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RESEARCH ARTICLE

ANTIMICROBIAL ACTIVITY AND CHARACTERIZATION OF MARINE *STREPTOMYSIS CHEONANENSIS*

***Y. S. Y. V. Jagan Mohan[1], B. Sirisha[1] and T. Ramana[2]**

1. Department of Biotechnology, College of Science and Technology, Andhra University, Visakhapatnam 530 003, India.

2. Dean of School of Life Sciences, GITAM University, Visakhapatnam 530045, India.

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Abstract

Marine environment is a potential source for development of novel natural pharmaceuticals. As marine environmental conditions are different from terrestrial ones, it is surmised that marine actinomycetes might produce novel bioactive compounds. These microbes have characteristics in common to both bacteria and fungi and yet they possess adequate distinctive features to classify them into a separate category. Actinomycetes were cultivated using a variety of media and selective isolation techniques from 15 marine samples collected from the Bay of Bengal. Out of 93 isolates, 11 strains showed high antimicrobial activity against test organisms. The strain AUBT - 902 was selected for further investigation due to its strong antibacterial and antifungal activity and was identified as a member of *Streptomyces* genus based on its morphological, cultural, physiological, utilization of carbon sources and biochemical characteristics. Phylogenetic analysis based on 16S rRNA gene sequences showed that the strain belongs to the genus *Streptomyces*, with the maximum similarity to *Streptomyces cheonanensis* (99.6%). On the basis of polyphasic evidence, the strain was recognized as a new isolate of *Streptomyces cheonanensis* from marine source.

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Introduction

Marine environment is a largely untapped source for the isolation of new microorganisms with potentiality to produce active secondary metabolites. The demand for new antibiotics continues to grow due to the rapid emergence of multiple antibiotic resistant pathogens causing life threatening infection. Although, substantial progress is being made within the fields of chemical synthesis and engineered biosynthesis of antibacterial compounds, nature still remains the richest and the most adaptable source for new antibiotics (Kpehn and Carter, *et al.,* 2005; Baltz, *et al.,* 2006). Marine sediment sources are valuable for the isolation of novel actinomycetes with the potential to yield useful new products (Goodfellow and Haynes, 1984). Marine sediment is an inexhaustible resource that has not properly been exploited. Few reports from the East Coast of India, suggests that soil is a major source of Actinomycetes (Sivakumar *et al.*, 2005; Vijayakumar *et al.*, 2007).

Actinomycetes are best known for their ability to produce antibiotics and are gram-positive bacteria, which comprise a group of branching unicellular microorganism (Waksman, S.A. *et al.,* 1954). They are free living saprophytic bacteria and a major source for production of antibiotics. They play a major role in recycling of organic matter (Unaogu *et al.,* 1994), production of novel pharmaceuticals, nutritional materials, enzymes, antitumor agents, enzyme inhibitors, immune-modifiers and vitamins. Around 80% of the total antibiotic production has been obtained from *Streptomyces* (Wellington, 1992).

Streptomyces are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of about half of the discovered bioactive secondary metabolites such as antibiotics, antitumor agents and immunosuppressive agents (Berdy J. *et al*., 2005). *Streptomyces* sps are widely recognized as industrially important organisms for their ability to elaborate different kinds of novel secondary metabolites (Bibb, M.J. *et al.,* 2005).

The taxonomy of actinomycetes has been subject to incessant controversy because of its filamentous, branching growth which resembles a fungal type of morphology (Woznicka W. *et al.,* 1964). Actinomycetes represent a high amount of the soil microbial biomass and have the capacity to produce a wide variety of antibiotics and extracellular enzymes (Kavitha A. *et al.,* 2011). When conservative isolation techniques were applied, most of the isolates recovered on agar plates have been identified as genus *Streptomyces*, which are the dominant actinomycetes in soil (Iwai H. *et al.,* 1992). For the purpose of screening novel bioactive molecules, several factors must be considered: choice of screening source, pretreatment, selective medium, culture condition, and recognition of candidate colonies on a primary isolation plate.

Specialized growth media were developed to isolate specific actinomycete genera. Macromolecules such as starch, glycerol, chitin, casein, humic acid and amino acids were selected as the best carbon and nitrogen sources of rare actinomycetes (Cho S. H. *et al.,* 1994). Recently the rate of discovery of new compounds from terrestrial actinomycetes has decreased whereas the rate of reisolation of known compounds has improved. Thus, it is essential that new groups of actinomycetes from unexplored or under exploited habitats be persued as sources of novel bioactive secondary metabolites (Donia *et al.*, 2003). Although the diversity of life in the terrestrial environment is astonishing, the greatest biodiversity is in the oceans (Dubey *et al.*, 2005).

As marine environmental conditions are exceptionally different from terrestrial ones, it is surmised that marine actinomycetes have different characteristics from those of terrestrial counterparts, and therefore, might generate different types of bioactive compounds (Gesheva *et al.*, 2005). In fact more than 50% of the known natural antibiotics are produced from actinomycetes (Miyadoh, 1993). The most striking feature of 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 (Weber *et al.,* 2003).

