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RESEARCH ARTICLE

ANTIMICROBIAL ACTIVITY AND CHARACTERIZATION OF MARINE STREPTOMYSES sps.

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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 5 marine samples collected from the Bay of Bengal. Out of 42 isolates, 6 strains showed high antimicrobial activity against test organisms. The strain GIBT-201 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 geysiriensis (99.7%). On the basis of polyphasic evidence, the strain was recognized as a new isolate of Streptomyces geysiriensis from marine source.

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Introduction:-

Microbial diversity is a vast frontier and potential goldmine for the biotechnology industry because it offers countless new genes and biochemical pathways to probe for enzymes, antibiotics and other useful molecules (Singh & Agrawal 2002). Choice of natural materials like soils in researches is based on the assumption that samples from widely diverse locations are more likely to yield novel microorganisms and therefore hopefully, novel metabolites as a result of the geographical variation. Although the microbial diversity of life in the terrestrial environment is extraordinary, the greatest biodiversity is in the oceans (Dubey *et al.*, 2005).

More than 70% of our planet's surface is covered by oceans and life on Earth originated from sea. In some marine ecosystems, such as the deep sea floor and coral reefs, experts estimate that the biological diversity is higher than in the tropical rainforests (Edward *et al.*, 2006). As marine environmental conditions are extremely different from terrestrial ones, it is surmised that marine actinomycetes have different characteristics from those of terrestrial counterparts, and therefore, might produce different types of bioactive compounds (Fenical *et al.*, 1999; Gesheva *et al.*, 2005).

Actinomycetes are the most economically and biotechnologically valuable prokaryotes and are responsible for the production of about half of the discovered secondary metabolites. Recently the rate of discovery of new compounds from terrestrial actinomycetes has decreased whereas the rate of reisolation of known compounds has increased. Thus, it is excited that new groups of actinomycetes from unexplored or under exploited habitats be pursued as sources of novel bioactive secondary metabolites (Donia *et al.*, 2003). It had been emphasized that actinomycetes from marine sediments might be valuable for the isolation of novel strains which could potentially yield a broad spectrum of secondary metabolites.

Marine actinomycetes are a potential source of novel compounds as the environmental conditions of the sea are entirely different from the terrestrial conditions (Meiying and Zhicheng, 1998). Many researchers have isolated novel antibiotics from the marine environment (Sujatha *et al.*, 2005; Biabani *et al.*, 1997; Maskey *et al.*, 2003; Charan *et al.*, 2004; and Li *et al.*, 2005). It had been emphasized that actinomycetes from marine sediments might be valuable for the isolation of novel strains which could potentially yield a broad spectrum of secondary metabolites. Actinobacteria originally considered as an intermediate group between bacteria and fungi, but latter it has attained a distinct position (Pandey *et al.*, 2004). Actinobacteria are a well-defined group of Gram positive, free-living, saprophytic bacteria with high G+C content in their DNA (Pandey *et al.*, 2004). Among the genus of Actinomycetes group, *Streptomyces* is the major and more than 500 species of this genus have been reported by Euzéby (2008). Almost two third of the naturally occurring antibiotics are produced by *Streptomyces*. The genus *Streptomyces* was described for the first time by Waksman and Henrici in the year 1943 (Kim *et al.*, 2006).

Around 23000 bioactive secondary metabolites produced by microorganisms have been reported. Over 10000 of these compounds are produced by actinomycetes, representing 45% of all bioactive microbial. Screening of antibacterial producing actinomycetes metabolites discovered. Among actinomycetes, around 7600 compounds are produced by *Streptomyces* species. Hence the best alternate to meet the increasing demand of safe and cost effective drugs is natural products from marine actinobacteria (Behal, 2003).

The present study deals with screening for the isolation of actinomycetes to produce antibiotics, isolated from the marine sediments from South East Coast of India for determination of their antimicrobial activity.

Materials and Methods:-

Sample Collection:-

Totally 5 marine sediment samples were collected from various depths of Bay of Bengal. The samples were collected in sterile air tight bags and accurately labelled indicating the date of collection and transferred to the laboratory for further study.

