International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 2 Number 6 (2013) pp. 236-245**
http://www.ijcmas.com

Original Research Article

Isolation and Identification of Actinomycetes *Isoptericola variabilis* From Cauvery River Soil Sample

M.Rajesh Muthu[1], R.Subbaiya[1*], M.Balasubramanian[1], P.Ponmurugan[1] and Masilamani Selvam[2]

[1]Department of Biotechnology, K.S.Rangasamy College of Technology, Tiruchengode-637 215, Tamil Nadu, India.
[2]Department of Biotechnology, Sathyabama University, Jeppiaar Nagar, Chennai-600 019, Tamil Nadu, India
******Corresponding author e-mail:* ramsubbubio@gmail.com

A B S T R A C T

Five actinomycetes strains were isolated from the 25 soil samples of Cauvery river. The isolated strains were subjected to different biological identification. Collected soil samples were air dried for 2-4 days. After 4 days of air drying it has been washed two times with the help of distilled water to remove the unwanted particles from the soil sample. The pure sample was subjected to basic biological procedures like serial dilution, pour plate technique and spread plate technique, that is to know the specific growth of the actinomycetes. Isolated actinomycetes culture was identified by 16s rRNA sequencing and it was confirmed by using bioinformatics tool as BLAST (*Isoptericola variabilis*).

Keywords

Actinomycetes; BLAST; *Isoptericola variabilis.*

Introduction

Actinomycetes are a group of bacteria which possess many important and interesting features (Hirsch*et al*.,1983) It is the best known for their ability to produce antibiotics and are gram positive bacteria which branching unicellular microorganism. Actinomycetes of about 100 genera exist in soil (Yokota *et al*.,1997). In their natural habitat such as forests, river, the interact in various ways with higher plants. In this study soil sample were collected in different habitats in the Cauvery river sample to investigate the diversity of actinomycetes. A large number of actinomycetes have been isolated and screened from soil in the past decades, accounting 70-80% of relevant secondary metabolites available commercially (Monisha Khanna *et al*.,2011). Actinomycetes which are profilicproducts of antibiotics and important supplier to the pharmaceutical industry.

Actinomycetes have provided many important bioactive compounds of high commercial value and continue to beroutinely screened for new bioactive compounds. The researches have been remarkably successful and approximately two thirds of naturally occurring antibiotics, includ-ing many of medical importance, have been isolated from actinomycetes, (Okami *et al.,* 1988). (Goodfellow *et al*., (1988) reviewed the literature on isolation of actinomycetes and suggested thatonly 10% of the actinomycetes are isolated from nature. Most of the antibiotics in use today are derivatives of natural products of actinomycetes and fungi, (Butler *et al.*, 2006). Actinomycetescan be isolated from soil and marine sediments. Althoughsoils have been screened by the pharmaceutical industry for about 50 years, only a miniscule fraction of the surface of theglobe has been sampled, and only a small fraction of actinomycetes taxa has been discovered (Baltz *et al.,* 2005).

Actinomycetes are the most economically valuable prokaryotes which are well known to produce chemically diverse metabolites with wide range of biological activity (Balagurunathan*et al*., 2007). Recent days the discovery of known metabolites and actinomycetes are increasing due to the exploitation of natural ecosystems. Exploitation of less and unexplored ecosystems for actinomycetes is highly necessary for the discovery of novel bioactive metabolites. Actinomycetes are important sources of new bioactive compounds such as antibiotics and enzymes (Vining *et al*.,1992) which have diverse clinical effects and are active against many organisms (Bacteria, Fungi, Parasites etc.). In fact more than 50% of the known natural antibiotics are produced from actinomycetes (Miyadoh, *et al*., 1993). The most striking feature of the actinomycetes is their ability to produce a wide variety of secondary metabolites. These natural products have been extraordinary sources of lead structures in the development of newer drugs (Kutzner, *et al*., 1986).

Actinomycetes are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of about half of the discovered bioactive secondary metabolites, notably antibiotics (Berdy *et al*., 2005), anti-tumor agents (Crag *et al*.,2005), immunosuppressive agents (Mann *et al*., 2001) and enzymes (Oldfield *et al*., 1998). Because of the excellent track record of actinomycetes in this regard, a significant amount of effort has been focused on the successful isolation of novel actinomycetes from terrestrial sources for drug screening programs in the past fifty years. Recently, the rate of discovery of new compounds from terrestrial actinomycetes has decreased, whereas the rate of re-isolation of known compounds has increased (Fenical *et al*., 1999). Thus, it is crucial that new groups of actinomycetes from unexplored around exploited habitats be pursued as sources of novel bioactive secondary metabolites.