In the present investigation an effort was made to screen different marine sediments of the east coast of the Bay of Bengal, India, which is a large, assorted and an unscreened ecosystem for the isolation of potent antibiotic - producing actinomycetes, their distribution pattern and characterization of a potential isolate.

Materials and Methods

Sample Collection

Totally 15 marine sediment samples were collected from various depths of the Bay of Bengal ranging from 50 to 250mts. The samples were collected in sterile air tight bags and accurately labeled indicating the date of collection and the depth and transferred to the laboratory for further study.

Pretreatment of Samples

The sediment samples were air dried. Appropriate selective media such as Starch casein agar, Glycerol yeast extract agar, Actinomycetes isolation agar, Glucose yeast extract malt extract agar, Chitin agar media and antibacterial antibiotic (Rifampicin) at 5 µg/ml, antifungal antibiotic (Nystatin) at 25 µg/ml were used for actinomycetes growth endorsement and also for prevention of fungal contamination.

Actinomycetes Isolation and Maintenance

Actinomycetes were isolated by serial dilution method from the sediments. Stock solution is prepared by diluting 1g of sediment in 9 ml of sterile saline water and shaken well by using vortex mixer. From the stock solution, 1 ml was used to prepare 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} concentration by serial dilution method. Finally, 0.1 ml of suspension from 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were used to spread on starch-casein agar medium aseptically. 0.1 ml of aliquot of water was spread evenly over the sterilized starch casein agar plates by using L-shaped glass rod. For each sample three plates were used and incubated at 30°C for 7 to 14 days. The plates were observed periodically for the growth of actinomycetes. The pure colonies were selected, isolated and maintained in starch casein agar slants at 4°C for consequent studies.

Test Organisms: *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (MTCC 441), *Bacillus cereus* (MTCC 430), *Pseudomonas aeruginosa* (MTCC 424), *Escherichia coli* (MTCC 443), *Proteus vulgaris* (MTCC 426), *Saccharomyces cerevisiae* (MTCC 170), *Candida albicans* (MTCC 227), *Aspergillus niger* (MTCC 961), and *Aspergillus flavus* (MTCC 3396).

Primary Screening

Antimicrobial activities of the isolates were tested preliminarily by cross streak method (Lemos, 1985). In this method a loop full of inoculum was streaked in the middle of the petridish containing modified nutrient agar

medium. After inoculation, petridishes were incubated at 28°C for 3 days for the growth of actinomycetes and then 24hrs old bacterial cultures were inoculated near the growth line of actinomycetes in the same petridish. The cross streaked plates were incubated at 28°C for 24 hrs. The inhibition zone (Cleaning zone) produced between the actinomycetes and the bacteria were measured.

Secondary Screening

The selected isolates were further tested in the secondary screening by shake flask studies to confirm their antimicrobial activity. The spore suspension of the selected isolates were inoculated into the soya bean medium and kept in the shaker. After 96hrs, the culture broth was separated from the mycelium by centrifugation at 5000rpm and tested for antimicrobial activity.

Agar well diffusion Method

100ml of sterilized Potato dextrose agar medium (PDA) in 250ml conical flask was seeded with 50µl of standardized test bacteria, swirled gently and aseptically poured into Petri dishes and allowed to solidify. Sterile cork borer (6 mm diameter) was used to make wells in the plate. About 100 µl of the sample was carefully dispensed into wells. The experiment was repeated for three times (Pandey, 2004). Extracts were allowed to diffuse for about 2h before incubating. Plates were incubated at 37°C for 24h. The diameter of the inhibition zone for each strain was recorded. Among the selected strains the most potent strain was selected for further analysis.

Morphological and cultural characters of the selected actinomycete strain was studied by inoculating into sterile International Streptomycetes Project ISP 1, 3-7, 9 media (Ohshima *et al.*, 1991). The media were sterilized and poured into sterile Petri dishes. After solidification of the media, the culture of the selected strain was streaked on the media surface by simple method aseptically and incubated at 28°C for 7 days (Shomura *et al.*, 1987). Morphological characters such as colony characteristics, type of aerial hyphae, growth of vegetative hyphae, fragmentation pattern and spore formation were observed.

Taxonomic Identification of Actinomycete Isolate

Polyphasic taxonomic approach was used to identify the active marine isolate. Morphological, cultural, physiological and biochemical characterization of the strain were studied by following the methods of Shirling and Gottlieb (1966). The morphological characteristics of the strain AUBT–902 were assumed by Scanning Electron Microscopy (model JSM-6610; JEOL, Ltd., Japan) of 14 day old cultures on ISP 2 medium.