Pre-treatment of samples:-

The sediment samples were air dried. Appropriate selective media such as starch casein agar, glycerol yeast extract agar, Actinomycete 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 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. 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 growing 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 50 μ l 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 μ l 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 most potent strain was selected for further analysis.

Morphological and cultural characters of the selected actinomycetes strain was studied by inoculating into sterile International Streptomyces Project ISP 1, 3-7, 9 media (Ohshima *et al.*, 1991). The media were sterilized and poured into sterile petridishes. After solidification of the media, 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 GIBT-201 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 of 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).

Molecular analysis:-

The 16S rRNA gene was amplified and analyzed as described by Li *et al.*, (2007) and the 16S rRNA gene sequence (1450 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 enissocaesilis* ACCA1 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 as 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 (1450 bp) has been deposited in the Gen bank under the accession number **KU296926**.

Results and Discussion:-

A total of 42 isolates of actinomycetes were isolated as pure cultures from 5 marine sediments collected from Bay of Bengal, India. Of these isolates, 6 isolates having distinct activity of both antibacterial and antifungal.

Among these isolates, one isolate (**GIBT-201**) showed significant antimicrobial activity against selected bacterial and fungal test organisms and was characterized by polyphasic taxonomy.

Characterization of actinomycete isolate GIBT-201:-

The active isolate was gram⁺, 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 GIBT-201 on various media.

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

TABLE-2: Morphological, cultural, physiological and biochemical characteristics of GIBT-201.

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	+
H ₂ S 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 GIBT-201

Carbon source 1% (w/v)	Growth dry wt (mg/ml)	Antimicrobial compound yield (µg/ml)
Arabinose	2.0	22
Fructose	2.9	142
Galactose	1.5	95
Glucose	3.0	185
Glycerol	2.5	115
Lactose	0.9	42
Maltose	2.8	110
Mannitol	0.4	52
Mannose	1.6	65
Meso-inositol	1.8	60
Rhamnose	2.9	45
Starch	1.3	75
Sucrose	1.8	50
Xylose	0.8	35

TABLE-4: Effect of different nitrogen sources on growth and antibiotic production by isolate GIBT-201

Nitrogen source	Growth dry wt. (mg/ml)	Antibiotic yield (µg/ml)
Inorganic source 1 % (w/v)		
Ammonium citrate	0.9	52
Ammonium nitrate	3.4	225
Ammonium sulphate	0.5	65
Monosodium Glutamate	0.8	85
Potassium nitrate	2.9	150
Sodium nitrate	3.2	165
Amino acids 0.05 % (w/v)		
Alanine	2.4	132
Arginine	2.3	95
Asparagine	2.7	82
Aspartic acid	2.4	95
Glutamic acid	3.0	105
Hydroxyproline	2.8	115
Leucine	2.2	95
Methionine	3.1	85
Phenylalanine	2.4	60
Serine	2.3	105
Threonine	2.8	120
Tryptophan	1.9	65
Tyrosine	1.8	90

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 enissocaesilis* ACCA1 (Fig.2). The almost complete 16S rRNA gene sequence of the strain was determined and has been deposited in the GenBank database (Accession No. KU296926). 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 geysiriensis* strain (GenBank Accession No. AB184661).

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 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 were shown in Table 4. The highest antibiotic production was obtained in 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 antibiotic. The results also showed that the addition of 0.5 g/l of magnesium sulphate to the culture medium was optimal for antibiotic production (Fig. 6). The environmental requirements and tolerance of GIBT-201 for growth and antibiotic production has been studied in detail. GIBT-201 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 GIBT-201 showed a broad antimicrobial spectrum against Gram (+) and (-) bacteria and fungi when tested with crude culture filtrate. Finally, the antibiotic production was tested employing the modified medium and optimized cultural conditions.

Fig. 1 Scanning electron micrograph of *Streptomyces geysiriensis* GIBT-201.

