Int J Pharm Bio Sci 2012 Oct; 3(4): (P) 331 - 342

Research Article Bio Pharmaceutics

International Journal of Pharma and Bio Sciences

ISSN
0975-6299

ANTIBIOTIC PRODUCTION FROM MARINE *STRPTOMYCYES SP*

RITESH KUAMR *[1], AMIT KUMAR SHRIVASTAV[2], ANIL KUMAR SINGHA[3], PANKAJ KUMAR[4] AND NIRMALA.A [5]

[1-5]Department of Biotechnology, Vinayaka Missions University, Aarupadi Veedu Institute of Technology, Chennai-603104, Tamilnadu, India.

ABSTRACT

Rapidly emerging strains of bacteria resistant to most advanced antibiotics have become issues of very important public health concern. Research currently directed towards marine actinomycetes presents a vast potential for new compounds that could be able to safely and effectively target resistant species. In this regard seven putative *Streptomyces* strains were selected and assessed for antibiotic production and activity against a wide range of plant fungal pathogen (*Rhizoctonia solani*) and human pathogenic bacteria (*Staphylococcus aureus, Vibrio cholera, Salmonella typhi, Klebsiella pneumonia).* In conclusion, the secondary metabolites of marine organisms displayed appreciable antimicrobial activity. The secondary metabolites were of low molecular compound with stable free radical scavenging activity. The compound(s) present within this extract showed activity against *Staphylococcus aureus, Vibrio cholera, Salmonella typhi, Klebsiella pneumonia.* The antagonistic secondary metabolite produced by this actinomycetes needs to be studied further to identify its chemical nature and characterization of its biological activity. The chromatogram developed with 10% ethyl acetate in hexane revealed the presence of ten major compounds at R_f value of 0.21, 0.33, 0.68, 0.78, 0.78, 0.88, 0.90, 0.95, 0.95 and 0.96 as visualized under iodine vapour and UV illumination. However, further studies are undertaken to purify, characterize and test the active molecule for its antibacterial activity and its mechanisms of action.

KEY WORDS: **Antibiotic, *Staphylococcus aureus, Vibrio cholera, Salmonella typhi, Klebsiella pneumonia***

A.NIRMALA
Department of Biotechnology, Vinayaka Missions University, Aarupadai Veedu Institute of Technology, Chennai-603104, Tamilnadu, India.

**Corresponding author*

INTRODUCTION

Natural products remain the most prolific source of new antimicrobials, and the chemical diversity of natural compounds is still unmatched by combinatorial chemistry approaches[1,2]. While the latter has been successfully applied for lead optimization, it basically failed to deliver genuinely new pharmacophores, especially in the field of antimicrobials[2], mainly due to limitations in the structural variety of compounds represented in combinatorial libraries. Most of the antibiotics in clinical use today have been developed from compounds isolated from bacteria and fungi, with members of the actinobacteria being the dominant source[3]. Traditionally, most of these antimicrobials have been isolated from soil-derived actinomycetes of the genus *Streptomyces.* Actinomycetes, the filamentous bacteria, are primarily saprophytic microorganisms of the soil, where they contribute significantly to the turnover of complex biopolymers, such as lignocelluloses, hemicelluloses, pectin, keratin, and chitin[4]. Actinomycetes are the strongest antagonists among microorganisms. The antibiotic substances they produce display antibacterial, antifungal, antitumor, antiprotozoic, and antiviral properties. Of the ten thousand known antibiotics produced by microorganisms over a decade ago, about 70% are of actinomycete origin of them, representatives of the genus Streptomycin account for two thirds[5]. The actinomycetes have provided many important bioactive compounds of high commercial value including antibiotics. Due to the special attributes of the marine environment, marine actinomycetes are thought to have distinct physiological, morphological and chemotaxonomic characteristics and unique production of secondary metabolite and bioactive compounds. Actinomycetes are a group of bacteria that live in soil and decompose organic matter such as cellulose. In the realm of drug discovery, these microorganisms are widely recognized for their ability to produce secondary metabolites (chemicals) with commercially viable antibiotic activity. Streptomycin, the first treatment for tuberculosis, was derived from the largest genus of these bacteria, Streptomyces. Erythromycin and tetracycline are two other examples of common medicines derived originally from these microorganism's metabolites. The first antibiotics were of natural origin, e.g. penicillins produced by fungi in the genus Penicillium, or Streptomycin from bacteria of the genus Streptomyces. Currently, antibiotics are obtained by chemical synthesis, such as the sulfa drugs (e.g. sulfamethoxazole), or by chemical modification of compounds of natural origin. Many antibiotics are relatively small molecules with a molecular weight of less than 1000 Da. The classical definition of an antibiotic is a compound produced by a microorganism which inhibits the growth of another microorganism[6] . Antibiotics can be grouped by either their chemical structure or mechanism of action[7].In this paper, we report on the anti bacterial and antifungal potential of antibiotic compound produced by Actinomycetes sp isolated from the marine environment.