For Scanning Electron Microscopy (SEM), the strain was fixed with 1.5% Glutaraldehyde and dried out with graded series of ethanol washes followed by drying in desiccators (EMITECH-K850-CPD). Samples were fixed to SEM stubs using carbon tape followed by thin coating with platinum and examined with Scanning Electron Microscope (JOEL; JSM-6610LV). Aerial mycelium, spore mass colour, substrate mycelium pigmentation and coloration of diffusible pigments of the strain were recorded on ISP media (Fig.1). The phenotypic properties of the strain were studied using standard procedures (Shirling and Gottlieb, 1966; Williams et al., 1983). Physiological tests such as growth at different temperatures (15, 25, 37, 42, 50° C), pH (5.2, 8.0 9.0 10.0) and NaCl concentrations (2, 5, 7, 10% w/v) were performed according to the method described by Williams et al., 1983. In addition, carbon source utilization and acid production were studied using media and methods described by Gordon *et al.,* 1974. The colours were determined by comparing with colour chips from the ISCC-NBS colour charts standard samples No. 2106 (Kelly, 1964).

Chemotaxonomy

The procedure of Lechevalier and Lechevalier (1980) was used for the analysis of whole sugars. Cell wall amino acids were analyzed by the methods of Jiang et al., 2001. Phospholipid analysis was carried out as described by Lechevalier et al., 1981. Menaquinones were determined using the procedure of Collins (1985). Fatty acid composition was determined by following the procedures of Sasser (1990) and Kampfer and Kroppenstedt (1996) by using the standard MIDI (Microbial identification) system.

Molecular Analysis

The 16S rRNA gene was amplified and analyzed as described by Li *et al.,* (2007) and the 16S rRNA gene sequence (1457 bp) of the strain was determined. The variable γ region (position 158-277) of the 16SrRNA gene sequence and the almost complete 16SrRNA gene sequence of the strain was compared with the closest related sequences of reference organisms. Sequence data were aligned with CLUSTAL_X (Thompson *et al.,* 1997). The evolutionary tree

rooted with *Streptomyces sp*. 102H11-6 (Accession No. EU181239.1) as the outer group, was inferred by using maximum-likelihood method by Felsenstein (1981) with PHYLIP package. The topology of the resultant tree was evaluated based on 1000 resamplings (Felsenstein, 1985). Dendroscope program was used to display, edit and print Phylogenetic trees (Huson *et al.,* 2007).

The chromosomal DNA was extracted as described by Marmur (1961) and the genomic DNA G+C content of the strain was determined by thermal denaturation method of Marmur and Doty (1962).

Nucleotide Sequence Accession Number

The almost complete 16SrRNA gene sequence of strain (1457 bp) has been deposited in Gen bank under the accession number KC757350.

Results and Discussion

A total of 93 isolates of actinomycetes were isolated as pure cultures from 15 marine sediments collected from the Bay of Bengal, India. Of these isolates, 36 isolates exhibited antimicrobial activity, 11 isolates having distinct activity of both antibacterial and antifungal.

Among these isolates, one isolate (AUBT-902) showed significant antimicrobial activity against selected bacterial and fungal test organisms and was characterized by polyphasic taxonomy.

Characterization of Actinomycete Isolate AUBT-902

The active isolate was gram^{+ve}, non-acid fast, non-motile, filamentous actinomycete. Morphological observations of the culture of the strain on different ISP media are presented in Table-1. Substrate hyphae of the strain were extremely branched and long, spiral spore chains were borne on the aerial hyphae. The oval spores (μm) were non-motile with smooth spore surfaces. Detailed physiological characters of the strain shown in Table-2.

TABLE-1: **Cultural characteristics of isolate AUBT-902 on various media.**

Medium	Growth	Sporulation	Diffusible Pigment	Colony Colour	
				Aerial mycelium	Substrate mycelium
Yeast extract agar (ISP2)	Good	Good	Black	Dark grey	Pale yellow
Oat meal agar (ISP3)	Moderate	Moderate	Reddish brown	Light grey	White
Inorganic salt –starch agar (ISP4)	Good	Good	Black	Grey	Creamy white
Glycerol-asparagine agar(ISP5)	Good	Good	Reddish brown	Dark grey	Pale yellow
Peptone-yeast extract iron agar (ISP 6)	Moderate	Moderate	Black	Grey	White
Tyrosine agar (ISP7)	Good	Good	Black	Dark grey	White
Czapek's agar	Poor	Poor	--	White	--
Nutrient agar	Moderate	Moderate	Brown	Grey	Creamy white

TABLE-2: Morphological, cultural, physiological and biochemical characteristics of AUBT-902.

Test	Results
Spore chain morphology	Spiral
Colony colour on ISP2	Light grey
Diffusible pigments	Yellow
Melanoid pigmentation	+
Growth at temperature	35°C
Growth at pH	6.5-7.5
Starch hydrolysis	+
Casein hydrolysis	-
Gelatin liquefaction	+

H2S production	+
Methyl red	+
Voges-Proskauer	-
Nitrate reduction	+
Indole	-
Catalase	+
Urea	+
Utilization of carbon sources	
Glucose	+
Arabinose	-
Sucrose	+
Xylose	+
Inositol	-
Mannitol	+
Fructose	+
Rhamnose	+
Raffinose	-
G+C content mol%	72.5%

+: Positive; - : Negative.