Materials and Methods

Soil Sample Collection

Soil Sample were collected by sterile method from various locations visited throughout this scientific expedition to Cauvery river sample from an area of Erode. Soil samples were air-dried under room temperature for about 10 days before isolation. Soil samples were collected 10cm below the soil surface of Cauvery river.

Isolation of Actinomycetes

0.5g of soil samples was suspended in 9.5 ml of sterile distilled water and was 1000-fold diluted ,0.1ml of the dilutions was spread on humic acid +modified B vitamins agar (HV) medium, pH 7.2, supplemented with cycloheximide. The plates were incubated at 28ºC for 2 weeks. Marine environment represents one such largely untapped ecosystem from which rare actinomycetes genera having a potential for producing novel metabolites have been discovered.

Morphological Identification

Microbes are largely characterized on the basis of their morphological characters. The macroscopic and macroscopic studies of an actinomycetes growing on agar can provide useful and rapid clues for identification of their respective genus.

Macroscopic characters include colony characteristics such as size, shape, color, consistency on different media, the absence or presence of aerial mycelium and extent of spore formation. Cultures are observed for microscopic feature including fragmentation of substrate and arial mycelium, presence of sclerotic, spore chain morphology and spore surface ornamentation. On the basis of spore chains, the strains can be placed into groups.

For example, the species belonging to the genus Streptomyces are divided into three groups broadly i.e. rectiflexibiles, ratinaculiberti and spirals. Characteristics of the spore bearing hyphae and spore chains can be determined by light microscopy using coverslip culture and slide culture techniques. Actinomycetes are also observed by the phase-contrast microscopy for study of spore surface ornamentation. Genera of purified isolate can be identified based on morphological comparisons to the existing description of known genera as given in Bergey's Manual Determinative Bacteriology.

Molecular Technique for Selective Isolation Actinomycetes

In microbial screening program, a large number of isolates are screened to increase the probability of finding novel antimicrobial compounds, since it is generally known that morphologically similar appearing strains have more chances of production the same secondary metabolites compared to morphologically discrimination is important to save efforts, time and resources. However, there are limitations in the use of traditional morphological methods alone for identifying strains.

Firstly, some genera of actiomycetes do not produce distinct aerial mycelium or show specific coloration, therefore morphological analysis of such strains would not help in their distinction as separate genera.

Secondly, many actinomycetes genera show morphological variation at different stages of growth when cultured on various media causing confusion during their taxonomic identification. As a result, result, reliable identification of actinomycetes may not be possible.

Thirdly, different strains of actinomycetes belonging to the same genus appear morphologically alike on the isolation plates and would be eliminated during screening programs decreasing the chances of recovering potentially useful isolates.

Furthermore, the traditional biochemical method and phenotypic studies including cell wall analyses, polar lipids analyses and fatty acids methyl ester analyses which are used for the identification of the aerobic filamentous actinomycetes require extensive labra and time. In many cases such methods can not identified an isolate to the level of single genus.

Another strategy that should be adopted during screening programs is to avoid rediscovery of known compounds derived from the commonly occurring soil Streptomyces species. Streptomyces have been extensively screened and exploited for secondary metabolite production therefore, the probability isolating novel antimicrobial compounds from species of Streptomyces very low.

The chances of isolating new bioactive compounds from rarer, non-streptomyces appear more promising. Therefore methods which can distinguish Streptomyces from morphologically similar actinomycetes as well as identify rare actinomycetes up to the genera level would be extremely useful.

Screening of Antibacterial Activity of Actinomycetes

Preliminary screening: Antibacterial active of actinomycetes was subjected to primary screening by cross streak plate technique straight-line inoculation of the actinomycetes isolates was made on modified nutrient agar medium and incubated at 28ºC for 4 to 5 days .

Secondary screening: The selected antagonistic actinomycetes isolates were inoculated into starch-casein broth, and incubated at 28ºC in a shaker for 7 to 10 days.

Colony morphology on different media: The selected two antagonistic actinomycetes were cultured on four different cultured media, namely nutrient agar, actinomycetes isolation agar, glycerol yeast extract ager and starch casein agar, and incubated for 7 to 10 days at 28ºC.

Physiological and biochemical characterization: The physiological characteristics of the isolates such as, growth at different pH(2,4,6,8 and 10),temperature(20,25,30,40 and 45C) and NaCl concentration (1.5,3,5 and 7g/l) were recorded in starch casein broth.

