HPLC analysis

The high performance liquid chromatographic (HPLC) technique performed for seperation identification of the antimicrobial compound utilizing a Shimadzu HPLC UV-VIS detector and LC-18 column. A 20 µl supernatant injected separately into the HPLC system maintaining flow rate 2.5 mL/min with a back pressure 250 psi. The compounds thus read at 235 nm. The samples run for 20 min after which the retention time RT was noted. RT was compared with standard thereby identifying the antimicrobial compounds.

Results: Antibacterial studies

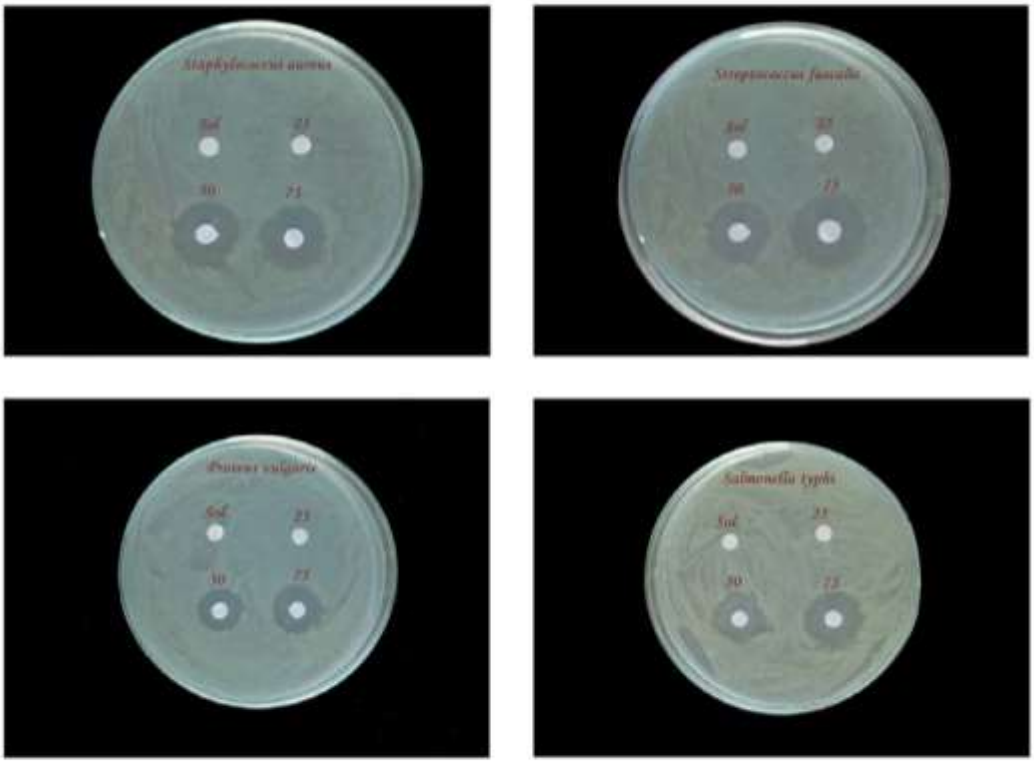
This present study involved ethyl acetate extracts of three species of marine actinobacteria being screened for four clinical bacterial strains causing diseases in humans. The actinobacterial extract screened for its activity based on zone of inhibition comparing with the standard drug ciprofloxacin. The average values of inhibition zone of actinobacterial extracts screened against bacteria as shown. .Maximum activity found with *Streptomyces alboniger* against *Staphylococcus aureus* as also inferred by Remya and Vijayakumar, (2008). Similarly *Streptomyces coelicolor* exerted maximum antibacterial activity against *Staphylococcus aureus* and *Streptococcus faecalis*; *Streptomyces griseus* against *Staphylococcus aureus* and *Proteus vulgaris.* The zone of inhibition localized 14-18 mm near to the standard drug ciprofloxacin. The inhibition zone 17 to 20 mm is exhibited. The actinobacterial extracts 25, 50 and 75 µg/mL screened for antibacterial activity seen only at concentrations of 50 and 75 µg/mL but not with 25 µg/mL wherein the activity found maximum at the concentration of 75 µg/mL for all extracts.