TABLE-3: Effect of carbon sources on growth and antibiotic production by isolate AUBT-902

Carbon source 1% (w/v)	Growth dry wt (mg/ml)	Antibiotic yield (µg/ml)
Arabinose	2.1	22
Fructose	2.8	158
Galactose	1.6	96
Glucose	3.1	192
Glycerol	2.6	125
Lactose	0.8	46
Maltose	2.7	105
Mannitol	0.5	55
Mannose	1.5	72
Meso-inositol	1.9	68
Rhamnose	2.8	48
Starch	1.4	84
Sucrose	1.9	55
Xylose	0.9	42

TABLE-4: Effect of different nitrogen sources on growth and antibiotic production by isolate AUBT-902.

Nitrogen source	Growth dry wt. (mg/ml)	Antibiotic yield (µg/ml)
Inorganic source 1 % (w/v)		
Ammonium citrate	0.8	48
Ammonium nitrate	2.8	180
Ammonium sulphate	0.6	59
Monosodium Glutamate	0.9	82
Potassium nitrate	2.8	145
Sodium nitrate	3.1	162
Amino acids 0.05 % (w/v)		
Alanine	2.5	135
Arginine	2.4	103
Asparagine	2.8	78
Aspartic acid	2.5	105
Glutamic acid	3.1	109
Hydroxyproline	2.9	121
Leucine	2.1	95

Methionine	3.0	90
Phenylalanine	2.5	55
Serine	2.4	110
Threonine	2.9	130
Tryptophan	1.8	69
Tyrosine	1.9	85

Chemotaxonamic tests showed that the cell wall contained LL-diaminopimelic acid and traces of glycine. No diagnostic sugars were detected in whole-cell hydrolysates. The predominant menaquinones were MK-9 (H_4)-69.7%, MK-9 (H_6)-14.1% and MK-9 (H_8)-16.2% and the diagnostic phospholipids were phosphatidylethanolamine and phosphatidycholine. Fatty acids comprised iso-$C_{13:0}$ (0.40%), iso-$C_{14:0}$ (1.19%), iso-$C_{15:0}$ (20.88%), anteiso-$C_{15:0}$ (17.65%), $C_{15:1}$ω6c (0.76%), $C_{16:1}$isoH (1.05%), iso-$C_{16:0}$ (13.57), iso-$C_{17:0}$ ω9c (5.82%), anteiso-$C_{17:0}$ (1.68%), iso-$C_{17:0}$ (8.19%), anteiso-$C_{17:0}$ (11.97%), iso-$C_{17:1}$ ω8c (0.63%), iso-$C_{18:3}$ ω6c (0.96%). In addition, the sum of $C_{16:1}$ ω7c/$C_{15:0}$iso 2-OH was 5.12% and $C_{18:2}$ ω6c, ω9c/anteiso-$C_{18:0}$ was 0.64%. The DNA G+C content was 72.5%.

Analysis of the γ region sequences of the 16S rRNA from *Streptomyces species* showed that the strain was grouped into a branch with type strain of *Streptomyces sp.* 102H11-6 (Accession No. EU181239.1) (Fig.2). The almost complete 16S rRNA gene sequence of the strain was determined and has been deposited in the GenBank database (Accession No. KC757350). This sequence was compared with the corresponding partial 16S rRNA sequence of the type strains of representative members of the genus *Streptomyces* retrieved from the public database by using BLAST (Altschul *et al.,* 1997). Phylogenetic analysis revealed that the strain is a member of the genus *Streptomyces*. The comparative analysis of 16S rRNA gene sequence and the estimation of Phylogenetic relationships showed that the strain formed a distinct lineage in the tree and showed the closest level of sequence similarity of 99.6% with *Streptomyces cheonanensis* strain VC-A46 (GenBank Accession No. NR043208).

Optimization of antibiotic production was carried out in batch culture. This strain was able to grow in all the tested carbon sources (Table 3). However, maximum antibiotic production was obtained in the medium supplemented with glucose as a sole carbon source followed by fructose and glycerol. The results also showed that glucose level of 12.5 g/l resulted in maximum antibiotic production (Fig. 3).

The results of nitrogen source utilization are shown in Table 4. The highest antibiotic production was obtained in the culture medium containing ammonium nitrate as a nitrogen source, followed by cultures containing sodium nitrate, potassium nitrate and alanine. The results also showed that the concentration of ammonium nitrate (Fig. 4) greatly influenced the production of the antibiotic with maximum antibiotic yield being obtained in cultures supplemented with 2.0 g/l of ammonium nitrate. As shown in Fig. 5, K_2HPO_4 at a concentration of 1.2 g/l gave maximum yield of the antibiotic. The results also showed that the addition of 0.5 g/l of magnesium sulfate to the culture medium was optimal for antibiotic production (Fig. 6). The environmental requirements and tolerance of AUBT-902 for growth and antibiotic production has been studied in detail. AUBT-902 showed a narrow range of incubation temperature for relatively good growth and antibiotic production (Fig. 7). In terms of its optimum temperature (30°C) for growth, the organism appeared to be mesophilic in nature. The increase of the incubation temperature from 20°C to 30°C increased the growth of the cells and the production of the antibiotic by 6 - fold. The maximum antibiotic activity was obtained at a pH of 7.2 (Fig. 8) suggesting its inclusion in the neutrophilic actinomycetes group. The results also indicated that an incubation time of 96 h as optimal. Optimization of medium components and physical parameters (pH, temperature, time) allowed an improvement in the concentration of antibiotic.