16s rRNA Approach

DNA preparation method: Growth from mature slant culture of the actinomycetes were inoculated aseptically into 250 ml Erlenmeyer flasks each containing 30 ml of Nutrient broth medium (beef extract 2 g/l, yeast extract 2 g/l, peptone 5 g/l and sodium chloride 8 g/l, pH 7.1 after sterilization) and incubated in a rotary shaker at 30°C for 2 days at 180 rpm. Cultures were centrifuged at 10,000 rpm for 10 minutes.

The 0.1 g of mycelium was transferred in to sterile porcelain dish and crushed with liquid nitrogen. The crushed mycelium was transferred into fresh tube containing 500 µl of TE buffer supplemented with lysozyme (20 mg/ml). The tube was incubated at 37°C for 30 minutes.

Added 20 µl of 10% SDS (w/v) and 20 µl of proteinase K into the tube and incubated at 55°C for 30 minutes. The lysate was cooled down and extracted once with equal volume of phenol: chloroform solution (v/v, 1:1) at 10,000rpm for 5 minutes. The aqueous phase was transferred carefully to a fresh tube and DNA was precipitated by adding 70-90% ethanol and keeping at -20°C for 30 minutes. The pellet was formed by centrifuging at 10,000 rpm for 10 minutes.

The pellet was washed twice with 90% ethanol and dissolved the pellet in TE buffer.

To obtain RNA free DNA added 20 µl of RNase solution (20 µg/ml) and then incubated at 37°C for 1hr. The sample was once again extracted with equal volume of phenol: chloroform and precipitated as above.

The purity and concentration was checked in Bio photometer PCR amplification, sequencing and restriction analysis PCR amplification of the 16S rDNA of the *Streptomyces* sp. was performed using two primers: 9 F (5'-GAGTTTGATCCTGGCT CAG3') and 1 541R (5'-AAGGAGGTGATCCAACC3'). The final volume of reaction mixture of 25 µl contained 1X PCR buffer F (Genei), 1.5 mMof MgCl2, 200 µM of each dNTP, 20 pico-moles of each primer, 2.5U of Taq DNA polymerase and 100 ng of template DNA.

The amplification was performed on Eppendorf Thermo-cycler 96, according to the following profile: an initial denaturation step at 94°C for 2 min. followed by 30 amplification cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min and a final extension step of 72°C for 2 min.

The PCR product was detected by agarose gel electrophoresis and was visualized by ultraviolet (UV) fluorescence after ethidium bromide staining. The restriction digestion of 16S rDNA was performed according to the method described previously. Prior to sequencing, amplified products were purified using a HiPurATM PCR product purification spin kit according to the manufacturer's instructions.

Sequencing reactions of PCR products were perform with the ABI PRISM® BigDye® Terminator version 3.1 Cycle Sequencing Kit according to the manufacturer's instructions using 1541R primer.

Results and Discussion

Isolation of Actinomycetes Culture

A total of 10 actinomycetes were isolated from 25 soil samples collected from Cauvery river, the average number of isolates per sample was 1.5. The Actinomycetes culture was isolated by using Starch casein nitrate agar medium. Antifungal (Nalidixic acid) and Antibacterial (Cycloheximide) chemicals are used to avoid the contamination while during the isolation process.