MATERIALS AND METHOD

Media used

Various media were used in the course of study for different purposes such as isolation, screening and optimization. The Medias were listed as follows:

Starch Casein Agar[8] Nutrient agar, Potato dextrose agar, Seed culture medium, Antibiotic Production Medium, Trace Element Solution etc.,

Maintenance of actinomycetes

The isolated actinomycetes were maintained on starch casein agar slants by frequent sub

culturing. And they were named as ABTRI 1, 2,3,4,5,6,7.

Primary screening against plant fungal pathogens

The isolated marine actinomycetes were screened for antifungal activity against fungal pathogen *Rhizoctonia solani.* PDA and NA medium were thoroughly mixed and autoclaved then poured into petriplates and allowed to solidify. Seven actinomycetes isolates were streaked separately on the side corner of each petriplate. And Mycelial discs of 8mm diameter were taken from the mother culture of actively growing region of the fungal pathogens. Then the mycelia disc of fungal pathogen was placed in the center of actinomycetes isolates streaked plate; while the mycelia disc alone served as control. All the plates were incubated at 25˚C up to 5 days and the zone of inhibition was measured.

Primary screening against human bacterial pathogens

Nutrient agar medium was prepared and poured into sterile Petri plates and allowed to solidify. 24 hrs old Bacterial cultures (*Staphylococcus aureus, Vibrio cholera, Salmonella typhi, Klebsiella pneumonia)* were inoculated by aseptic swabbing plate method. Then with the help of a sterile cork borer, wells were cut measuring 0.9 cm in diameter. About different concentrations of the actinimycetes culture filtrate was added into the wells of each plate under aseptic conditions and incubated at room temperature. The antibiotic production was confirmed by measuring the clear zone around the well after 24 hrs.

Selection of potential actinomycetes

Among the isolated actinimycetes, the strain ABTRI-6 was selected for further analysis owing to its superior antifungal and antibacterial activity in primary screening.

Genus level identification of marine actinomycetes

The cover slip culture technique[9] was used for studying the morphological characteristics of the marine isolates. Sterile glass cover slips were inserted at an angle of 45^0c into solidified SCA medium in petriplate. A loop full of inoculums of the isolate was streaked along the line where the cover slip meets the agar and then the plates were incubated at room temperature for 5 days. The organisms grow both on the medium and in a line across the upper surface of the cover slip. The cover slip was removed and examined under a light microscope and photographed. The morphology was observed and motility test was carried out using Gram's staining and hanging drops method, respectively.

Preparation of seed culture inoculums

About 100ml of seed broth was prepared and sterilized. The broth was cooled completely and was inoculated with two to three loops of actinomycetes from the master plate. The flask was placed in shaker at 200 rpm in room temperature. After 5 – 7 days, this seed culture could be used for proceeding steps.

Optimization of metabolite production

The metabolite production was optimized by taking 8 different 250ml flasks containing 100ml of antibiotic production medium and it was autoclaved simultaneously. After autoclaving, the flasks were labeled by numbering them from 1 to 8 along with consequent dates. The first day flask was inoculated with 1 to 1.5ml of seed medium and is kept in shaker at 200rpm. By the next day, the same time as inoculated for the first day, the second flask was inoculated with seed medium. The flask was then placed in shaker. Consequently the ten flasks were inoculated each day and were placed in shaker at 200rpm. After completion of 8 days all the flasks were harvested from shaker and was filtered using a filter paper in ten different flasks labeled as 1 to 8. From the filtrate 10ml was taken and was added to 20ml of ethyl acetate and was kept in shaker at 100rpm for a minimum of 30min. After 30min, the solution