Strain AUBT-902 showed a broad antimicrobial spectrum against gram $^{+ve}$ and gram $^{-ve}$ bacteria and fungi when tested with crude culture filtrate. Finally, the antibiotic production was tested by employing the modified medium and optimized cultural conditions. The results indicated the antibiotic titer was very high under optimized conditions (Fig. 9, 10).

Fig. 1 Scanning electron micrograph of Streptomyces cheonanensis AUBT-902.

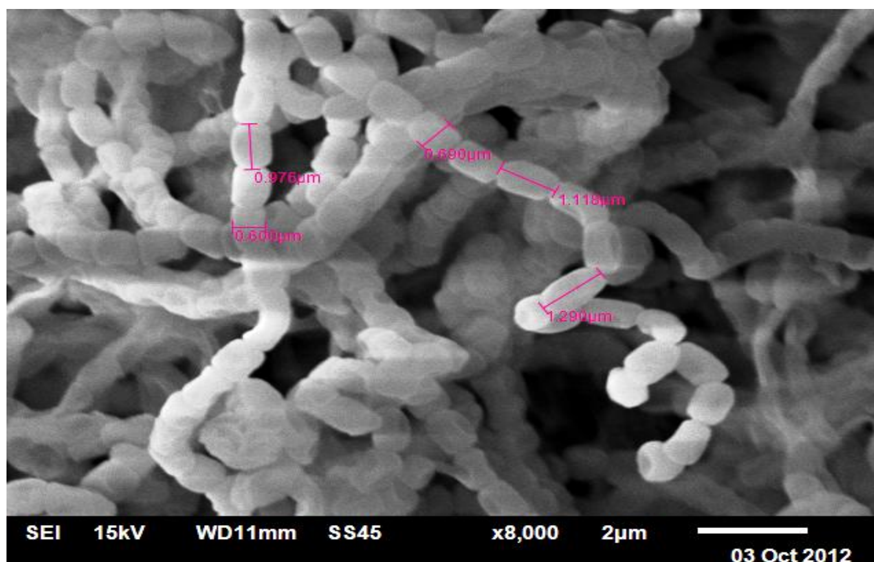


Fig. 2 Phylogenetic dendrogram obtained by maximum likelihood method of 16S rRNA sequences, showing the position of strain Streptomyces cheonanensis among Phylogenetic neighbours. Numbers of branch nodes are bootstrap values (1000 resamplings).

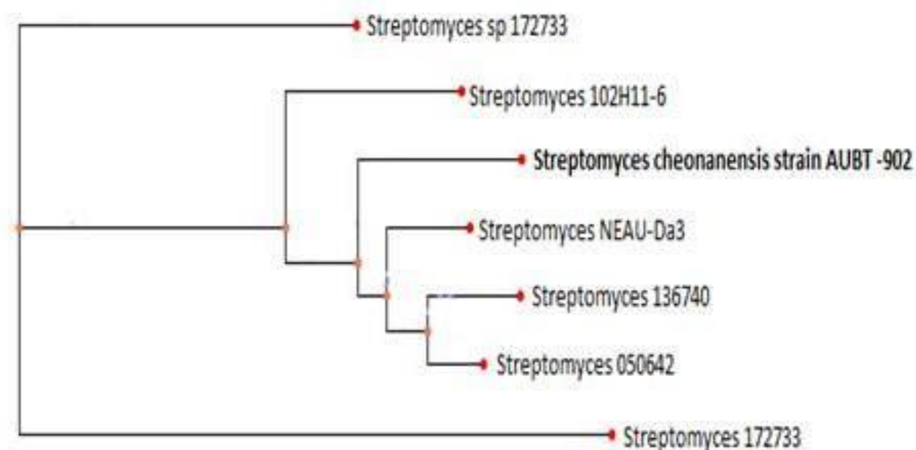


Fig. 3 Effect of glucose concentration on the growth and production of antibiotic by *Streptomyces cheonanensis* AUBT-902.