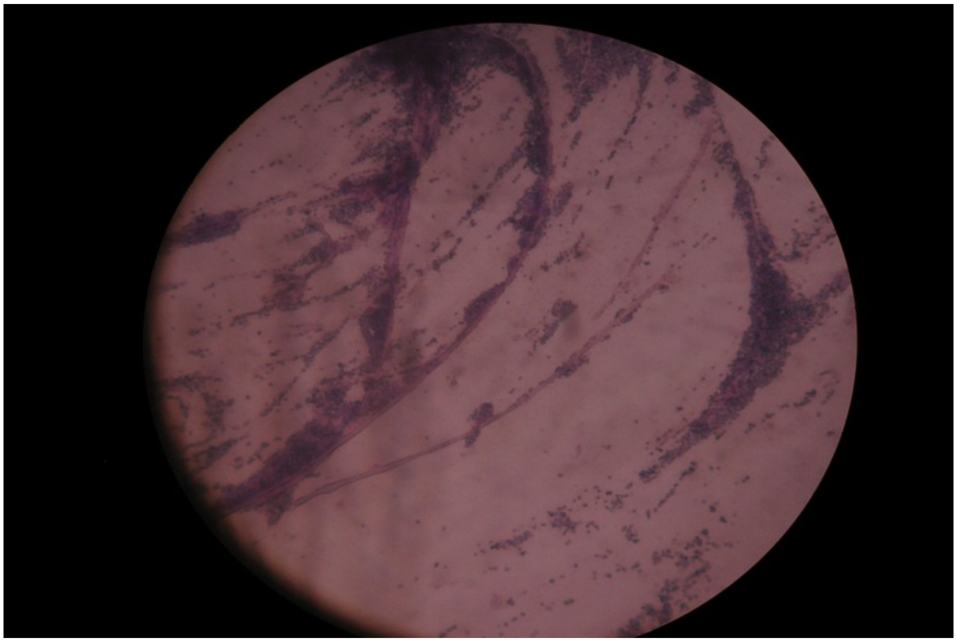
Starch Casein Nitrate agar media is the specific media to isolate the actinomycetes. It is also evident that different physiological characteristics are influencing the growth rate of the actinomycetes (Figure 1). In the present study, the assessment of physiological characteristics of the isolated strain shows that could grow well at 30 and 40ºC temperature, pH 7.0 to 9.0 respectively. However, the strain had maximum growth at NaCl concentration of 1g/l.

Identification of actinomycetes

Gram staining procedure has been carried out to identify the actinomycetes and with the help of microscopical examination it has been confirmed that gram positive bacteria (Figure 2).

Figure.1 Actinomycetes strain identified from soil sample and the growth was observed after 6-8 days

Figure.3 Actinomycetes stained with gram staining and it shows the purple colour appearance represents the gram positive organism



DNA Isolation and Preparation method

Growth from mature slant culture of the actinomycetes were inoculated aseptically into 250 ml Erlenmeyer flasks each containing 30 ml of Nutrient broth medium (beef extract 2 g/l, yeast extract 2 g/l, peptone 5 g/l and sodium chloride 8 g/l, pH 7.1 after sterilization) and incubated in a rotary shaker at 30°C for 2 days at 180 rpm. Cultures were centrifuged at 10,000 rpm for 10 minutes. The 0.1 g of mycelium was transferred in to sterile porcelain dish and crushed with liquid nitrogen. The crushed mycelium was transferred into fresh tube containing 500 µl of TE buffer supplemented with lysozyme (20 mg/ml). The tube was incubated at 37°C for 30 minutes.

Added 20 µl of 10% SDS (w/v) and 20 µl of proteinase K into the tube and incubated at 55°C for 30 minutes. The lysate was cooled down and extracted once with equal volume of phenol: chloroform solution (v/v, 1:1) at 10,000rpm for 5 minutes. The aqueous phase was transferred carefully to a fresh tube and DNA was precipitated by adding 70-90% ethanol and keeping at -20°C for 30 minutes. The pellet was formed by centrifuging at 10,000 rpm for 10 minutes. The pellet was washed twice with 90% ethanol and dissolved the pellet in TE buffer.