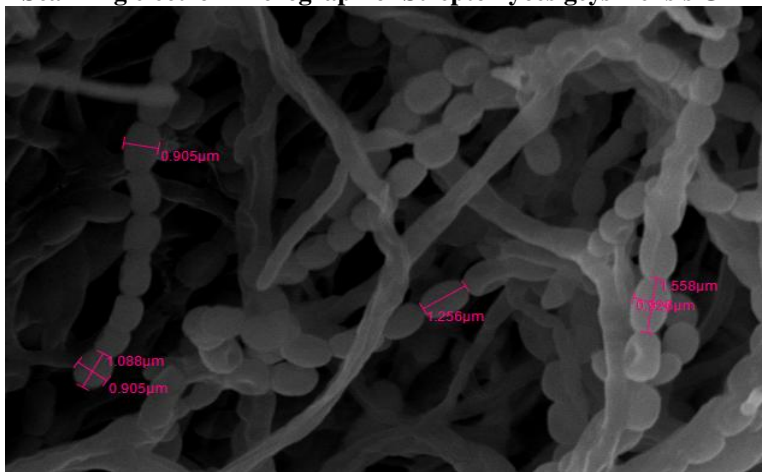


Fig. 2 Phylogenetic dendrogram obtained by maximum likelihood method of 16S rRNA sequences, showing the position of strain *Streptomyces geysiriensis* among Phylogenetic neighbours. Numbers of branch nodes are bootstrap values (1000 resamplings).

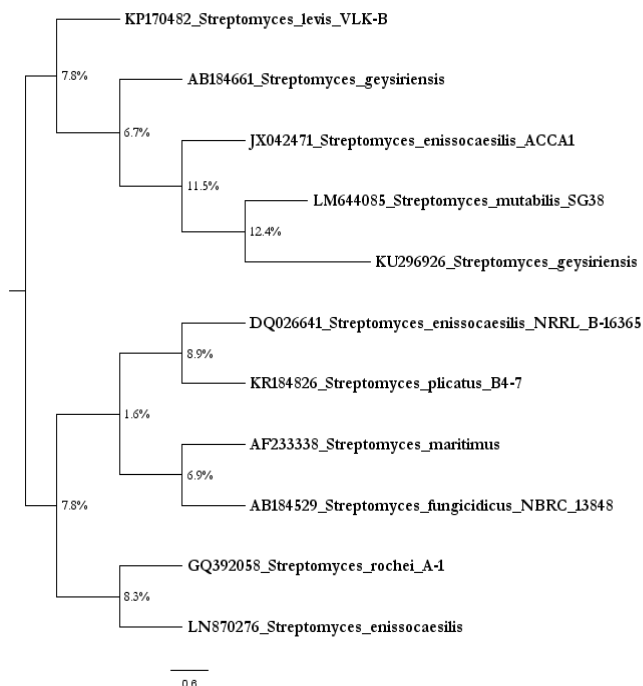


Fig. 3: Effect of Glucose concentration on Growth and Production of Antibiotic by *Streptomyces geysiriensis* GIBT-201

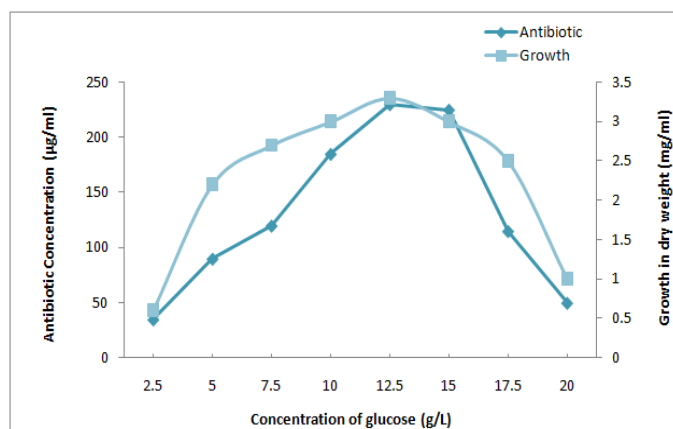


Fig 4: Effect of Ammonium nitrate concentration on Growth and Production of Antibiotic by *Streptomyces geysiriensis* GIBT-201