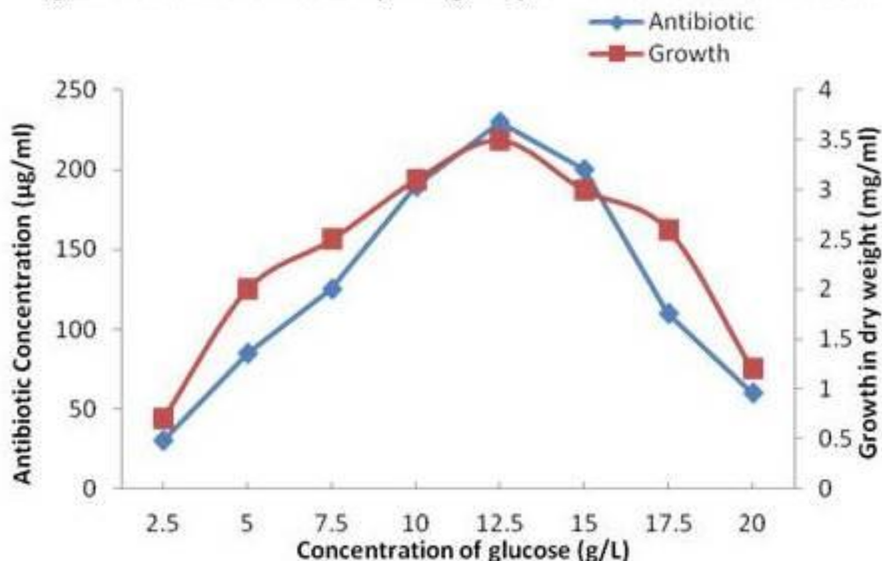


Fig. 4 Effect of ammonium nitrate concentration on the growth and production of antibiotic by *Streptomyces cheonanensis* AUBT-902.

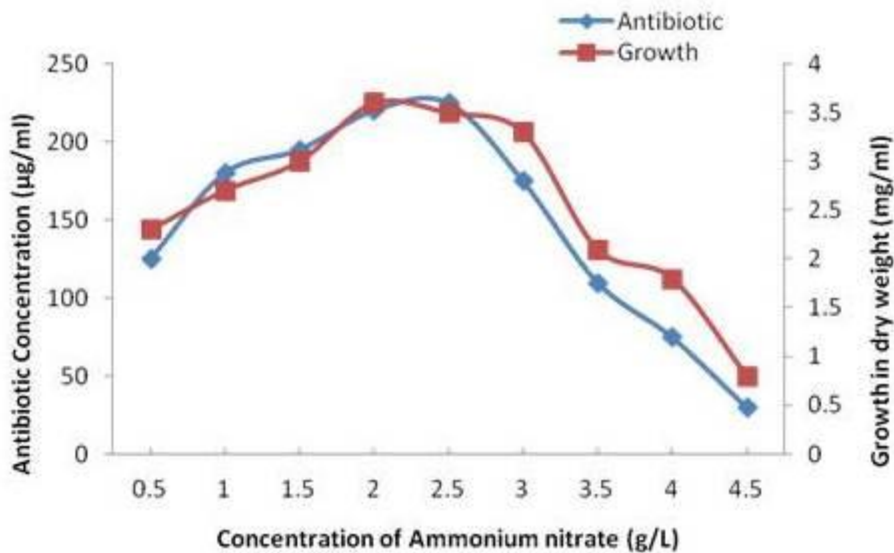


Fig. 5 Effect of K_2HPO_4 concentration on the growth and production of antibiotic by Streptomyces cheonanensis AUBT-902.

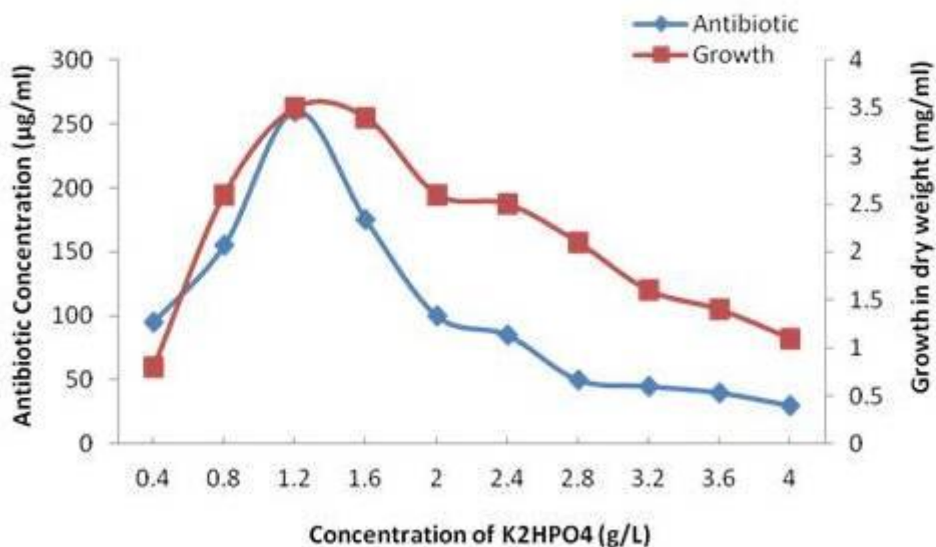


Fig. 6 Effect of $MgSO_4.7H_2O$ concentration on the growth and production of antibiotic by Streptomyces cheonanensis AUBT-902.