Effect of Streptomyces alboniger : extracts atdifferent concentrations (25, 50 and 75 g/ml) against bacteria



Sol - Ethyl acetate (showed nil effect against the microorganisns under test)
25,50 & 75 - Different concentrations of *S.alboniger* extract

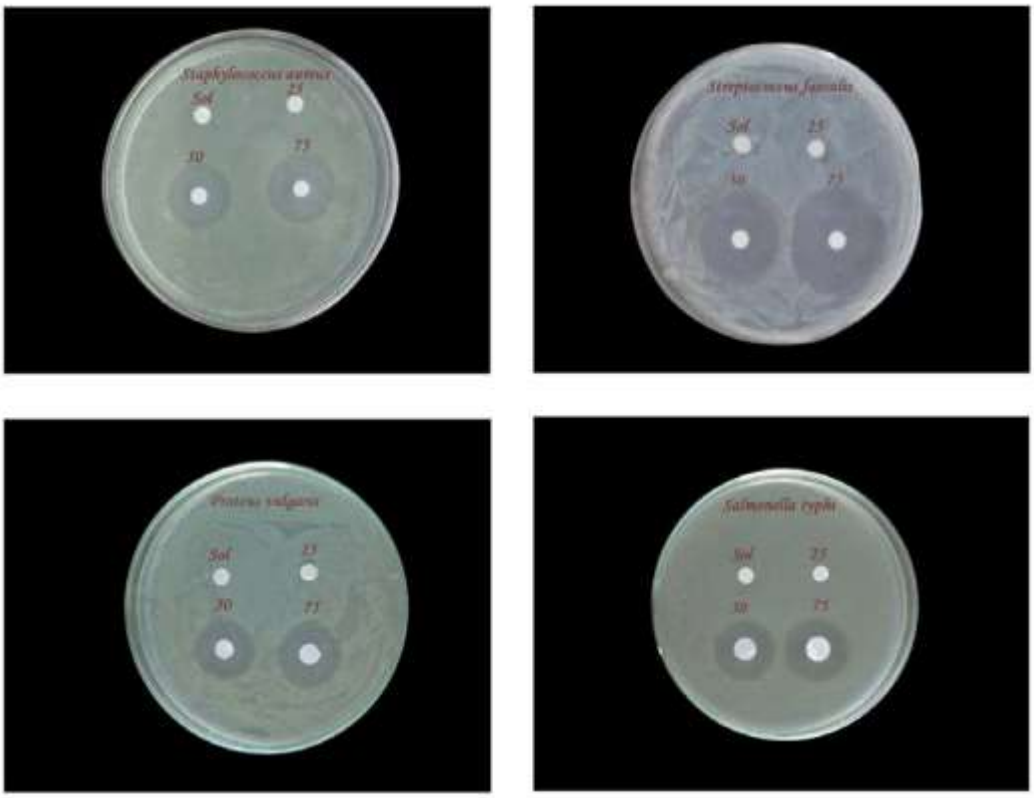
Effect of Streptomyces coelicolor : extracts at different concentrations (25, 50 and 75 g/ml) against bacteria



Sol - Ethyl acetate (showed nil effect against the microorganisms under test)

25,50 & 75 - Different concentrations of *S.coelicolor* extract

Effect of Streptomyces griseus: extracts at different concentrations (25, 50 and 75 g/ml) against bacteria



Sol - Ethyl acetate (showed nil effect against the microorganisms under test)
25,50 & 75 - Different concentration of *S.griseus* extract

Analysis of the antibacterial compounds identified:

HPLC being used in analytical estimation of different antibiotics in research. In this present investigation HPLC profile of antibacterial compounds identified from *Streptomyces alboniger, Streptomyces coelicolor* and *Streptomyces griseus* is shown

Bioactive compounds identified in the ethyl acetate extract of species of *Streptomyces*

S. No	Name of marine actinobacteria	RT (min)	Name of the bioactive compounds	Molecular formula	MW	Peak area%	Compound nature	Activity based on scientific literature
1	*Streptomyes alboniger*	16.41	Pamamycin	C36H63NO7	621	6.86	Antibiotic	Antibacterial
2	*Streptomyces coelicolor*	3.313	Cephalexin	$C_{16}H_{17}N_3O_4S.H_2O$	365.41	100	Antibiotic	Antibacterial
3	*Streptomyces griseus*	10.36	Streptomycin	$(C21H39N7O12)2 - 3H_2SO_4$	1457.41	100	Antibiotic	Antibacterial