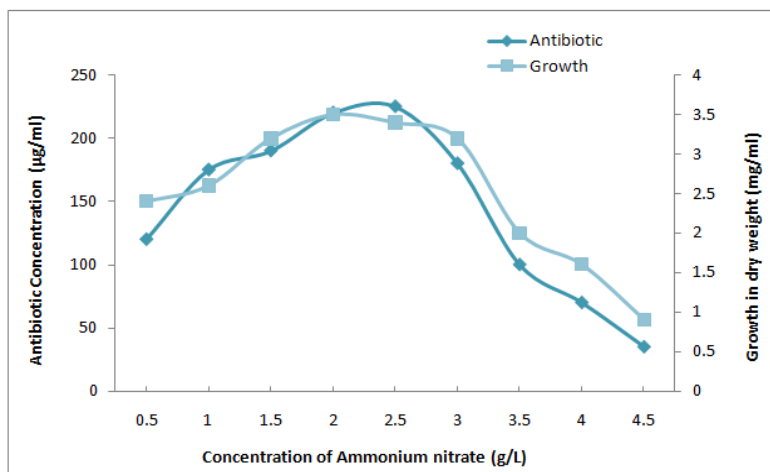


Fig 5: Effect of K_2HPO_4 concentrations on Growth and Production of Antibiotic by *Streptomyces geysiriensis* GIBT-201

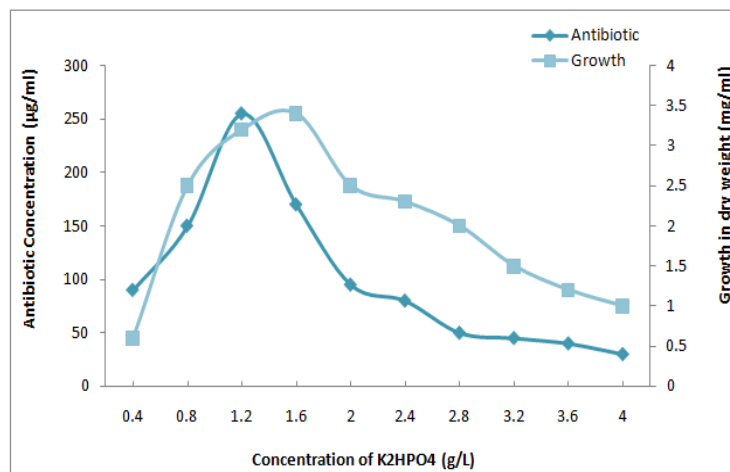


Fig 6: Effect of $MgSO_4$ concentrations on Growth and Production of Antibiotic by *Streptomyces geysiriensis* GIBT-201