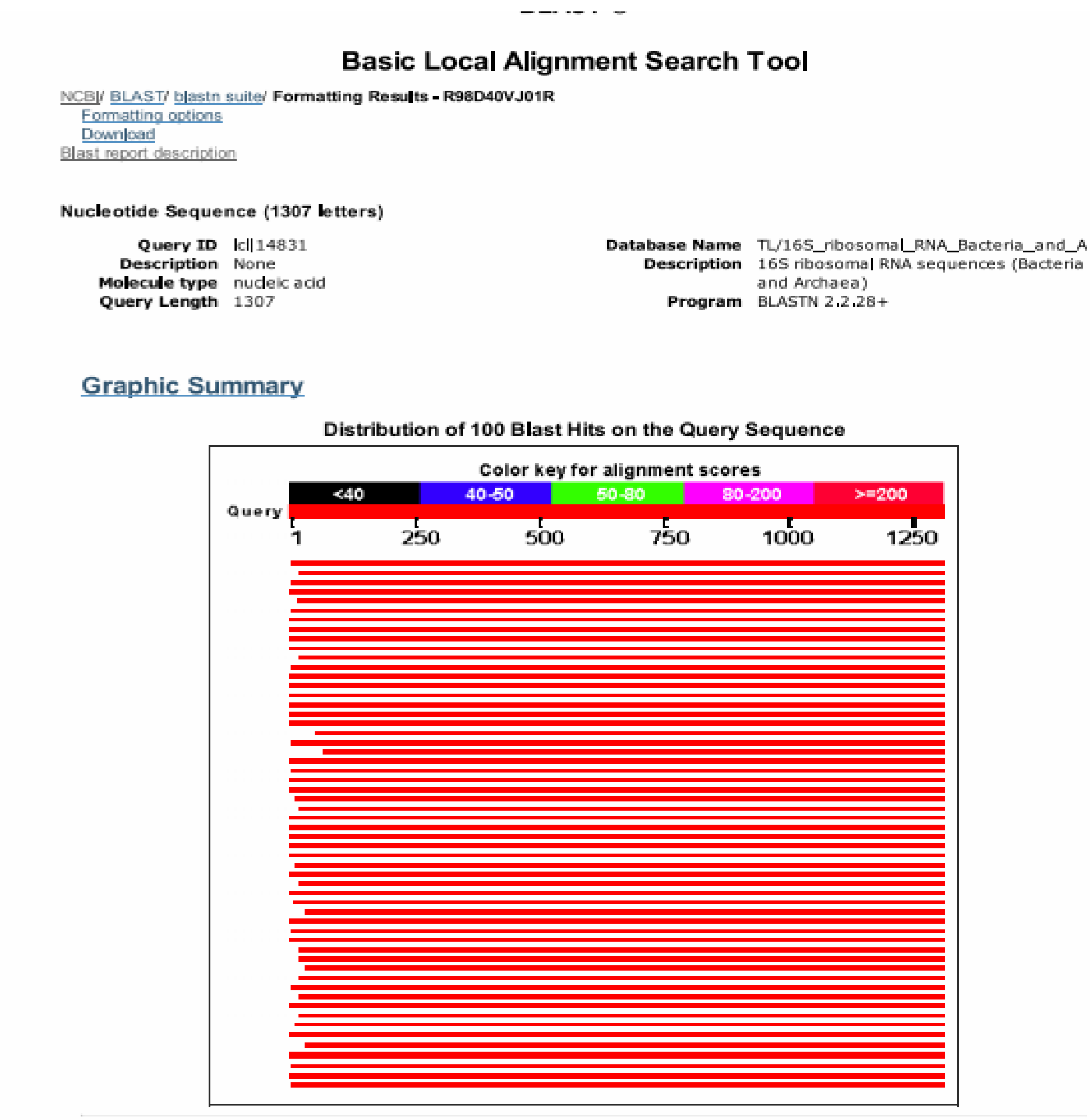
To obtain RNA free DNA added 20 µl of RNase solution (20 µg/ml) and then incubated at 37°C for 1hr. The sample was once again extracted with equal volume of phenol: chloroform and precipitated as above. The purity and concentration was checked in Biophotometer PCR amplification, sequencing and restriction analysis PCR amplification of the 16S rDNA of the Streptomyces sp. was performed using two primers: 9F (5'-GAGTTTGATCCTGGCTCAG3') and 1 5 41R (5'-AAGGAGGTGATCCAACC3'). The final volume of reaction mixture of 25 µl contained 1X PCR buffer F (Genei), 1.5 mMof MgCl2, 200 µM of each dNTP, 20 pico-moles of each primer, 2.5U of Taq DNA polymerase and 100 ng of template DNA. The amplification was performed on Eppendorf Thermo-cycler 96, according to the following profile: an initial denaturation step at 94°C for 2 min. followed by 30 amplification cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min and a final extension step of 72°C for 2 min. The PCR product was detected by agarose gel electrophoresis and was visualized by ultraviolet (UV) fluorescence after ethidium bromide staining. The restriction digestion of 16S rDNA was performed according to the method described previously. Prior to sequencing, amplified products were purified using a HiPurATM PCR product purification spin kit according to the manufacturer's instructions.In DNA isolation,1% agarose gel was prepared and genomic DNA, PCR product was ran through the gel electrophoresis. Lane 1 denotes the genomic DNA, Lane 2 represents the one kilo basepair DNA ladder and Lane 3 shows the PCR product.

BLAST analysis

Finally the bioinformatics tool as BLAST result reported that the identified culture was *Isoptericola variabilis.* Identified organism has been deposited at NCBI, Bethesda, Maryland, U.S.A and received the submission number as 1628659 (Figure 3).