mixture was poured into the separating funnel via a funnel. The solution was mixed / shacked thoroughly so as to disperse the metabolite into the solvent layer. The solvent layer was collected in 8 different petriplates and allowed the solvent to evaporate by air drying. The metabolite was retained in the plate and was collected from the plate using 500µl of ethyl acetate. The plate was swirled uniformly and from this 100µl of the metabolite along with the solvent was taken from each plate and was added to the appropriate wells made in petriplates containing NA medium which was swabbed with the following human pathogens (*Staphylococcus aureus, Vibrio cholera, Salmonella typhi, Klebsiella pneumonia*). The plate without metabolite served as control. The plates were incubated so as to obtain the zone of clearance.

Production of metabolite from positive strain of actinomycetes

Among different optimization of days the 6th day shows maximum activity, so it was selected for the production of metabolites Antibiotic production medium (1L/organism) was prepared in different conical flasks (150ml/flask) and was autoclaved. One ml of seed culture (ABTRI-6), for 100ml of medium was inoculated in production medium and incubated at 28^0c in environ shaker at 200 rpm for 6 days. After 6 days, when the broth becomes turbid, the contents were centrifuged at 10,000 for 20 min and supernatant was collected. To the supernatant twice the amount of Ethyl acetate (1:2) was added and incubated at 28^0c in environ shaker, overnight. This allows continuous exchange of compounds in the organisms to the solvent layer. The contents were then transferred to separating funnel and the aqueous phase was collected. The aqueous phase was then transferred to Soxhlet apparatus, for compound extraction. Here condensation reaction occurs were solvent is separately collected, leaving behind the crude extract containing the metabolite. The crude extract was then scrapped using Ethyl acetate and allowed to evaporate, to obtain the metabolite of interest. This compound was then used for further analysis.

Qualitative bioassay of the compound
Screening for antibacterial activity

Agar diffusion assay is used widely to determine the anti-bacterial activity of crude metabolite. The technique works well with defined inhibitors[10]. However when examining extract containing unknown components, there are problems leading to false positive and false negative results[11] . Nutrient agar prepared was poured in the Petri dish 24 hours growing culture (*Staphylococcus aureus, Vibrio cholera, Salmonella typhi, Klebsiella pneumonia*) were swabbed on it. The wells (10mm diameter) were made by using cork borer. The different concentrations of the crude metabolite were loaded in the wells. The plates were then incubated at 37 ^{o} C for 24 hours. The inhibition diameter was measured.

Thin layer chromatography[12]

It is used to separate the compound present in the crude metabolite. The separation of the compound also depends on the usage of the solvent. Here the solvent used 10% ethyl acetate in hexane. The concentration (1mg/ml) of the metabolite was spotted on the TLC plates and dried. It was then run with different solvent ratio. The spots were identified both in the UV light, far light and in the iodine chamber. Then R_f value was calculated. R_f value is calculated by distance traveled by the solute to the distance traveled by the solvent.

RESULT AND DISCUSSION

Screening of actinomycetes against plant fungal pathogens

From the 7 Actinomycetes isolates, 4 Actinomycetes isolates (2, 3, 5, & 6,) were exhibited antifungal activity against plant

pathogen *Rhizoctonia solani.* And they were selected for antibacterial activity (Fig-2).

Screening of Actinomycetes against human bacterial pathogens

The actinomycetes isolates (2, 3, 5, & 6,) exhibited antimicrobial activity against *Staphylococcus aureus, Vibrio cholera, Salmonella typhi, Klebsiella pneumonia.* The antimicrobial activity was found to be higher in 6th isolate and was identified (Fig-3) (Table -1).

Genus level identification of marine actinomycetes

The colonies are rough and elevated and the morphological observation in light microscope and scanning electron microscope reveals chains of spores which is a specific characteristic of *Streptomyces* species (Fig-1).

Optimization of Metabolite Production

The metabolite production was found to be higher at the sixth day and the zone of inhibition was measured (Fig-4) (Table-2).

Metabolite production - Solvent Extraction

By solvent extraction procedure, the Metabolite was extracted to its full form as the solvent ethyl acetate is middle polar solvent. The metabolite was obtained in the petriplates which was prone to air drying.