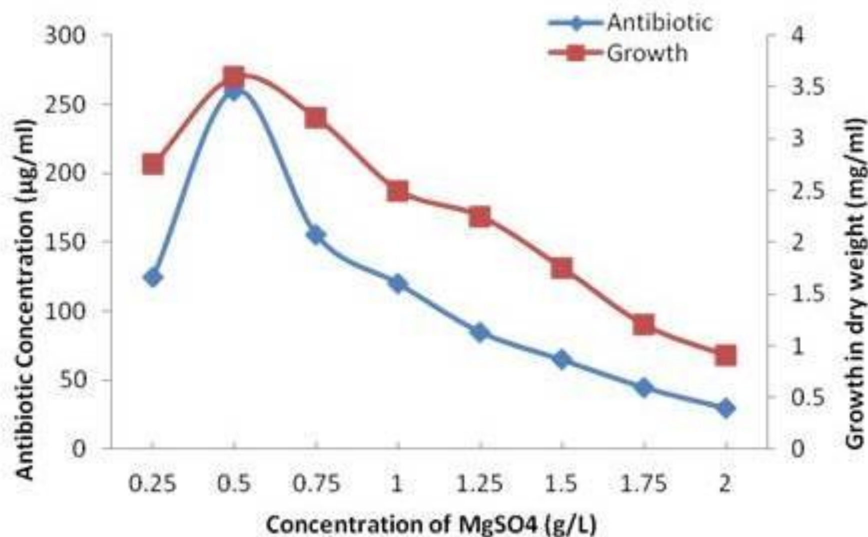


Fig. 7 Effect of incubation temperature on the growth and production of antibiotic by Streptomyces cheonanensis AUBT-902.

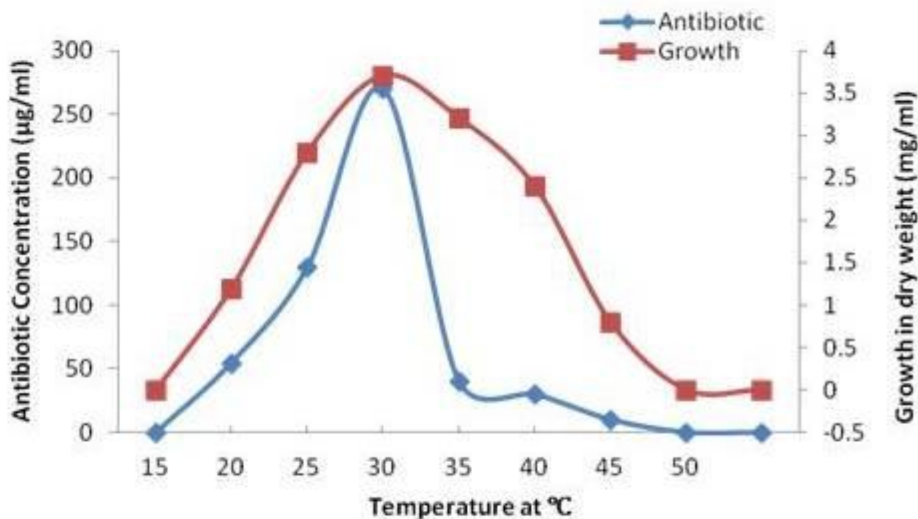


Fig. 8 Effect of pH of the medium on the growth and production of antibiotic by Streptomyces cheonanensis AUBT-902.