The present investigation concluded that the physiological characteristics of actinomycetes varied depending up on the available nutrients in the medium and the physical conditions. Upon the growth of both the experimental isolates on various

Figure.3 Represents the BLAST result for the organism and it shows the percentage of coverage and identification



Isoptericola variabilis 225 strain 225 16S ribosomal RNA, complete sequence	2152	2152	100%	0.0	96%	NR_074510.1

media, SCN agar was observed to be the best medium for maximal growth. Further, the colour of diffusible pigments and the aerial and substrate mycelia produced by the two isolates varied with different media. Thus, it was concluded on the basis of the present and previous studies that the nutrient compositions of the medium greatly influence the growth and morphology of organisms. Other biologically active compounds are produced by the *Isoptericola variabilis*. When compare with the marine sediments, river soil sample has the better activity and it has been observed that numerous numbers of colonies has been seen. Pigments produced by the soil sample have the better activity. During the DNA isolation sample has shown the better activity within the time period followed by the isolation it has been immediately subjected to the 16s rRNA sequencing and it has shown the exact and expected result.

Acknowledgement

The authors are thankful to, The Management, Head, Department of Biotechnology, K.S.R College of Technology, Tiruchengode, India, Progen Biotech India Pvt Ltd. Salem, for their encouragement and constant support to to carry out this work.

References

Butler, M.S., and Buss, A.D. 2006. Natural products: The future scaffolds for novel antibiotics? Biochem. Pharmacol.71:919—29.

Baltz, RH., 2005. Antibiotic discovery from actinomycetes: Will a renaissance follow the decline and fall? . SIM News.55:186—96

Balagurunathan, R., and Radhakrishnan, M. 2007.Actinomycetes: Diversity and their importance. In: Microbiology – Applications and Current Trends. P.C. Trivedi (editor), Pointer publishers, Jaipur, India.pp. 297-329.

Berdy, J., 2005. Bioactive microbial metabolites. J. Antibiot .(Tokyo).58:1-26.

Cragg, G.M., D.G.I. Kingston and Newman, D.J. 2005. Anticancer Agents from Natural Products. Taylor & Francis.

Fenical, W., D. Baden, M. Burg, C.V. de Goyet, J.D. Grimes, N.H. Katz M,Marcus, S. Pomponi, P. Rhines, P. Tester and Vena, J. 1999. Marine-derived pharmaceuticals and related bioactive compounds. In From Monsoons to Microbes: Understanding the Ocean's Rolein Human Health. Edited by Fenical W. National Academies Press.pp. 71-86.

Goodfellow, M., and Haynes, J.A.1984. Actinomycetes in marine sediments.In: Ortiz-Ortiz T, Bojalil LF, Yakoleff V, editors. Biological, biochemical and biomedical aspects of actinomycetes. London:Academic Press. pp.453—72.

Hirsch, C.F., and Christensen, D.L. 1983, Novel Methods for Selective Isolation of Actinomycetes . Appl. Environ. Microbiol. 925-929.

Kutzner, K.J., 1986. The family Streptomycetaceae. In: Starr MP, Stolp H, Trper HG, Balows A, Schlegel HG (eds) The prokaryotes, A Handbook on Habitats, isolation and identification of bacteria. 2, Spriner-verlag, New York.pp.2028-2090.

Miyadoh, S., 1993. Research on antibiotic screening in Japan over the late decade: a producing microorganisms approach. Actinomycetologica. 7: 100 – 106.

Mann, J,, 2001. Natural products as immunosuppressive agents. Nat .Prod .Rep.18:417-430.

Monisha Khanna., RenuSolanki and Rup Lal. 2011, Selective Isolation of rare Actinomycetes producing novel Antimicrobial Compounds. Inter. J. Adv. Biotechnol. Res.2(3):357-375.

Okami, Y., and Hotta, K. 1988. Search and discovery of new antibiotics. In:Goodfellow M, Williams ST, Mordarski M, editors. Actinomycetes in biotechnology. New York: Academic Press Inc.pp. 33—67

Oldfield, C., N.T. Wood, S.C.Gilbert, F.D. Murray and Faure, F.R. 1998. Desulphurisation of benzothiophene and dibenzothiophene by actinomycete organisms belonging to the genus *Rhodococcus*, and related taxa. Antonie Van Leeuwenhoek.74:119-132.

Vining, L.C., 1992.Secondary metabolism, inventive evolution and biochemical diversity – a review. Gene. 115: 135-140.

Yoko yamamota, Tankonari kouchiwan and Yoshikuni Hodoki 1998.Distribution and Identification of Actinomycetes layering cyanobacteria in a eutrophic lake.10: 391-397.