Qualitative bioassay of the compound

Screening for antibacterial activity

The crude metabolites isolated from *Streptomyces sp* showed inhibitory activity against *Staphylococcus aureus, Vibrio cholera, Salmonella typhi, Klebsiella pneumonia (Fig-5) (*Table-3).

Thin Layer Chromatography

The chromatogram developed with 10% ethyl acetate in hexane revealed the presence of ten major compounds at R_f value of 0.21, 0.33, 0.68, 0.78, 0.78, 0.88, 0.90, 0.95, 0.95 and 0.96 as visualized under iodine vapour and UV illumination (Fig-6).

DISCUSSION

Streptomyces are Gram positive filamentous bacteria which belong to the order Actinomycetales[13]. The natural habitat of most *Streptomyces* is soil but can be also found in aquatic environment. The most intriguing trait of *Streptomyces* have a capacity to produce secondary metabolites including antibiotics and bioactive compounds valued in human and veterinary medicine, agriculture, and unique biochemical tools[14], and now uncommonly-used streptomycin takes its name directly from *Streptomyces*[15].Among microorganisms, the Actinomycetes are known to produce a number of bioactive compounds with diverse biological properties. Therefore, the members of the genus Streptomyces are in the limelight for over 60 years in the academic as well as industrial research area. The search for novel biologically important metabolites is a continuous process to fulfill the everlasting demand for new biologically active molecules that possess antimicrobial and therapeutic properties to combat the plant and human pathogens and also to treat other human ailments. It is an established fact that the soils are an excellent source for the isolation of microorganisms with diverse potential. Actinomycetes are one of the prominent microbial groups of soil populations together with bacteria and fungi. Even though they are distributed in different habitats, soil is considered the most important habitat for actinomycetes[16]. In this study, screening of 6 isolates for their antimicrobial activity on agar medium using dual culture technique resulted in choosing an isolate identified as *Streptomyces sp*. This is very commonly adapted method to screen a large number of microorganisms for antimicrobial activity and also to determine their biocontrol potential[17] . All the 7 actinomycetes were initially screened for their antifungal activity against *R. solani*.

Among all the 7 isolates only 4 isolates (2, 3, 5, & 6,) exhibited good antifungal activity against *R. solani*. Among the four isolates (2, 3, 5, & 6,), 6th isolate exhibited maximum activity against bacterial pathogens and hence selected for further studies.

The inhibition of plant and human pathogens in the invitro plate assays clearly indicated the possible production of extracellular substances by the isolate 6. Based on the in vitro assay results, further studies were designed to investigate the production of antimicrobial secondary metabolites in 6th isolate. The morphological characteristics of the isolate 7 were studied to establish its identity. The isolate 6 showed elevated, convex leathery colony with filamentous margin. It exhibited pleomorphism with well-defined reddish brown substrate mycelium and highly branched pink coloured aerial mycelium with monopodial branching bearing grey spores. Many of such morphological characteristics are common to most of the streptomycetes[18-20]**.** The metabolite production was found to be higher at the sixth day and the zone of inhibition was measured. Nutrition plays an important role in the production of secondary metabolites because, limiting the supply of an essential nutrient is an effective means of restricting growth but it can have specific metabolic and regulatory effects. To achieve high product yields, it is a prerequisite to design a proper production medium for an efficient fermentation process. Disc diffusion and agar diffusion are the most frequently used methods. In such assays, the susceptibility of an organism to a sample would result in a zone of inhibition, with a magnitude related to the amount of bioactive compounds present in the fermentation broth[21] **.** A number of compounds have been produced during fermentation. This was known by performing TLC. However, further studies are planned to purify, characterize and test the active molecule for its antibacterial activity and its mechanisms of action.

Almost all the antibacterial agents, isolated from the actinomycetes are aromatic or saturated organic compounds, they are most often obtained initially from ethyl acetate extraction[22] . In the direct extraction experiment, ethyl acetate extracted broad range of compound as determined by TLC[23]. The TLC profile of secondary metabolite in chromatogram developed using 10% ethyl acetate in hexane as solvent system revealed the presence of ten compounds. In conclusion, the secondary metabolites of marine organisms displayed appreciable antimicrobial activity. The secondary metabolites were of low molecular compound with stable free radical scavenging activity. The compound(s) present within this extract showed activity against *Staphylococcus aureus, Vibrio cholera, Salmonella typhi, Klebsiella pneumonia.* The antagonistic secondary metabolite produced by this actinomycetes needs to be studied further to identify its chemical nature and characterization of its biological activity. The chromatogram developed with 10% ethyl acetate in hexane revealed the presence of ten major compounds at R_f value of 0.21, 0.33, 0.68, 0.78, 0.78, 0.88, 0.90, 0.95, 0.95 and 0.96 as visualized under iodine vapour and UV illumination