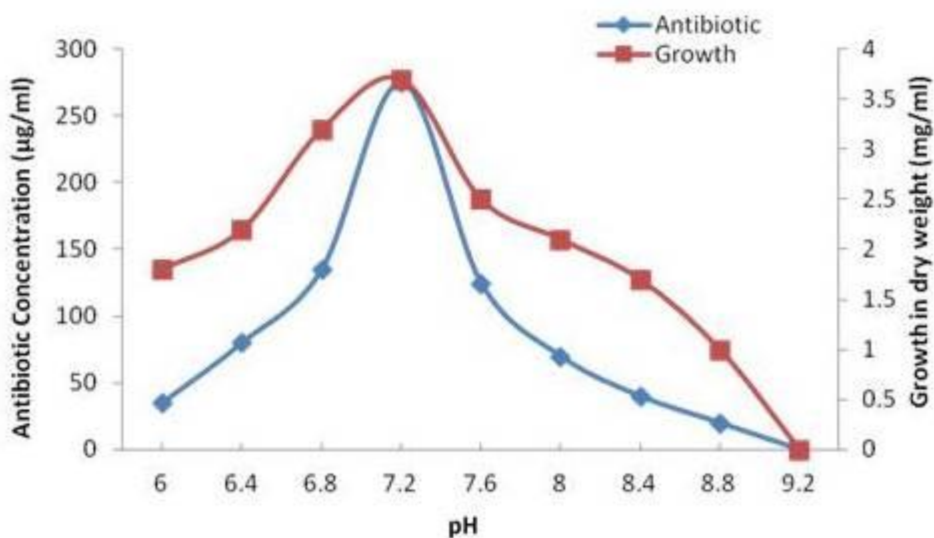


Fig. 9 Zone of inhibition against bacteria by isolate AUBT–902

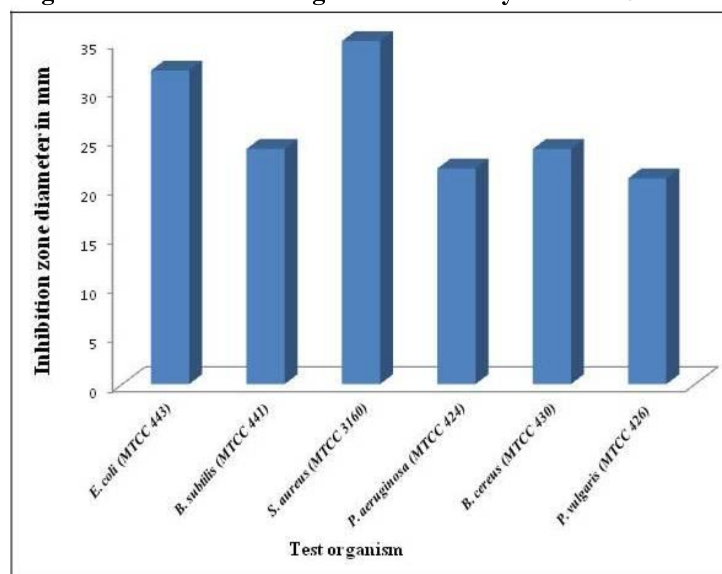
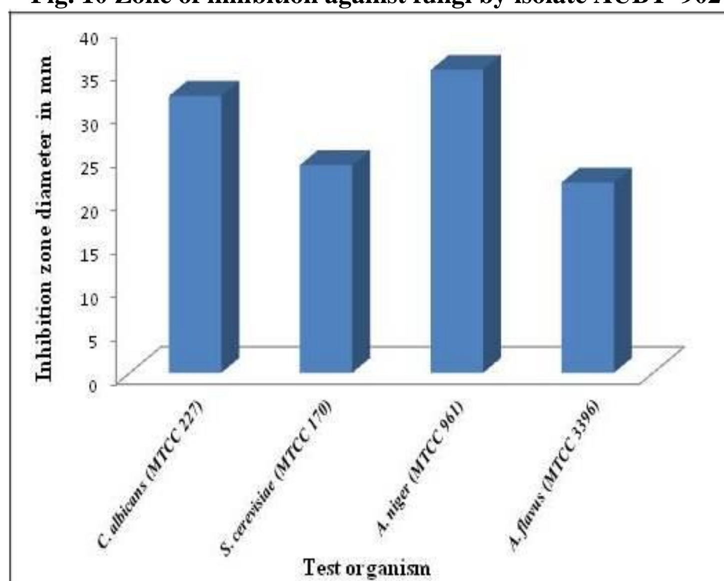


Fig. 10 Zone of inhibition against fungi by isolate AUBT–902



Discussion

Marine environment is a potential source for development of novel natural pharmaceuticals. As marine environmental conditions are different from terrestrial ones, it is surmised that marine actinomycetes might produce novel bioactive compounds. These microbes have characteristics in common to both bacteria and fungi and yet they possess adequate distinctive features to classify them into a separate category. The first report on marine actinomycetes was made by Oskay, *et al.,* 2004 from the salt mud. Actinomycetes especially Streptomyces have been reported from the marine sub habitats such as marine soil (Dhanasekaran, 2005). Previous investigations indicate the tremendous potential of marine actinomycetes, particularly *Streptomyces species* as a useful and sustainable source of new bioactive natural products (Dhanasekaran, 2006; Huang, 2008). *Streptomyces* strains have been known for their valuable potential as sources for antimicrobial agents.

Among all the isolates, one isolate (AUBT - 902) showed significant antimicrobial activity against selected bacterial and fungal test organisms. The strain was further identified as a member of *Streptomyces* genus based on its

morphological, cultural, physiological, utilization of carbon sources and biochemical characteristics. The identification of the potent isolate in this study was based on 16S rRNA gene sequence analysis. Analysis of 16S rRNA gene sequences has been proved to be a powerful method for phylogenetic characterization of microorganisms (Thenmozhi, 2010). It helps to elucidate the evolutionary relationship among microorganisms.

Microorganisms have a metabolic capacity to utilize a variety of carbon sources and to adapt to changes in osmotic strength, stress conditions, oxygen, and nutrient limitations (Postma, 1993). Glucose and ammonium nitrate were found to be the best carbon and nitrogen sources, respectively, for growth and antibiotic biosynthesis by *Streptomyces cheonanensis*. The maximum antibiotic biosynthesis by *Streptomyces* cheonanensis AUBT-902 was obtained in medium supplemented with 12.5g/l glucose as carbon source and 2.5g/l ammonium nitrate as nitrogen source at pH 7.2 after 96hrs of incubation.

Conclusion

This is the first report of *Streptomyces cheonanensis* from marine sediment, producing bioactive compound. There is an exponential increase in the number of new bioactive secondary metabolites produced by the actinomycetes isolated from marine sources. They have the ability to produce potent, distinctive, adapted, exceptional bioactive secondary metabolites.

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