Discussion

Adaptation of marine actinobacteria to extreme climatic conditions as high salinity, pressure and temperature has modified their physiological conditions to survive and elaborate novel bioactive metabolites**.** Actinobacteria of marine origin offer a promising basis to discover newer biologically active components by Betancur *et al.*, (2017). The marine sediments are often considered to be dominant in origin with respect to actinobacteria involved in the production of benefecial bioactive metabolic products by Girao *et al.*, (2019). Studies pertaining to the marine sediments by Elliah *et al.*, (2002, 2004) of the river Krishna acted as a vivid actinomycetes source in the screening of bioactive metabolites. The studies of Valli *et al.*, (2012) validated that most of the marine antagonistic strains were of actinobacterial origin**.** Thus the exploitation of marine actinobacteria in antibiotic production stands good in research in the fight against increased pathogen toxicity.

Amongst the marine microorganisms actinomycetes is the most investigated and researched group particularly the genus *Streptomyces* which is credited as having produced the maximum number of antibiotics by Dharmaraj, (2010), Tenebro *et al.*, *(*2021). This study reveals the major marine species thus isolated of coastal Machilipatnam sediments being *Streptomyces*. The findings based on the studies by Barka *et al.*, *(*2016) Cumsille *et al.*, (2017) imply the availability of *Streptomyces* as intraspecies

exhibiting distinctive adptability thereby outcompeting other bacteria in the production of antibiotics. The present study recorded the isolation of 27 species of which three species screened and shown potent antibiotic activity. The three species thus isolated and screened as part of the present investigation characterized by the presence of spores with surface morphology being smooth in accordance to the streptomycetes specification by Tresner *et al.*, (1967). The observations pertaining to this work has implicated that there were three species with good specific activity in defense of bacteria both Gram positive and negative bacteria. Studies of the ethyl acetate extracts, of *Streptomyces alboniger, Streptomyces coelicolor,* and *Streptomyces griseus* shown apt effects to 50, 75 µg/ml concentrations. This lead to the identification of pamamycin, cephalexin and streptomycin based on HPLC study involving the above three sample extracts. Amongst which pamamycin being characterized by certain exclusive characteristics stimulates the aerial mycelium in *Streptomyces alboniger*

The studies of Hashimoto *et al*., (2011) explained the effect of the aerial mycelium in the antibiotic production in *Streptomyces sp.* wherein the induciung compound was pamamycin-607. It induced the production of puromycin in *Streptomyces alboniger* and streptomycin in *Streptomyces griseus* and virginiamycin M1 in *Streptomyces sp.*91. Hoz *et al*., (2017) described four paulomycin derivatives from *Streptomyces albus* J1074 whereas its biosynthesis was studied by Aranzazu *et al*., (2016). Victor *et al*., (2021) studied with referance to the production of 2 antibiotics from *Streptomyces coelicolor* A3(2*)*. Odumosu *et al*., (2017) emphasized and studied the antimicrobial activities of *Streptomyces coelicolor* AOBKF977550 in the African estuaries. Hence *Streptomyces sp*. being the most abundant is often explored more as there is an everlasting requirement for research in antibiotics by Procopio *et al*., (2012). *Streptomyces coelicolor* studies on the antibacterial compounds were found to show retention time 3.313 with respect to the absorption peaks.

Research studies by Yague *et al*., (2013) reflected in the pre sporulation stages of *Streptomyces* in optimizing the differentiation of hyphae in the production of antibiotics. The observations of Manteca and Sanchez, (2009) included differential studies of mycelium and also the production of antibiotics in *Streptomyces coelicolor.* The first isolation of *Streptomyces griseus,* given by Krainsky, (1914) during outbreak of first world war by Waksman and Henrica, *(*1948) from the soils of Russia as studied by Kuznetsov *et al*., (1987). Streptomycin an antibiotic far ranging thereby inhibiting bacteria both Gram positive and negative studied by Al-Ansari *et al*., (2019). Among the different actinobacterial strains isolated from coastal regions of Guntur district Andhra Pradesh India one potent strain with broad-spectrum antagonistic activity identified as *Streptomyces rectiverticillatus* VJMS-8 by Swapna and Vijayalakshmi, (2020). This work made the use of *Streptomyces griseus* extracts of ethyl acetate able to cause the growth and inhibition of bacteria both Gram positive and negative bacteria which inferred the presence of streptomycin in the extract in confirmation with the HPLC analysis. The initial and early studies of Carvajal, (1946) are still in support that few strains of *Streptomyces griseus* researched from other sources produced streptomycin with variable function.

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