This article can be downloaded from www.ijpbs.net

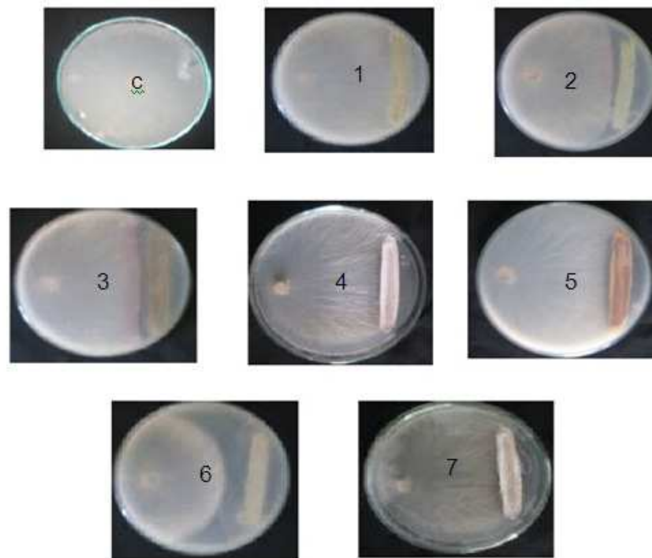
RESULTS

Figure 1
Isolation of marine actinomycetes

7 actinomycetes were isolated from marine soil sample

PRIMARY SCREENING AGAINST FUNGI

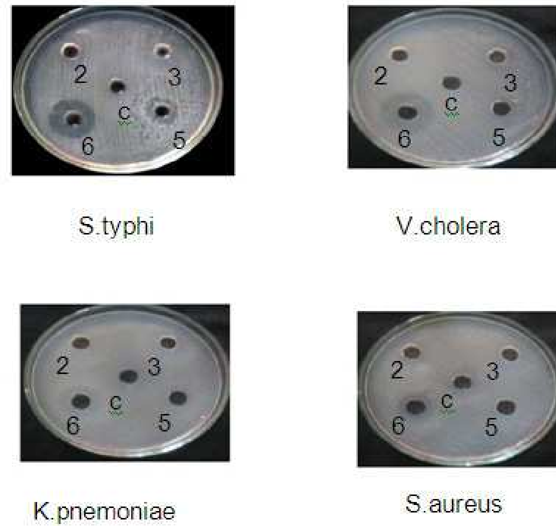
Figure 2
The actinomycetes isolates were exihibited antifungal activity against fungal pathogens of Rhizoctonia solani



Isolates2,3,5&6 Actinomycetes exihibits good activity against Rhizoctonia solani

PRIMARY SCREENING AGAINST BACTERIA

Figure 3
The actinomycetes isolates were exihibited antibacterial activity against human bacterial pathogens



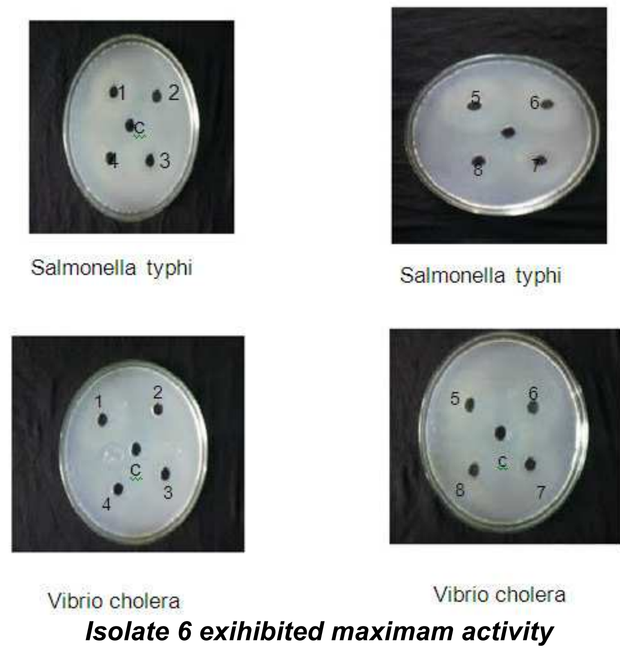
Isolate 6 only exihibited antibacterial activity against human bacterial pathogen