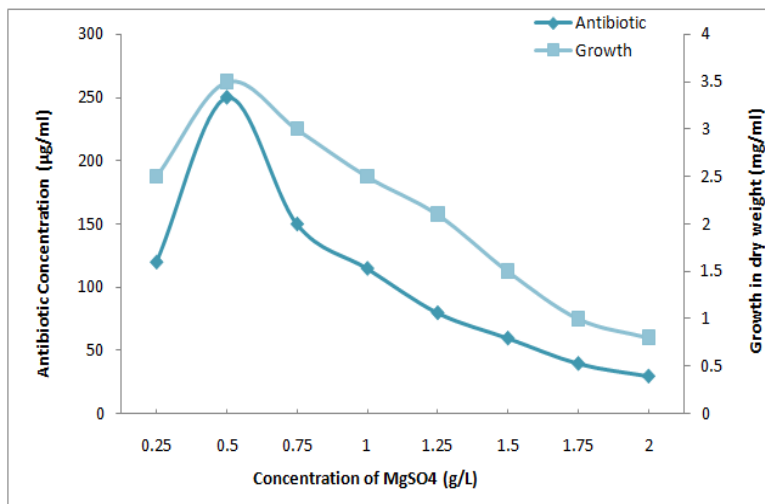
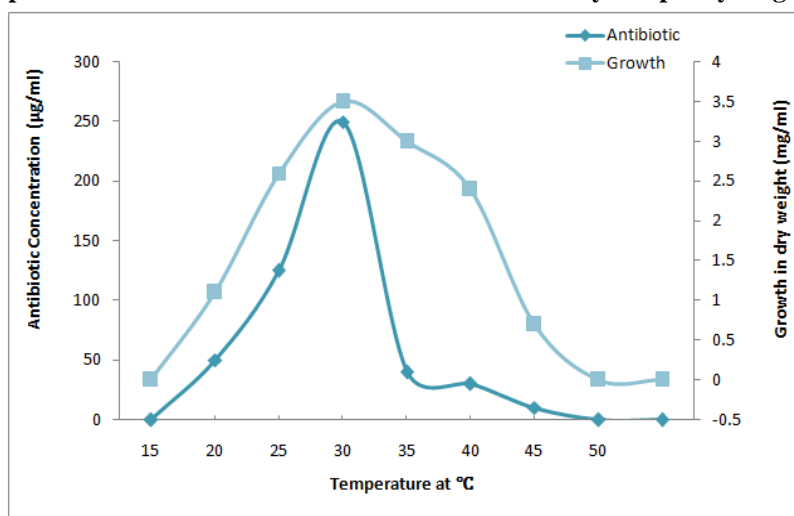
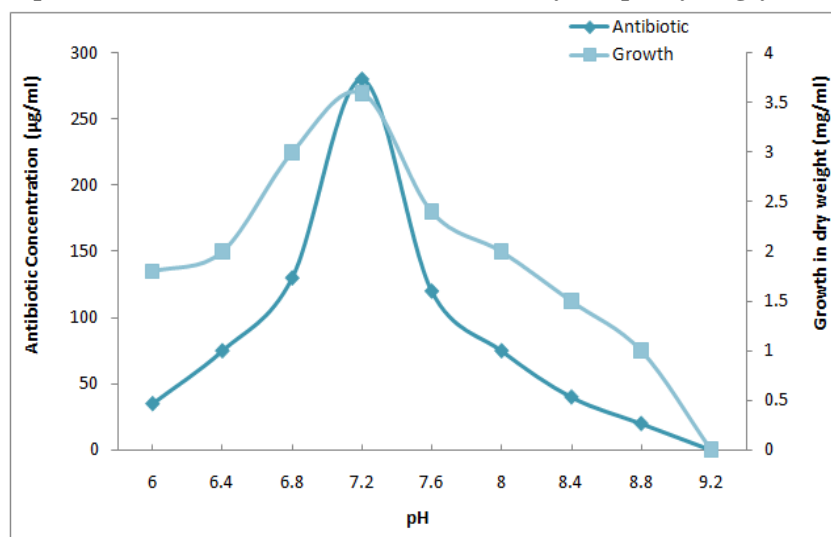


Fig 7: Effect of Temperature on Growth and Production of Antibiotic by *Streptomyces geysiriensis* GIBT-201**Fig 8: Effect of pH on Growth and Production of Antibiotic by *Streptomyces geysiriensis* GIBT-201**

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, 2005; Huang, 2008). *Streptomyces* strains have been known for their valuable potential as sources for antimicrobial agents.

Among all the isolates, one isolate (GIBT-201) 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 bacteria 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 nutrients limitations (Postma, 1993). Glucose and ammonium nitrate were found to be the best carbon and nitrogen sources, respectively, for growth and antibiotic GIBT-201 biosynthesis by *Streptomyces geysiriensis*. The maximum antibiotic biosynthesis by *Streptomyces geysiriensis* GIBT-201 was obtained in medium supplemented with 12.5g/l glucose as a carbon source and 2.5g/l ammonium nitrate in nitrogen source at pH 7.2 after 96hrs of incubation.

Conclusion:-

It is the first report of *Streptomyces geysiriensis* 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 source. They have the ability to produce potent, distinctive, adapted, exceptional bioactive secondary metabolites.

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