Table 1
Primary screening of antibacterial activity against human pathogenic bacteria (Zone of inhibition in mm)

Organism	Isolate1	Isolate2	Isolate5	Isolate6
Salmoonella typhi	_	_	15	21
Vibrio cholera	_	_	_	18
Staphylococcus aureus	_	15	16	19
Klebsiella pneumoniae	14	16	17	21

DAY OPTIMIZATION

Figure 4
(Zone of inhibition (mm)



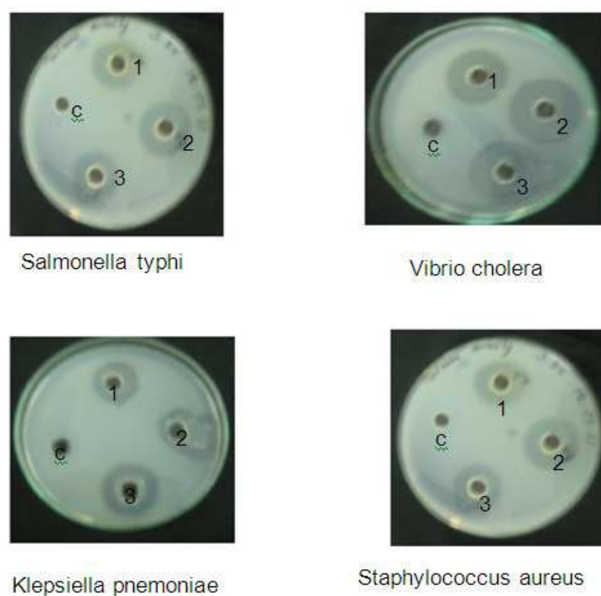
Isolate 6 exihibited maximam activity

Table 2
DAY OPTIMIZATION RESULT
(Zone of inhibition (mm)

Organism	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8
Salmonella typhi	_	_	13	14	17	19	15	16
Vibrio cholera	_	_	12	13	15	18	16	14

ANTIBACTERIAL ACTIVITY

Figure 5



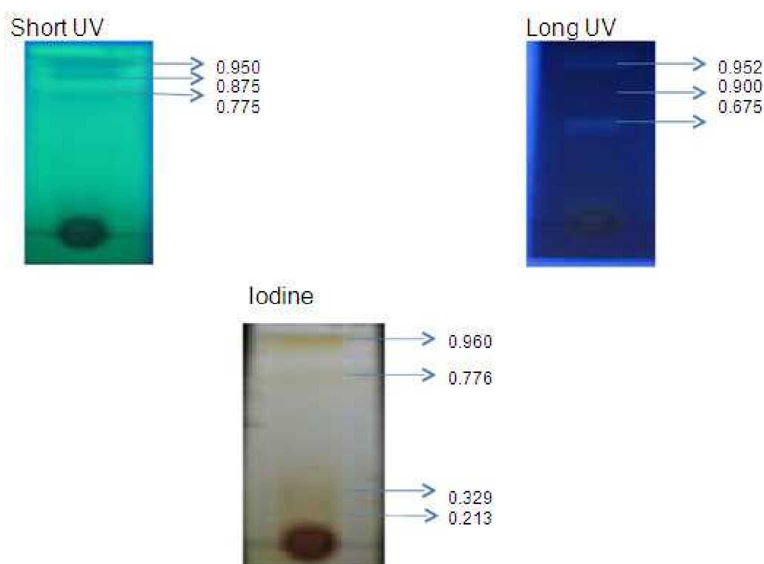
C-control
1-50µl
2-75µl
3-100µl

Table 3
Antibacterial activity using metabolites

Organism	50µl	75µl	100µl
Salmonella typhi	19	19	21
Vibrio cholera	15	17	18
Staphylococcus aureus	15	16	18
Klebsiella pneumoniae	19	19	19

Thin layer chromatography

Figure 6



REFFERENCE

1. Cragg GM, Grothaus PG, Newman DJ, Impact of natural products on developing new anti-cancer agents. Chem. Rev. 109:3012–3043,(2009).
2. Newman DJ, Cragg GM, Natural products as sources of new drugs over the last 25 years. J. Nat. Prod. 70:461–477,(2007).
3. Pela´ez F, The historical delivery of antibiotics from microbial natural products—can history repeat? Biochem. Pharmacol. 71:981–990, (2006).
4. Vijayakumar R, Studies on the diversity of actinomycetes in the palk region of bay of Bengal ,India,Actinomycetologica,21;59-65, (2007).
5. Miyadoh S, Research on antibiotic screening in Japan over the last decade: A producing microorganism approach. Actinomycetologica 9: 100-106,(1993).
6. Mayer KH, Hopkins J D, Gilleece E S , Chao L, O' Brien TF, Molecular evolution, species distribution, and clinical consequences of an endemic aminoglycoside resistance plasmid. Antimicrob. Agents Chemother. 29: 628-633,(1986).
7. Kümmerer K, Antibiotics in the aquatic environment – A review – Part I Chemosphere 75: 417–434,2009.
8. Kuster E, Williams S, Selection of media for the isolation of Streptomyces. Nature 202: 928-929,(1964).
9. Williams ST, Sharpe ME, Holt JG, Bergy's Mannual of Systematic Bacteriology. Vol. 4. Williams and Wilkins Co., Baltimore. USA. pp. 2451-2507,(1989).
10. Hewitt W and Vincent, The agar diffusion assay in theory and application of Microbiology Assay ,Academic press,New York,NY., 38-79,(1989).
11. Ellof JN, A sensitive and quick micro plate method to determine the minimal inhibitory concentration of plant extract for bacteria. Plant Medica 64: 711-713,(1998).
12. Skipski VP, Peterson RF, Barclay M ,Quantitative analysis of phospholipids by thin-layer chromatography. Biochem. J. 9 0 374-378,(1964).
13. Madigan M, Martinko J, Brock Biology of Microorganisms (11th ed. ed.).Prentice Hall, (2005).

14. Omura S, Tanaka Y, Macrolide antibiotics. In: Biotechnology Vol. IV. (Eds. Pape, H. and Rehn, H.J.), VCH Verlagsgesellschaft, Weinheim. pp. 360-391,(1986).
15. Kämpfer P,The Family Streptomycetaceae, Part I: Taxonomy. The prokaryotes: a handbook on the biology of bacteria (Dworkin, M et al, eds.). Berlin: Springer. pp. 538–604. ISBN 0-387-25493-5,(2006).
16. Lee JY, Hwang BK, Diversity of antifungal actinomycetes in various vegetative soils of Korea. Can. J. Microbiol. 48: 407-416,(2002).
17. Aghighi S, Shahidi BGH, Saadoun I , First report of antifungal properties of a new strain of Streptomyces plecatus (strain 101) against four Iranian phytopathogenic isolates of Verticillium dahliae, a new horizon in biocontrol agents. Biotechnology, 3: 90-97, (2004).
18. Williams ST, Bergeys Maunal of Determinative Bactriology,4.Williams and Wilkins ,Baltimore.USA,(1989).
19. Fourati-Ben Fguira L, Fotso S, Ben Ameur-Mehdi R, Mellouli L, Laatsch H, Purification and structure elucidation of antifungal and antibacterial activities of newly isolated Streptomyces sp. strain US80. Res. Microbiol. 156: 341-347, (2005).
20. Sujatha P, Bapi Raju KVVSN, Ramana T, Studies on a new marine Streptomycete BT-408 producing polyketide antibiotic SBR-22 effective against methicillin resistant Staphylococcus aureus. Microbiol. Res. 160: 119-126, (2005).
21. Selvakuma D, Dey S, Das D, Production and bioassay of bialaphos biosynthesized by Streptomyces hygroscopicus NRRL B-16256. Bioprocess. Engg. 20: 459-462, (1999).
22. Hewitt W, Vincent S, In: Theory and application of microbiological assay. Academic Press, San Diego, 39, (1989).
23. Cowan MM , Plant Products as Antimicrobial Agents. Clin. Microbiol. Rev. 12(4), 564-582, (1999).

This article can be downloaded from www.ijpbs.net